STUDIES ON MITOGENIC SIGNAL TRANSDUCTION BY THROMBIN IN VASCULAR CELLS

A thesis presented by

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ABSTRACT

The serine protease α-thrombin may be an important mediator of vascular disease states such as atherosclerosis, restenosis and pulmonary hypertension that are characterized by the hyperactive division of cell types of the blood vessel wall. Fundamental to an understanding of the role of thrombin in these conditions will be the identification of intracellular signal transduction processes activated by thrombin in vascular cells that are involved in proliferation. The aim of this study was to define protein kinase signalling pathways implicated in the mitogenic effects of thrombin on bovine pulmonary arterial (BPA) fibroblasts.

In BPA fibroblasts, thrombin was found to stimulate the time and concentration-dependent activation of 1) components of the mitogen-activated protein (MAP) kinase cascade including p42/p44 MAP kinases, MEK-1/2, and c-Raf-1, by a protein kinase C (PKC)-independent and pertussis toxin-insensitive mechanism; 2) the 70 kDa ribosomal S6 kinase ($p70^{s6k}$) in a manner consistent with an involvement of PKC, a heterotrimeric G protein of the $G_{i/o}$ family, and a phosphoinositide 3-kinase different from p85/p110; 3) a MAP kinase-activated protein kinase 2 (MAPKAP kinase-2) -associated kinase activity, that may be attributable to the MAP kinase homologue p38. All three signalling mechanisms contributed to a strong mitogenic effect of thrombin in BPA fibroblasts as thrombin-stimulated DNA synthesis was inhibited to varying extents when components of these pathways were specifically blocked by pharmacological intervention. The signalling events evoked by thrombin in BPA fibroblasts did not appear to be a cellular consequence of activation of the cloned tethered ligand thrombin receptor, nor the recently described proteinase-activated receptor-2 (PAR-2), and occurred by an undefined mechanism, possibly involving a novel receptor species. In contrast, both of the currently defined proteolytically-activated receptors coupled to activation of the MAP kinase cascade in rat aortic smooth muscle cells.

The results presented demonstrate that multiple signal transduction mechanisms are employed by thrombin to control cell cycle re-entry in a single cell type. In addition, they highlight roles for different protease-sensitive cell surface proteins in initiating signalling events in vascular cells.

PUBLICATIONS

Papers:

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Abstracts:

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Abbreviations

AII	angiotensin II
ADP	adenosine diphosphate
AP-1	activator protein-1
ATF2	activating transcription factor-2
ATP	adenosine triphosphate
BPA	bovine pulmonary arterial
BSA	bovine serum albumin
cAMP	cyclic adenosine3',5'-monophosphate
cdk	cyclin-dependent kinase
cDNA	complimentary DNA
CRE	cAMP-responsive element
CREM	cAMP-responsive element modulator
DAG	*sn*-1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
FAK	focal adhesion kinase
FCS	foetal calf serum
FGF	fibroblast growth factor
FKBP	FK506-binding protein
FRAP	FKBP-rapamycin-associated protein
GAP	GTPase activating protein
G-protein	GTP-binding protein
Grb2	growth factor receptor binding protein-2
GSK	glycogen synthase kinase
GSH	glutathione
GST	glutathione-S-transferase
GDP	guanosine diphosphate

GTP	guanosine triphosphate
5-HT	5-hydroxytryptamine
HOG1	high osmolarity glycerol response 1
Hsp27	27 kDa heat shock protein
IGF	insulin-like growth factor
IL-1	interleukin-1
IP_3	inositol 1,4,5 trisphosphate
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
LPA	lysophosphatidic acid
MAP	mitogen-activated protein
MEK	MAP kinase or ERK kinase
MEKK	MEK kinase
MAPKAPK-1	MAP kinase -activated protein kinase-1
MAPKAPK-2	MAP kinase -activated protein kinase-2
MLCK	myosin light chain kinase
mRNA	messenger RNA
mSos	mammalian homologue Son of Sevenless
NCS	newborn calf serum
NF-κB	nuclear factor-kappa B
$p70^{s6k}$	p70 ribosomal protein S6 kinase
PA	phosphatidic acid
PAGE	poly acrylamide gel electrophoresis
PAK	p21-activated kinases
PAP	phosphatidic acid phosphohydrolase
PAR	proteinase-activated receptor
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGE_1	prostaglandin E_1
PI	phosphatidylinositol
PI 3-kinase	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate

PI-3,4-P_2	phosphatidylinositol-3,4-bisphosphate
PI-3,4,5-P_3	phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	4β-phorbol 12-myristate 13-acetate
PTX	pertussis toxin
RA	rat aortic
RK	reactivating kinase
RNA	ribonucleic acid
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SAP	stress-activated protein
SEK	SAP kinase or ERK kinase
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulphate
SH-2	Src homology domain-2
SH-3	Src homology domain-3
SRE	serum-response element
SRF	serum response factor
TAK-1	β (TGF-β)-activated kinase
TEMED	N, N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
TNF	tumour necrosis factor
TOR	target of rapamycin
TRAP	thrombin receptor-activating peptide
TRE	TPA response element

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Thrombin (EC 3.4.21.5) is a glycoprotein which is representative of a subclass of serine proteases that appears to have a number of physiological functions. The most extensively studied action is in the blood clotting cascade in which thrombin is a key enzyme that converts the soluble plasma protein fibrinogen to the insoluble fibrin matrix to form the basis of a clot, activates factor XIII to initiate fibrin cross-linking and feeds back to promote clotting by activating factors V, VIII and XI.

Besides acting on soluble substrates in the circulation, thrombin also exerts hormone-like activity on a large variety of cell types, not only those that play a critical role in thrombosis and haemostasis but others that may contribute to inflammatory and proliferative responses under normal and pathological conditions. This dual function of thrombin as both an enzyme and agonist is unique and makes understanding the molecular mechanisms by which thrombin activates cells an intriguing issue. The recent cloning and characterization of a thrombin receptor has gone a long way towards explaining the nature of the interaction between thrombin and its target cells, although the biochemical consequences of receptor activation are not entirely clear. In this thesis, some of the early cellular signalling events that are evoked by thrombin will be investigated with particular attention to signal transduction pathways believed to be involved in mitogenesis as the mechanisms by which thrombin evokes growth-promoting effects in a number of cells is a major area of uncertainty.

1.2 THROMBIN, THE THROMBIN RECEPTOR, AND CELL ACTIVATION

1.2.1 Structure of α-thrombin

α-Thrombin is generated in the final stages of the blood clotting cascade by the cleavage of the circulating zymogen prothrombin, a process requiring Factors Va and Xa, Ca^{2+} and membrane phospholipid. Human α-thrombin comprises of two polypeptide chains of 36 (A-chain) and 259 (B-chain) residues linked by a disulphide bridge. The native α-thrombin is susceptible to limited proteolytic degradation by autolysis or other serine proteases to form β-, γ-, ε- and ζ-thrombin derivatives (Fenton *et al.*, 1977) which generally exhibit reduced proteolytic activity toward a range of substrates (Guillin *et al.*, 1995). The highly refined X-ray crystallographic structure of α-thrombin revealed that the protein is a intricately

structured globular protein that is ellipsoid in shape (Stubbs & Bode, 1993). Thrombin belongs to the trypsin-like serine protease family, but displays greater specificity than other serine proteases for its substrates. While in all cases thrombin cleaves peptide bonds of substrates C-terminal to an arginine residue, preferences are made for specific amino acid residues residing up to four positions either side of the cleavage site. The unique specificity of thrombin for substrate selection may be ascribed in part to the depth and narrowness of the active site cleft, over which project two prominent insertion loops preventing access to all but a small number of proteins. Another structural feature which may also determine the selection of substrates by thrombin is a cluster of basic amino acids within a loop encompassed by Lys^{65} and Lys^{77} which form an anion binding site that is distinct from the active site and interacts with positively charged acidic regions identified in several ligands including fibrinogen, fibrin, hirudin and the thrombin receptor (see Section 1.2.2).

1.2.2 The thrombin receptor

Despite considerable research efforts, the mechanism by which thrombin evoked cellular responses has been a long-standing enigma up until recently. While early studies using traditional ligand-binding approaches identified the presence of high-affinity thrombin binding sites on the surface of a number of cells (Carney & Cunningham, 1978) these sites were unlikely to represent a functional receptor as none could evoke a second messenger response and modified thrombins which did not act as agonists were able to bind at these sites as efficiently as thrombin. The molecular cloning of a thrombin receptor in several mammalian species and cell types including human platelets (Vu *et al.*, 1991), hamster lung fibroblasts (Rasmussen *et al.*, 1991), rat aortic smooth muscle cells (Zhong *et al.*, 1992) and *Xenopus laevis* (Gerstzen *et al.*, 1994) provided a framework for understanding how most, if not all, of the cellular effects of thrombin are transmitted. This receptor displays structural features characteristic of the superfamily of receptors that are coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins), possessing seven hydrophobic transmembrane spanning domains, three intracellular- and three extracellular loops, a C-terminal intracellular tail and a long N-terminal extracellular domain. However, unlike other receptors of this class which become activated by classical occupancy mechanism based on reversible interactions with ligands, a unique model for thrombin receptor activation has been proposed. The N-terminal exodomain contains a putative thrombin cleavage site between residues Arg^{41} and

Ser^{42}. Thrombin interacts specifically with two regions either side of this site. Residues immediately N terminal to Arg^{41} (LDPR) serve as a cleavage recognition sequence recognized by the active centre while further away from the N-terminus, the amino acids 52-60 (DKYEPFWED) dock with the anion-binding exosite on the surface of thrombin, in a manner similar to the leach anticoagulant and specific thrombin inhibitor hirudin. Proteolytic cleavage of exodomain by bound thrombin reveals a new N-terminus containing a receptor-activating sequence (Vu *et al.*, 1991). This sequence serves as a "tethered ligand", interacting with other sites elsewhere in the receptor. These sites have not been precisely mapped, but are thought to include Asn^{75} and Ser^{93} in the N-terminus near the first transmembrane domain, as well as residues in the second extracellular loop (Bahou *et al.*, 1993, Gerzten *et al.*, 1994). Consistent with a role for the cloned receptor, most of the cellular effects of thrombin require the enzyme to be proteolytically active and structure-activity studies have shown that an intact thrombin cleavage site is necessary for receptor activation by thrombin (Vu *et al.*, 1991).

1.2.3 Thrombin-receptor -activating peptides (TRAPs)

A convincing piece of evidence to support the proposed model for thrombin receptor activation is the demonstration that synthetic peptides corresponding to the first 6 to 14 amino acids adjacent to the putative cleavage site (SFLLRN and SFFLRN of the human and rodent receptors, respectively) mimic many of the actions of thrombin on cells. Presumably these thrombin-receptor activating peptides (TRAPs) bypass the requirement for receptor proteolysis and activate the receptor directly. Structure activity studies have identified the structural determinants within TRAPs critical for receptor activation. While substitutions outwith the first six amino acids C-terminal to the receptor cleavage site have little effect on the efficacy of the peptide, within this region, only very conservative replacements are allowed (Vouret-Craviari *et al.*, 1992, Scarborough *et al.*, 1992). Protonation of the free N-terminal amino group has been found to be an absolute requirement for agonist function (Scarborough *et al.*, 1992).

TRAPs have served as a useful tools in addressing the involvement of the cloned tethered ligand receptor in specific cellular responses to thrombin (Vu *et al.*, 1991, Huang *et al.*, 1991). A role for this receptor has been implicated where peptides have been shown to effectively reproduce the effects of thrombin, as has been reported for a number of early signalling events associated with G-protein-linked effector systems including the stimulation of

phospholipid turnover by PI-PLC (Hung *et al.*, 1992a, 1992b, Vouret-Craviari *et al.*, 1992), PLD and PC-PLC activities (McKenzie *et al.*, 1992) and inhibition of adenylyl cyclase (Hung *et al.*, 1992b, Vouret-Craviari *et al.*, 1992) that are described in greater detail in other sections of this chapter (1.4.2.1, 1.4.2.3 & 1.4.4). In other instances, different cellular responses to receptor activation by thrombin and TRAPs have been observed, such as the inability of peptides to mimic the mitogenic effect of thrombin in certain cell systems (Vouret-Craviari *et al.*, 1992). These findings have been interpreted as evidence for the possible existence of more than one type of thrombin receptor, or the inability of TRAPs to evoke signals generated by an interaction of thrombin with the receptor further to those produced by proteolysis.

1.2.4 Cellular effects of thrombin *in vitro* and *in vivo*

The first, and best characterized example of a cellular action of thrombin is on blood platelets. Thrombin is the most potent physiological activator of platelets, causing aggregation and degranulation by the release reaction, which contributes to haemostatic plug formation. Subsequently, it has been shown that thrombin interacts with a diverse array of cell types, both vascular and perivascular cells, as well as cells of neuronal origin, evoking a host of biological responses via mechanisms independent of platelets or the coagulation cascade. Most of these non-thrombotic cellular actions of thrombin have been defined *in vitro* and are reviewed more comprehensively elsewhere (Coughlin *et al.*, 1992, Grand *et al.*, 1996). Briefly, thrombin has been shown to have a number of effects on the vascular endothelium. These include stimulating endothelial secretion of a number of biologically active mediators that act on other cells including prostacyclin, platelet activating factor (PAF), plasminogen-activator inhibitor, von Willebrand factor and platelet-derived growth factor (PDGF) (reviewed by Coughlin *et al.*, 1992). Thrombin promotes an inflammatory response by exerting a chemoattractant effect on peripheral blood monocytes and cells belonging to the monocyte/macrophage family (Bar-Shavit *et al*, 1983) and causing adhesion of inflammatory cells to the vessel wall by an endothelium-dependent mechanism (Hattori *et al.*, 1989). Thrombin has also been shown to be a strong mitogen for a number of cell types in culture, most notably those of mesenchymal origin such as various types of fibroblast (Chen & Buchanan, 1975, Carney *et al.*, 1978, Vouret-Craviari *et al.*, 1993) and vascular smooth muscle cells (McNamara *et al.*, 1993, Molloy *et al.*, 1996) but also a number of other types

including endothelial cells (Herbert *et al.*, 1994), macrophage-like tumour cell lines (Bar-Shavit *et al.*, 1986) and lymphocytes (Chen *et al.*, 1976).

Whether these non-thrombotic actions of thrombin are important *in vivo* remains to be established. However, the multiple effects of thrombin on a broad variety of cells has led to the suggestion that the protease may orchestrate inflammatory and proliferative responses as part of the postclotting program for tissue repair following wounding or vascular damage, which may also be important under normal conditions. Of particular interest is the possibility that thrombin may be a causal factor in vascular disease states in which hyperplasia of cell types of the blood vessel wall is a hallmark in their pathogenesis. These include 1) atherosclerosis and restenosis (an undesirable consequence of balloon coronary angioplasty), where vascular lesion development is characterized by smooth muscle cell proliferation and migration leading to neointimal formation and lumenal narrowing; and 2) chronic hypoxic pulmonary hypertension, where pulmonary vascular remodelling is characterized by a significant thickening of the blood vessel wall and a narrowing of the lumen due in part to neomuscularization of the media of small diameter vessels and a prolonged and intense fibroblast proliferative response in the adventitial layer (Meyrick & Reid, 1980, Stenmark *et al.*, 1988). A role of thrombin in both of these conditions is indicated by the finding that antithrombin treatment with hirudin, a potent and specific thrombin inhibitor, reduces restenosis after angioplasty in a rabbit model of atherosclerosis (Sarembock *et al.*, 1991) and vascular remodelling in an endotoxin model of pulmonary hypertension in the pig (Hoffman *et al.*, 1990).

1.3 NUCLEAR EVENTS REGULATING CELL DIVISION

Despite the evidence presented above to exemplify the mitogenic behaviour of thrombin for different cell types both *in vitro* and *in vivo*, the specific role of thrombin on cell proliferation at the cellular level is not well understood. Ultimately, thrombin modulation of growth responses in cells is brought about by altered expression of genes associated with the early stages of proliferation. The activation of transcription factors is likely to be an important part of the mechanism by which thrombin promotes these changes in the nucleus. The following section outlines the events involved in cell division and describes how thrombin may regulate the processes involved by transcriptional activation.

1.3.1 The cell cycle

Dividing cells pass through an orderly cyclic sequence of events that by convention, is divided into four distinct phases; G1; a gap phase prior to DNA replication; S phase, a discrete period of DNA synthesis; G2, another gap phase prior to mitosis; and M, the mitotic phase. Cells that are not actively dividing exist in a state of temporary arrest or quiescence called G0. All transitions of the cell cycle such as conversion of quiescent cells to a state of active proliferation, commitment to DNA replication, initiation of DNA replication and entry into, and exit from mitosis, are controlled through phosphorylation of specific targets brought about by a family of serine/threonine protein kinases called cdks (cyclin-dependent kinases). The catalytic subunit of cdks are inactive and require for their activation, association with positive regulatory subunits called cyclins (for a more extensive description of cell cycle regulation see review by Dorée & Galas, 1994). In addition, the activity of cdks is also regulated by activating and inhibitory phosphorylation, interaction with a number of low molecular weight protein inhibitors and proteolytic degradation of cyclin subunits. These various levels of control ensure that the different cyclins are expressed and bind to different cdks at specific times of the cell cycle. Thus, cyclin D-cdk4 complexes may be required early in G1 for growth arrested cells to exit G0. In mammalian systems, cdk2 associated with cyclin E is required during G1 for cells to enter S phase beyond a "restriction point", a key checkpoint in late G1 where progression through the cell cycle may be arrested if the cell has not completed a particular process. DNA replication occurs two or more hours after the restriction point and is believed to involve cyclin A-cdk2 kinase (reviewed by Roberts, 1993). Another important checkpoint occurs at the G2/M phase transition. Passage through this phase of the cell cycle is believed to involve cdc2 kinase complexed with cyclins A and B as most of the events associated with entry into mitosis including chromosome condensation, nuclear envelope breakdown, cytoskeletal changes and formation of the mitotic spindle have been shown to be caused by this complex (reviewed by Nigg, 1993).

1.3.2 Transcriptional activation by thrombin

Movement through the cell cycle coincides with the expression of different families of genes. Expression of cyclins, cdks and kinase-inhibitory molecules are likely to be modulated by mitogen-responsive transcription factors although it is currently unclear how

transcriptional and cell cycle components are co-ordinated. One of the earliest nuclear events that follows the addition of growth factors to quiescent cells is the expression of "immediate-early" proto-oncogenes that are the normal cellular homologues of transforming genes carried by viruses that are intimately involved in regulating G0 to G1 transition. Thrombin receptor activation leads to the rapid and transient induction of members of this family including c-*jun*, c-*fos*, *fos*B and *fra*-1, whose mRNA levels increase rapidly and transiently within 15-30 minutes in vascular smooth muscle cells and fibroblasts (Seuwen *et al.*, 1990, Rothman *et al.*, 1994, Yalkinoglu *et al.*, 1995). Expression of the proto-oncogene c-*myc* by thrombin occurs later after about 2 hours and is maximal at 4 hours (Seuwen *et al.*, 1990, Weiss & Ives, 1991). One or more products of immediate early genes may act as transcription factors for a second group of growth-related genes called "delayed early" genes that require new protein synthesis for their expression. Formation of either heterodimeric complexes between Fos and Jun proteins or homodimeric Jun protein complexes form activator protein-1 (AP-1) complexes which bind to and activate the induction of a number of genes containing the AP-1 binding site, the tetradecanoyl phorbol acectate (TPA) response element (TRE), in their promoters (reviewed by Karin, 1995). The prolonged elevation of c-*jun* mRNA and associated increases in c-Jun protein stimulated by thrombin in 1321N1 astrocytoma cells leads to AP-1 formation and transcriptional activation of AP-1 responsive genes (Trejo *et al.*, 1992). The precise functions of early genes in promoting cellular proliferation is unclear. While overexpression of c-*jun*, c-*fos* and c-*myc* has been shown to be oncogenic and oligonucleotide antisense to c-*fos* and c-*myc* block proliferation (reviewed by Gorski & Walsh, 1995), expression of these genes does not always correlate with cell division (Seuwen *et al.*, 1990), suggesting that other regulatory steps are involved. Another transcriptional regulator that is activated by thrombin is nuclear factor-kappa B (NF-κB) (Nakajima *et al.*, 1994). NF-κB induces many genes with the κB sequence in their regulatory elements, including c-*myc*, and has been shown to be required for thrombin-stimulated growth as antisense treatment inhibits proliferation of vascular smooth muscle cells by thrombin (Nakajima *et al.*, 1994). The early transcriptional events described are followed by induction of the "late -G1" genes at the end of G1 phase or at the beginning of S phase, which are directly implicated in the a series of events leading to DNA synthesis.

1.4 EARLY CELLULAR SIGNALLING EVENTS MEDIATED BY THROMBIN

Intracellular signalling molecules and second messengers relay signals initiated by receptors at the cell surface to the nucleus where genes involved in cell cycle control are expressed. Identification of the signal transduction pathways evoked by thrombin is therefore of central importance in understanding how the protease acts as a mitogen. The following section reviews the early literature on thrombin-evoked signalling events that have been identified from extensive studies in diverse cell types, most notably, platelets, endothelial cells and vascular smooth muscle cells, and the evidence provided for their role in cell growth stimulation.

1.4.1 Coupling of the thrombin receptor to G proteins

The thrombin receptor is typical of many receptors possessing seven transmembrane domains in coupling to G proteins. These proteins exist in heterotrimeric complexes made up of α, β and γ subunits. In their inactive state, GDP is bound to the α subunits bound to $\beta\gamma$ in a heterotrimer and can associate with the inactive receptor. On agonist binding, the receptor undergoes a conformational change which allows the exchange of GTP for GDP on the α subunit. In its GTP-bound state, the α subunit dissociates from $\beta\gamma$ dimers and the receptor. It is now established that both α and $\beta\gamma$ components of the G protein serve to regulate effector proteins in their free state. The G protein switches itself off when the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP causing the reassociation of the subunits into a heterotrimer. Studies have endeavoured to identify the particular heterotrimeric G proteins that couple the thrombin receptor to its effectors in different cell types. More than 20 distinct G protein α subunits have been cloned and classified into the four major subgroups on the basis of sequence homology of their α subunits: G_s, G_i, G_q and G_{12}. At least 4 β and 6 γ subunits have also been described (reviewed by Helper & Gillman, 1992). Specificity of signal transduction by a particular agonist is thought to depend on the combination of G proteins and effector systems present in a given cell type and whether these signalling proteins are able to couple to the receptor for that agonist. The cloned thrombin receptor has been shown to couple to at least two classes of G proteins, G_q and G_i proteins (Hung *et al.*, 1992b).

1.4.1.1 Role of G proteins in thrombin-stimulated DNA synthesis

Bordetela pertussis toxin (PTX) catalyses the ADP-ribosylation and inactivation of the α subunit of the G_i subfamily including three forms of α_i (α_{i11}, α_{i12} and α_{i1}) and two forms of α_o (α_{o1} and α_{o2}), and has been used as a specific pharmacological tool to dissect the role of this class of G-protein in a number of biological responses to G-protein-coupled agonists (Spiegel, 1992). Studies demonstrating inhibition of thrombin-stimulated DNA synthesis by PTX have implicated pathways coupled to G_i or G_o in the regulation of mitogenesis by thrombin in CCL39 fibroblasts (Chambard *et al.*, 1987, Hung *et al.*, 1992a) and human vascular smooth muscle cells (Kanthou *et al.*, 1992). In agreement with these observations, thrombin-stimulated DNA synthesis is completely blocked by microinjection of anti-$G\alpha_{i2}$ antibody in mouse Balb 3T3 fibroblasts (Lamorte *et al.*, 1992). In another study, however, it has been shown that TRAP -stimulated DNA synthesis in CCL39 fibroblasts is inhibited by microinjection of anti- α_o rather than anti- α_i antibodies (Baffy *et al.*, 1994) indicating that in this cell type, G_o is most likely to represent the PTX-sensitive component of thrombin receptor-mediated mitogenesis. Microinjection studies have also demonstrated that $G\alpha_q$ plays an important function in thrombin receptor-mediated mitogenesis in Balb/c3T3 fibroblasts (Lamorte *et al.*, 1992, Baffy *et al.*, 1994). Thus, thrombin requires functional coupling to at least three distinct subtypes of G proteins, PTX-sensitive $G\alpha_i$ and $G\alpha_o$ and PTX-insensitive $G\alpha_q$ subunits to transduce mitogenic signals.

1.4.2 Phospholipid-derived second messenger pathways in thrombin responses

The formation of phospholipid-derived second messengers is one of the most widespread signal transduction mechanisms under the control of a multitude of hormones, including thrombin. These signalling systems were some of the first to be characterized and as such were originally considered important in mediating the mitogenic action of thrombin.

1.4.2.1 Thrombin stimulation of phosphatidylinositol (PI) -specific phospholipase C (PLC) activity

One of the earliest events detected following exposure of a variety of cells to thrombin is the activation of a phosphatidylinositide-specific phospholipase C (PI-PLC). PI-PLC

hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to form the second messenger molecules inositol-1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG). These products of PI turnover serve as second messengers; IP_3, in combination with one of its intermediate breakdown products, inositol-1,3,4,5-tetrakisphosphate, promotes the release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1989) while DAG activates certain protein kinase C (PKC) isoforms by increasing enzyme sensitivity to Ca^{2+} and phospholipids (see Section 1.4.3). Thrombin has been shown to effectively stimulate IP_3 and DAG formation and evoke Ca^{2+} transients in several cell types including fibroblasts (Paris & Pouysségur, 1986, Hung *et al.*, 1992a, 1992b), platelets (Brass *et al.*, 1986), endothelial cells (Lampugnani *et al.*, 1989, Brock & Capasso, 1988) and vascular smooth muscle cells (Huang & Ives, 1989, Kanthou *et al.*, 1996).

1.4.2.2 Role of G protein α and βγ subunits in thrombin-evoked PI-PLC activity

It has been established for a while that activation of PI-PLC by thrombin involves a G protein. A role for the G_q subtype in coupling the thrombin receptor on thrombin binding to the activation of a PI-PLC has been inferred by studies demonstrating that microinjection with antibodies directed against $G\alpha_q$ suppress Ca^{2+} mobilization by thrombin in Balb 3T3 fibroblasts (La Morte *et al.*, 1992) and by SFLLRN in CCL39 fibroblasts (Baffy *et al.*, 1994). While the identity of the PLC isoforms that couples to $G\alpha_q$ on activation of the thrombin receptor is not known, biochemical studies using intact cells or cell-free systems have shown that members of the $G\alpha_{q/11}$ class of G protein subunits interact with the PI-PLC-β1 isoform on activation of other G-protein coupled receptors (Berstein *et al.*, 1992, Wu *et al.*, 1992). A role for PLC-β1 in thrombin-stimulated PIP_2 hydrolysis is supported by the finding that inositol phosphate production and Ca^{2+} mobilization are reduced in a CCL39 fibroblast derivative that expresses constitutively low levels of PLC-β1 (Fee *et al.*, 1994).

In some cell types such as platelets (Brass *et al.*, 1986), CCL39 fibroblasts (Paris & Pouysségur, 1986, Hung *et al.*, 1992b) and vascular smooth muscle cells (Huang & Ives, 1989), the activation of PLC by thrombin is either partially or completely sensitive to inhibition by PTX. As the $G\alpha_q$ subtype is resistant to PTX, these observations suggested that in certain cells, other mechanisms are involved in PLC activation involving PTX-sensitive G-proteins. A model has been proposed to accommodate for the PTX sensitivity of PLC activation in these systems whereby free G protein βγ dimers released from activated $G\alpha_i$ or

$G\alpha_q$ directly regulate certain PLC isoenzyme activities, including PI-PLC-β2 and β3 isoenzymes (Camps *et al.*, 1992, Katz *et al.*, 1992).

1.4.2.3 Thrombin stimulated hydrolysis of phosphatidylcholine by phosphatidylcholine-specific phospholipase C and phospholipase D

Quantitative discrepancies have been identified between that levels of DAG generated from phosphoinositide hydrolysis and the total mass of DAG accumulating in agonist-stimulated cells, suggesting that the majority of DAG could arise from the breakdown of phospholipids other phospholipids including phosphatidylcholine (PC). Thus far, two alternative phospholipid pathways leading to DAG production from PC have been identified. In the first, a phospholipase D (PLD) hydrolyses PC to yield choline and phosphatidic acid (PA) which can subsequently be converted to DAG by phosphatidic acid phosphohydrolase (PAP). The second pathway described more recently involves the activation of a phosphatidylcholine-specific PLC (PC-PLC) which liberates DAG directly along with phosphorylcholine (reviewed by Exton, 1994).

Thrombin has been shown to stimulate PC hydrolysis in HC9 and CCL339 fibroblasts (Rangan *et al.*, 1991, McKenzie *et al.*, 1992). In these cells, the time course for DAG accumulation by thrombin is biphasic. While the initial, rapid and transient peak is thought to be due to phosphoinositide hydrolysis since it is accompanied by increases in IP_1 and Ca^{2+}, a more slowly developing, but prolonged accumulation that follows results primarily, if not exclusively, from PC breakdown and associated with an increase in the release of choline and/or phosphorylcholine, but not Ca^{2+} (Pessin & Rabin, 1989, Ha & Exton, 1993). The contribution of either PLD or PC-PLC to the sustained increase in PC-derived DAG levels lasting for several hours may differ between cell types. In CCL39 fibroblasts, thrombin-stimulated PC-PLC activity is sustained after PLD activity is rapidly desensitized within 5 minutes (McKenzie *et al.*, 1992, Wright *et al.*, 1992), while in human endothelial cells, PLD activity evoked by thrombin is sustained for at least 30 minutes (Garcia *et al.*, 1992).

1.4.3 Protein kinase C

The effects of phospholipid-derived DAG are mediated through the activation of PKC. However, PKC exists as a growing family of serine/threonine protein kinase isoforms

that are differentially expressed in various cells and subcellular localities and can be subdivided into three groups based on their structural and biochemical properties. Members of the PKC family so far examined are dependent on phosphatidylserine, but show clearly different requirements of Ca^{2+} and phospholipid metabolites for their activation. The classical or conventional PKC isoforms (cPKC) include α, βI, βII and γ are regulated by Ca^{2+} and DAG. The novel or non-classical isoforms (nPKC) δ, ε, η and θ are insensitive to Ca^{2+} as they lack the Ca^{2+}-binding C2 domain although they respond to DAG. The atypical PKC subgroup (aPKC) λ and ζ, isoforms are insensitive to Ca^{2+} and DAG or phorbol ester. (Further structural features and of individual PKC isoforms are outlined in reviews by Nishizuka, 1988, and Hug & Sarre, 1993).

The specific regulatory requirements of the different members of the PKC family produce distinct PKC activation patterns with respect to the subspecies of PKC that becomes activated, the extent and duration of the response and perhaps their intracellular localization. In particular, a number of reports have demonstrated differential functions of DAG derived from PI-4,5-P_2 and PC in the activation of PKC. In IIC9 fibroblasts, translocation of the Ca^{2+}-dependent PKCα isoform from the cytosol to the plasma membrane by thrombin, regarded as a parameter of enzyme activation, parallels the initial peak in DAG formed from phosphoinositide, but not from PC hydrolysis (Leach *et al.*, 1991, Ha & Exton, 1993). On the other hand, translocation of the Ca^{2+}-independent isoform PKCε, also occurred rapidly but was maintained during the persistent phase of DAG production derived from PC (Ha & Exton, 1993). The presence of Ca^{2+} accompanying the initial DAG peak, rather than different molecular species of DAG derived from PI-4,5-P_2 and PC, varying in their fatty acid composition, accounted for the differential activation of the PKC isoforms (Ha & Exton, 1993).

1.4.3.1 Role of PKC in mitogenic signal transduction

PKC has been implicated as a major component of cellular signalling networks regulating a variety of cell functions including cell growth and differentiation (reviewed by Nishizuka, 1988). The role of PKC in mitogenesis has received much attention ever since the discovery that the enzyme was the major receptor for tumour promoters of the phorbol ester family, cell permeable analogues of DAG. Exogenous application of phorbol esters has been reported to mimic the effect of growth factors, and downregulation of PKC by prolonged

PMA pretreatment has been shown to inhibit the mitogenic effects of a number of agonists in different cell types. While the tissue-specific distribution and distinct modes of activation suggests functional diversity of the PKC family members, information on their specific biological roles including the control of proliferation, however, is still limited as few of the physiological substrates of PKC have been identified, and of those that have, no clear difference in substrate specificity among the members has been demonstrated. Furthermore, phorbol esters are not selective in the PKC species that they activate or downregulate, potentially exerting pleiotropic effects, and the lack of specific inhibitors has prevented the dissection of responses attributable to particular isoforms. Despite this, studies in which distinct PKC isoenzymes have been overexpressed have revealed that PKCβ1 and ε exhibit transforming characteristics (Housey *et al.*, 1988). Other studies using similar approaches complimented by data with a selective pseudosubstrate peptide inhibitor indicated that the atypical PKC isoform ζ is involved in mitogenic signalling (Berra *et al.*, 1993). The precise role of PKC in regulating cellular proliferation has not been clarified. However, PKC can either directly or indirectly control the expression of certain genes by inducing transcriptional activation through TRE, serum-response element (SRE) (Hata *et al.*, 1993) and NF-κB (Hirano *et al.*, 1995) -responsive promoters in an isoenzyme-specific manner. These effects may be mediated by the integration of PKC in growth factor-initiated phosphorylation cascades, by phosphorylating and activating kinases that are intrinsic components of such pathways, including c-Raf-1 of the MAP kinase cascade (see Section 1.6.4.2) (Sozeri *et al.*, 1992, Kolch *et al.*, 1993) and elements upstream of the $p70^{s6k}$ pathway (Susa *et al.* 1992) (see Section 1.7.3.5).

1.4.3.2 Role of PLC/PLD-PKC pathway in thrombin-stimulated mitogenesis

Initial studies showing that stimulation of DNA synthesis by thrombin correlated with PI breakdown in CCL39 fibroblasts (Chambard *et al.*, 1987, Paris *et al.*, 1988) was interpreted to suggest that the PI-PLC/PKC pathway played an important role in the mitogenic action of thrombin. This notion, however was later reconsidered by the finding that growth factors activating receptor tyrosine kinases such as EGF and FGF strongly stimulated cell proliferation in the absence of PI turnover (Paris *et al.*, 1988). Subsequently, it became clear that strong activation of PI turnover is not sufficient to explain thrombin mitogenesis as studies have shown that in cell types in which thrombin is a potent mitogen, activation of

transfected muscarinic or 5-HT_2 receptors stimulated inositol phosphate generation, increased intracellular Ca^{2+} and induction of immediate early genes as strongly as thrombin, but had a negligible effect on DNA synthesis and cell proliferation (Seuwen *et al.*, 1990, Seuwen & Pouysségur, 1990).

It has been postulated that the initial burst of agonist-stimulated PI-PLC activity and DAG production that rapidly desensitizes and activates PKC transiently may not convey enough information to allow cells to re-enter the cell cycle. Prolonged DAG generation as a consequence of hydrolysis of PC (Section 1.4.2.3) by PLD and PC-PLC activities leading to the persistent activation of specific PKC isoenzymes may be necessary for regulating long-term physiological responses such as DNA synthesis and proliferation (reviewed by Asoaka *et al.*, 1992). In support of this, conditions that prevent the sustained increase in thrombin-stimulated DAG generation cause a blunting of the mitogenic response to thrombin (Rangan *et al.*, 1991). In other cell types, however, an increase in the rate of PC hydrolysis by either PLC or PLD does not appear to be sufficient on its own, nor essential to mitogenic signalling since there is no correlation between the mitogenic capability of thrombin and other agonists, with their ability to stimulate the rate of PC hydrolysis (McKenzie *et al.*, 1992). Furthermore, pretreatment with phorbol ester did not affect thrombin-stimulated DNA synthesis reinitiation in CCL39 fibroblasts (Seuwen *et al.*, 1990). Taken together these findings suggests that while PI/PC hydrolysis and PKC activation may mediate events associated with mitogenesis, they are insufficient for cell cycle progression, and that additional pathways are likely to be involved in mediating the growth-promoting effects of thrombin.

1.4.4 Thrombin-stimulated inhibition of adenylyl cyclase

In addition to phospholipases, another important membrane-bound effector generating a second messenger that plays a key role in signal transduction, is the adenylyl cyclase system which forms cyclic adenosine 3'5'-monophosphate (cAMP) from ATP. Thrombin has been shown to inhibit agonist-stimulated adenylyl cyclase activity in a number of cell types including platelets (Brass *et al.* 1991), fibroblasts (Magnaldo *et al.*, 1988, Hung *et al.*, 1992b), and vascular smooth muscle cells (Kanthou *et al.*, 1992). Thus, the elevation of intracellular cAMP caused by forskolin, which directly activates the catalytic subunit of adenylyl cyclase (Seamon & Daly, 1981) or prostaglandin E_1 (PGE_1), which activates a receptor coupled to stimulation of adenylyl cyclase, has been shown to be reduced by

thrombin in these cell types. This effect is likely to be mediate by $G_{i/o}$ as activated G_i subtype couples to inhibition of adenylyl cyclase activity and thrombin mediated inhibition of agonist-stimulated adenylyl cyclase activity is completely inhibited by PTX (Magnaldo *et al.*, 1988, Hung *et al.*, 1992b).

1.4.4.1 Role of inhibition of cAMP formation in thrombin-stimulated mitogenesis

The precise role of cAMP in the control of the proliferative response, has been the subject of a large and controversial literature. In a wide variety of cell types, an elevation of cAMP has been found to cause inhibition of growth, while in specific cell types, such as Swiss 3T3 cells and thyrocytes, it appears to have the opposite effect, conferring a positive mitogenic signal. In fibroblasts (Magnaldo *et al.*, 1989) and vascular smooth muscle cells (Kanthou *et al.*, 1996), re-initiation of DNA synthesis by thrombin is strongly inhibited by agents that elevate cAMP. It was initially envisioned that a chronic lowering of intracellular cAMP following G_i-dependent inhibition of adenylyl cyclase by thrombin may be of great importance for the mitogenic response (Magnaldo *et al.*, 1988) and explain the PTX-sensitivity of thrombin-stimulated mitogenesis (see Section 1.4.1.1). However, the effects of thrombin on cellular cAMP levels have been investigated in cells in which adenylyl cyclase has been stimulated, and variations of basal cAMP levels in thrombin-stimulated cells are very small. As such, it is doubtful whether PTX-sensitive inhibition of adenylyl cyclase provides a *bona fide* mitogenic signal.

1.4.5 Tyrosine phosphorylation of cellular proteins

The exact role of alterations in second messenger levels by PLC/PLD and adenylyl cyclase systems in thrombin-stimulated mitogenesis remains unclear, but what is apparent from early studies is that additional signalling events activated in parallel are likely to be important. Thrombin has been shown to cause rapid phosphorylation of several cellular proteins on tyrosine residues in platelets (Ferrell & Martin, 1988, Golden & Brugge, 1989), fibroblasts (Kohno & Pouysségur, 1986). and vascular smooth muscle cells (Weiss & Nuccitelli, 1992, Molloy *et al.*, 1996). In most cases, the identity of these substrates and the significance of their phosphorylation to the functional responses of thrombin is unclear. However, protein tyrosine kinase activity has been implicated as a critical biochemical event

in the mitogenic signal transduction cascade evoked by thrombin as the protein tyrosine kinase inhibitor herbimycin A has been shown to inhibit thrombin-induced DNA synthesis in rat aortic smooth muscle cells (Weiss & Nuccitelli, 1992). In addition, this agent did not affect thrombin-dependent intracellular Ca^{2+} release, further demonstrating that pathways distinct from PI-PLC-mediated phosphoinositide hydrolysis are involved in thrombin-induced mitogenesis.

1.5 OVERVIEW OF PROTEIN TYROSINE PHOSPHORYLATION CASCADES INVOLVED IN GROWTH

Protein phosphorylation and dephosphorylation mediated by protein kinases and phosphatases, respectively, has emerged in recent years as a major mechanism for propagating signals generated by extracellular stimuli to the nucleus or other cellular compartments in mammalian cells. It is now recognized that receptors for polypeptide growth factors, which belong to a family of receptors distinct from the G-protein-coupled receptors, possess intrinsic tyrosine kinase activity and that phosphorylation of proteins on tyrosine residues is believed to be a major signal transduction mechanism in cell growth stimulation by such agonists. Most of the proteins that are downstream targets in these pathways are themselves regulated by phosphorylation, not on tyrosine but on serine and/or threonine residues. In recent years, it has become apparent that many of the signalling pathways activated by G-protein-coupled receptor agonists, such as thrombin, are, in part, convergent with those activated by growth factors. This may explain the appearance of phosphotyrosyl proteins in extracts of thrombin treated cells, and the requirement for these proteins in thrombin-stimulated mitogenesis (see Section 1.4.5). The following section describes the mechanism by which growth factor receptors initiate the activation of signalling molecules to allow subsequent comparison with G-protein-linked effector mechanisms.

1.5.1 Activation of Growth factor receptors and binding to SH2 domain-containing signalling molecules

The archetypal growth factor receptor is characterized by a large extracellular ligand-binding binding domain, a single hydrophobic transmembrane section and a cytoplasmic carboxyl-terminal tail which contains a catalytic domain for intrinsic tyrosine kinase activity

(Fantl *et al.*, 1993). Following binding of the growth factor to its cognate receptor, tyrosine kinase receptors undergo dimerization leading to the activation of their intrinsic tyrosine kinase activity (Fantl *et al.*, 1993, Kazlauskas & Cooper, 1989). Autophosphorylation of specific tyrosine residues in the cytoplasmic domain of growth factor receptors creates docking sites for high affinity interaction with a number of target proteins or enzymes, including PI-PLCγ (Meisenhelder *et al.*, 1989), the 85 kDa subunit of phosphatidylinositol (PI) 3-kinase (see Section 1.6.5.2)(Kazlauskas & Cooper, 1989), GTPase activating protein (GAP) (Kaplan *et al.*, 1990), the adaptor protein Grb2 (see Section 1.6.5.1), phosphotyrosine phosphatase SHPTP-2 (Lechleider *et al.*, 1993), and members of the non-receptor Src family of tyrosine kinases (Kypta *et al.*, 1990). A common attribute of all of these regulatory proteins is the possession of a recognition sequence of approximately 100 amino acids that share considerable sequence similarity to the non-catalytic region of the src family of protein tyrosine kinases (Pawson & Gish, 1992) and termed Src homology-2 (SH-2) domains. These SH2 domains are structural features required to enable these effector proteins to interact specifically with defined phosphotyrosine residues on the activated receptor, and in doing so, become activated by either tyrosine phosphorylation, conformational change or translocation to the plasma membrane. The signal is further propagated and amplified by cascades of cytosolic protein kinases, which ultimately activate nuclear transcription factors in order to initiate metabolic processes necessary for growth.

1.6 THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

At the same time as researchers were studying interactions between effectors and activated tyrosine kinases receptors, biochemical and genetic approaches were being conducted to elucidate downstream events. These studies revealed that growth factors employed sequential protein kinase reactions to regulate cellular functions such as metabolic and transcriptional events. Most strikingly, this mechanism of signal transduction was well conserved in diverse responses to extracellular stimuli in evolutionary distinct organisms as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Xenopus laevis*. One pathway that has been identified as playing an important role in mitogenesis in mammals shows a remarkable degree of homology with the protein kinase pathway that links the pheromone receptor with sexual differentiation in budding yeast, and is referred to as the mitogen-activated protein (MAP) kinase cascade.

1.6.1 p42/p44 MAP kinase

Tyrosine phosphorylation of proteins in the molecular weight range of 40-45 kDa has long been recognized as a characteristic of quiescent mammalian cells stimulated by a number of diverse mitogens to re-enter the cell cycle. It became apparent that one of the most widely studied of these proteins, pp42, was likely to be identical to a novel serine/threonine kinase identified in insulin-stimulated 3T3-L1 cells shown to phosphorylate microtubule-associated protein 2 as well as *Xenopus* ribosomal protein S6 kinase II *in vitro* (Ray & Sturgill, 1987) and dubbed mitogen-activated protein (MAP) kinase (Rossomando *et al.*, 1989). Two forms of MAP kinase have been purified from fibroblasts with apparent molecular weights of 42 and 44 kDa and referred to as p42 and p44. Subsequently, cDNAs encoding highly related MAP kinases corresponding to p42 and p44, and a third isoform of 62 kDa have been isolated from the rat (Boulton *et al.*, 1990, 1991) and named extracellular signal-related kinase ERK 2, 1 and 3, respectively, to reflect the diversity of signals that can regulate them. Human MAP kinases that are similar to these rat isoforms have also been identified by molecular cloning (Gonzalez *et al.*, 1992). The identification of additional MAP kinase members (including JNK and p38 MAP kinase homologues described in Section 1.8.1) implies that family of kinases is extensive.

It is now apparent that the p42/p44 members of the MAP kinase family of serine/threonine-specific protein kinases become activated very early in response to a wide range of extracellular stimuli. These include growth factors that activate receptor tyrosine kinases, G-protein-coupled receptor agonists including thrombin (see Section 1.6.1.1), phorbol esters, cytokines and proto-oncogene products (reviewed by Blenis, 1993). The p42/p44 MAP kinases have been proposed to play a key role in integrating and transmitting extracellular signals to cytoplasmic and nuclear targets that regulate cellular responses including proliferation or differentiation.

1.6.1.1 Activation of p42/p44 MAP kinases by thrombin

In CCL39 cells, the 42 and 44 kDa MAP kinases have been identified (L'Allemain *et al.*, 1991a) as being identical to two previously described major proteins found phosphorylated on tyrosine in response to thrombin and growth factors (Koyno & Pouysségur,

1986). Tyrosine phosphorylation of p42 MAP kinase by thrombin corresponded to enzymatic activation (L'Allemain *et al.*, 1991a). Subsequently, activation of p42 and p44 MAP kinases by thrombin has been demonstrated in a number of other cell types including vascular smooth muscle cells (Malarkey *et al.*, 1996, Molloy *et al.*, 1996), platelets (Papkoff *et al.*, 1994) and astrocytes (Bhat *et al.*, 1995), but is is in fibroblasts that this effect has been most well characterized.

The kinetics of thrombin stimulated MAP kinase activation are biphasic consisting of an initial transient activation that is maximal after 5 minutes, followed by sustained activation lasting up to 4 hours (Meloche *et al.*, 1992). The two phases are believed to be mediated by different signalling pathways. In CCL39 fibroblasts, the initial phase can be inhibited by pretreatment of cells with high concentrations of phorbol ester, which almost totally down-regulates immunodetectable PKC, and can be mimicked by phobol ester, consistent with a role for PKC as the major transducer of the early signal (Kahan *et al.*, 1992). The late phase of activation is completely inhibited by PTX (Meloche *et al.*, 1992), although the mechanism which accounts for this is not known. Lowering of cAMP by G_i-dependent inhibition of adenylyl cyclase is not involved as in this cell type, thrombin-stimulated MAP kinase activation is not affected by parallel treatment of cells with PGE_1 (Kahan *et al.*, 1992). The second and sustained late phase of p44 MAP kinase activation correlates with mitogenicity since early removal of thrombin with the specific inhibitor hirudin, prevents DNA synthesis and eliminates the second phase of the p44 MAP kinase response (Meloche *et al.*, 1992). Furthermore, carbachol, which has no mitogenic potential, stimulates the first but not the second peak in the same cells expressing the m1 muscarinic receptor (Kahan *et al.*, 1992) and PTX, which inhibits thrombin-induced DNA synthesis, completely abolishes the late phase of p44 MAP kinase activation (Meloche *et al.*, 1992). Activation of both isoforms of MAP kinase by thrombin, as with other potent mitogens, causes their rapid translocation to the nucleus, suggesting that these protein kinases participate in orchestrating gene expression (Lenormand *et al.*, 1995). An essential role for MAP kinases in thrombin-stimulated cell cycle re-entry in fibroblasts has been indicated by the finding that inhibition of both MAP kinases by expression of either p44 MAP kinase antisense cDNA or dominant-negative allele prevents G0 to S-phase transition (Pagès *et al.*, 1993).

1.6.1.2 Cellular targets of p42 and p44 MAP kinase

Downstream of MAP kinase, several proteins thought to play important regulatory roles in cellular function including other protein kinases, lipases and transcription factors have been identified as substrates (reviewed by Davis, 1993). MAP kinase can phosphorylate and partially reactivates the dephosphorylated 90 kDa ribosomal protein S6 kinase II ($p90^{rsk}$) (Sturgill *et al.*, 1988). This kinase is a physiological target as insulin stimulation of $p90^{rsk}$ activity in 3T3 LI adipocytes is inhibited by MAP kinase oligonucleotide antisense (Sale *et al.*, 1995). It was initially thought that $p90^{rsk}$ was responsible for phosphorylating ribosomal protein S6 *in vivo*, although it now appears this function is likely to be served by another S6 kinase (see Section 1.7.1). Nevertheless, $p90^{rsk}$ has other functions in cell regulation including roles in glycogen synthesis (Sutherland *et al.*, 1993) and in transcriptional control (Chen *et al.*, 1996, see below). MAP kinase can also directly regulate gene expression by phosphorylating the ternary complex transcription factor Elk-1/$p62^{TCF}$(Gille *et al.*, 1992). Elk-1/$p62^{TCF}$ phosphorylation facilitates the formation of a ternary complex composed of itself and the serum response factor (SRF) (which itself can be phophorylated by $p90^{rsk}$) at the serum-response element (SRE) in the promoter of many genes, including the proto-oncogene c-*fos* (reviewed by Treisman, 1992). This complex mediates transcription of c-*fos*, one of the immediate early genes induced by many stimuli, including thrombin (see Section 1.3.2). In addition, both MAP kinase and $p90^{rsk}$ also phosphorylate the C-terminus of c-Fos proteins *in vitro* at major *in vivo* phosphorylation sites, leading to increased c-Fos stability and AP-1 activity (Chen *et al.*, 1996) (see Section 1.3.2), indicating that the MAP kinase/$p90^{rsk}$ pathway may modulate early genetic changes in response to mitogens at multiple levels. MAP kinase also phosphorylates and activates the cytosolic phospholipase A_2 (PLA_2) *in vitro* and in intact cells (Lin *et al.*, 1993). This enzyme catalyses the release of arachidonic acid from the *sn*-2 position of phospholipids, the rate-limiting precursor for the synthesis of prostaglandins, thromboxane and leukotrienes, key mediators controlling a number of cellular processes. A role for PLA_2 as a substrate of MAP kinase *in vivo* is supported by the finding that expression of a recombinant $cPLA_2$ in which the MAP kinase phosphorylation site Ser^{505} is mutated, does not become activated on agonist-stimulation (Lin *et al.*, 1993). While a number of other substrates for MAP kinase have been identified *in vitro* (Davis, 1993), few of these have been shown to be unequivocal physiological targets for the enzyme.

1.6.2 Regulation of p42/p44 MAP kinase by the dual specificity kinase MEK

Activation of MAP kinase requires phosphorylation of both tyrosine and threonine residues (Anderson *et al.*, 1990). The regulatory phosphorylation sites in mammalian MAP kinase have been identified as Thr^{183} and Tyr^{185} found within a TEY motif in the kinase subdomain VII and VIII (Payne *et al.*, 1991). It was originally thought that both of these phosphorylations required the integration of two protein kinases from distinct signalling pathways (Anderson *et al.*, 1990) or a protein kinase that enhances the rate of autophosphorylation (Ahn *et al.*, 1991) for activation of the kinase. It is now clear, however, that MAP kinase becomes activated by a single protein kinase which is unusual in exhibiting dual specificity and capable of phosphorylating both the regulatory threonine and tyrosine residues. This enzyme was identified initially as a MAP kinase activator purified from growth factor treated cells and tissue based on its ability to reactivate MAP kinase that had been inactivated with both tyrosine and serine/threonine phosphatases, or activate purified recombinant wild-type MAP kinase *in vitro* (Gomez & Cohen, 1991, Nakielny *et al.*, 1992) and was termed MAP kinase kinase or MEK (MAP kinase or ERK kinase). Subsequent molecular cloning of MEK homologues in the mouse (Crews *et al.*, 1992), rat (Wu *et al.*, 1993b) and human (Seger *et al.*, 1992) revealed that MEK constitutes a multiple enzyme family with sequence homology to the byr1 and STE7 gene products of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively that exhibited biochemical properties identical to the purified enzymatic activities. In addition to MEK1 a second cDNA encoding a closely related MAP kinase activator has been identified in humans and designated MEK2 (Zheng & Guan, 1993a). Overexpression of MEK in cultured cells increased MAP kinase activity (Seger *et al.*, 1992). While several laboratories have recently uncovered additional MEKs, these appear to function as dual-specificity kinases that selectively regulate defined members of the stress-activated family of MAP kinase homologues (see Section 1.8.2). The unique dual phosphorylation motifs each of these MAP kinases imparts exclusive selectivity for their activation by specific MEKs such that MEK1 and MEK2 are the only identified activators of p42/p44 MAP kinase.

1.6.3.1 Regulation of MEK by Raf-1

c-Raf-1, an ubiquitously expressed 74 kDa serine/threonine protein kinase encoded by the cellular homologue of the v-Raf oncogene, the transforming gene of murine sarcoma virus (Morrison, *et al.*, 1988) has long been recognized to be involved in mitogenic signalling. Many growth factors and oncogenes stimulate the phosphorylation and activation of c-Raf-1 (Kyriakis *et al.*, 1992). When introduced into cells as a constitutively activated form, Raf-1 can induce gene transcription, DNA synthesis and transformation (Rapp, 1991). Conversely, inhibition by Raf-1 antisense, or by expressing dominant negative mutants, suppresses serum-induced proliferative responses in NIH3T3 cells (Kolch *et al.*, 1991). Two related mammalian gene products, A-raf and B-raf have now been identified (Huleihel *et al.*, 1986, Ikawa, *et al.*, 1988) and show tissue-specific distribution (Storm *et al.*, 1990). A unique characteristic of the Raf kinases is the presence of three conserved domains (CR1, CR2 and CR3); the N-terminal CR1 region is rich in serine residues and contains a putative zinc binding region which is important for association with GTP-Ras (Section 1.6.4.1); CR2 is a region rich in serine and threonine residues; the C-terminal CR3 contains the kinase domain. The kinase activity of the C-terminal catalytic domain is regulated by the amino terminal CR1 and CR2 domains, and thus a truncated mutant of Raf-1 in which CR1 and CR2 has been removed is constitutively active (Heidecker *et al.*, 1990).

A substantial body of both genetic and biochemical evidence has shown that c-Raf-1 is at least one of the intermediates directly upstream of MEK in a linear cascade leading to activation of MAP kinase. Cells transformed with v-Raf, or overexpression of oncogenic mutants of c-Raf-1, display elevated MEK and MAP kinase activities (Dent *et al.*, 1992, Howe *et al.*, 1992, Kyriakis *et al.*, 1992). Furthermore, c-Raf-1 immunoprecipitated from mitogen-stimulated cells can directly phosphorylate and activate MEK *in vitro* (Dent *et al.*, 1992, Howe *et al.*, 1992, Kyriakis *et al.*, 1992). Other biochemical studies have demonstrated that Raf-1 forms a stable complex with MEK1 *in vivo* and *in vitro* (Huang *et al.*, 1993) and phosphorylates MEK1 exclusively on serine residues (Yan & Templeton, 1994, Zheng & Guan, 1994). Phosphopeptide mapping has identified Ser^{218} and Ser^{222} as the primary regulatory residues of MEK and phosphorylation of these sites is necessary and sufficient for activation of MEK by c-Raf-1 (Yan & Templeton, 1994, Zheng & Guan, 1994). Analogous to the activating region of MAP kinase, these two phosphorylation sites lie within an activation domain of MEK1 between kinase domains VII and VIII that is highly conserved in other

members of the mammalian MEK family kinases (see Section 1.8.2). In support of Ser^{218} and Ser^{222} as sites of activating phosphorylation, MEK mutants in which these residues are substituted by alanine are unable to become activated by Raf-1 (Yan & Templeton, 1994). In addition, substitution with negatively charged glutamic acid residues results in a constitutively active allele (Yan & Templeton, 1994). The latter mutants have been shown to promote cellular transformation of NIH3T3 fibroblasts (Cowley *et al.*, 1994, Mansour *et al.*, 1994) supporting a role for MEK and MAP kinase in the regulation of growth responses.

1.6.3.2 Raf-1 -independent regulation of MEK

A number of studies from several laboratories using cells transfected with both constitutively active and dominant negative c-Raf-1 mutants (Chao *et al.*, 1994, Porras *et al.*, 1994) or expressing c-Raf-1 antisense RNA (Kizaka-Kondoh & Okayama, 1993) have provided evidence that c-Raf-1 may not be involved in the MAP kinase pathway under many circumstances. Subsequently, it has been demonstrated that other serine/threonine kinases can serve as direct regulators of MEK. These include other members of the Raf family of proto-oncogene products. Biochemical approaches have identified B-Raf as an activator of MEK in neuronal tissue (Catling *et al.*, 1994) and NIH3T3 fibroblasts, together with two other identified kinases (Reuter *et al.*, 1995). A-Raf from agonist-stimulated Hela cells has been shown to selectively phosphorylate and activate MEK1 but not MEK2 (Wu *et al.*, 1996). Expression of A-Raf and B-Raf, however, is restricted to certain tissues (Storm *et al.*, 1990) and therefore roles for these Raf family members may be cell type-specific.

Apart from Raf family members, other kinase that activate MEK have been described. A MEK1 kinase (MEKK1) isolated as the mammalian homologue of *Saccharomyces cerivisiae* STE11 protein and *Schizosaccharomyces pombe* Byr2 protein has been shown to activate MEK in Rat1a, NIH3T3 and PC12 cells in a c-Raf-1-independent manner (Lange-Carter *et al.*, 1993) by phosphorylating MEK on the same serine residues that are phosphorylated by c-Raf-1 (Yan & Templeton, 1994). Originally it was thought that MEKK may be responsible for MEK activation by heterotrimeric G-protein coupled receptor agonists, such as thrombin. However, recent evidence suggests that this kinase is more likely to act upstream of another MEK family member responsible for activating the MAP kinase homologue c-Jun N-terminal kinase in the parallel stress-activated protein kinase pathway (see Section 1.8.3) (Minden *et al.*, 1994 Yan *et al.*, 1994). In addition to different forms of Raf

and MEKK, the proto-oncogene c-Mos, a germ cell specific serine/threonine kinase, has been shown to be another MEK kinase in extracts from *Xenopus* oocytes (Posada *et al.*, 1993). Although proteins immunologically related to c-Mos have been detected in somatic cells (Reuter *et al.*, 1995), there is no evidence as yet to indicate that they function as MEK activators in these cell types. The existence of more than one MEK kinase provides alternative pathways by which different agonists in specific cell types may converge on MEK to cause a generic, non-specific activation of MAP kinase, while potentially allowing the selective regulation of other protein substrates in the cell.

1.6.4.1 Role of Ras in Raf-1 activation

Several lines of evidence indicate that the proto-oncogenic product Ras, a member of the family of monomeric low molecular weight guanine nucleotide-binding proteins, plays a central role in signal transduction pathways involved in a number of cellular processes including cell growth (Mulcahy *et al.*, 1985, Cai *et al.*, 1990). Ras becomes activated on transition from a GDP-bound inactive state to the active GTP-bound conformation. Exchange of GTP is stimulated by treatment of cells with mitogens, and is switched off by hydrolysis of bound GTP to GDP catalysed by the intrinsic GTPase activity of the G protein. A number of studies have placed Ras upstream of c-Raf-1 in the MAP kinase cascade since expression of oncogenic Ras activates, and dominant negative Ras mutants prevent, the activation of Raf-1 (Troppmair *et al.*, 1992, Wood *et al.*, 1992, Porras *et al.*, 1994) and MAP kinase (de Vries-Smidt *et al.*, 1992, Wood *et al.*, 1992) in response to growth factors. Conversely, Raf-1 has been shown to play an important role in Ras function since dominant negative mutants of Raf or Raf antisense expression can block Ras-mediated cell growth and transformation (Kolch *et al.*, 1991, Bruder *et al.*, 1992). The mechanism by which Ras participates in Raf-1 activation is unclear but an initial step is believed to be the formation of a high-affinity complex between the N-terminal regulatory domain of Raf-1 with the effector domain of active GTP-bound Ras (Moodie *et al.*, 1993, Warne *et al.*, 1993, Zhang *et al.*, 1993b). The binding of Ras-GTP does not appear to be sufficient to activate Raf-1 directly as addition of Ras GTP to recombinant inactive c-Raf-1 *in vitro* does not alter c-Raf-1 kinase activity (Zhang *et al.*, 1993b, Leevers *et al.*, 1994). It is now believed that *in vivo*, the primary function of GTP-bound Ras is to cause the translocation of c-Raf-1 kinase to the plasma membrane where additional protein-protein or protein-lipid interactions regulate Raf-1 activity (see Sections 1.6.4.2 & 1.6.4.3).

Support for this model is provided by the demonstration that c-Raf-1 artificially directed to the plasma membrane by fusion of membrane-targetting sequences onto the Raf-1 carboxyl terminus bypasses the need for Ras in Raf activation (Leevers *et al.*, 1994, Stokoe *et al.*, 1994).

1.6.4.2 Role of protein kinases in Raf-1 activation

The processes which regulate enzymatic activation of Raf-1 localized to the membrane by GTP-Ras is complex and still not completely understood. Most studies have focused on the role of protein kinases as in many systems, activation of c-Raf-1 is accompanied by multisite phosphorylation. The serines Ser^{43}, Ser^{259} and Ser^{621} found in the amino terminus of the kinase domain appear to be important residues in regulating Raf-1 activity since enhancement of Raf-1 activity in response to mitogens correlates with increases in phosphorylation at these sites, and mutation of Ser^{259} and Ser^{621} can alter the activity of Raf-1 (Morrison *et al.*, 1993). Tyrosine phosphorylation of Raf-1 has also been detected to varying extents depending on the cell type and stimulus. The *in vivo* sites for this modification have been identified as Tyr^{340} and Tyr^{341} located in the Raf-1 kinase domain which mutational analysis has also revealed can dramatically alter the function of Raf-1 (Fabian *et al.*, 1993). B-Raf differs from Raf-1 and A-Raf in that negatively charged residues are found at these positions indicating that the mechanism of the regulation of this form may not be regulated by tyrosine phosphorylation. The identity of the Raf-1 protein kinase(s) that catalyse phosphorylations both on serine/threonine and tyrosine residues *in vivo* is uncertain. Evidence for an important role for PKC in the Raf-1 activation is available in some systems; phorbol esters can induce c-Raf-1 serine phosphorylation and activation and downregulation of PKC prevents activation of Raf-1 (Morrison *et al.*, 1988). It has also been shown that certain PKC isotypes can directly phosphorylate Raf-1 *in vitro*, although different reports are conflicting as to whether this results in Raf-1 activation (Sozeri *et al.*, 1992, Kolch *et al.*, 1993, MacDonald *et al.*, 1993).

c-Raf-1 has also been shown to be negatively regulated following phosphorylation by cyclic AMP-dependent protein kinase A (PKA) *in vitro* (Wu *et al.*, 1993a). This effect is believed to be caused by PKA-mediated phosphorylation of Ser^{43} within the c-Raf-1 N-terminal regulatory domain which substantially reduces Ras-GTP binding (Wu *et al.*, 1993a) possibly by folding a polypeptide flap consisting of residues 1 to 50 over the Ras docking site

(Chuang *et al.*, 1994). Inhibition of Raf-1 by PKA represents one of a number of inactivation mechanisms for the MAP kinase pathway and provides one possible explanation for the observation that elevated cAMP interferes with the ability of growth factors to stimulate growth in many cell types.

1.6.4.3 Role of phospholipids and 14. 3. 3 ζ polypeptides in Raf-1 activation

Other investigators have proposed that as well as phosphorylation events by kinases, the activity of Ras-bound Raf-1 is regulated by binding to one or more putative activator molecules including proteins and plasma membrane components. One member of a highly conserved class of acidic polypeptides which modulate the activity of a number of enzymes *in vitro*, termed 14.3.3. ζ, has been shown to associate with the N-terminal portion of Raf-1 (Fantl *et al.*, 1994) at a site distinct from the Ras-GTP binding site (Luo *et al.*, 1995). While in some observations, this interaction dramatically increases Ras-dependent Raf activity (Fantl *et al.*, 1994), other studies have shown that 14.3.3 ζ does not activate Raf-1 but participates in the process of Raf-1 activation by mechanisms that remain to be elucidated (Luo *et al.*, 1995).

In addition to regulatory peptides, other studies have shown that phospholipids may play a role in Raf-1 activation. In specific cell types, hydrolysis of PC by PC-PLC acts upstream of Raf to link Ras to Raf-1 activation during mitogenic stimulation (Cai *et al.*, 1993) although the nature of this coupling has not been established. Raf-1 has also been shown to be capable of binding via the zinc-co-ordinating cysteine-rich region in the N-terminus to phosphatidylserine-containing liposomes *in vitro* (Ghosh *et al.*, 1994). Furthermore, very recent evidence indicates that Raf-1 associates with phosphatidic acid by a distinct binding site which deletion mutagenesis studies have located to a carboxy terminus of the protein (Ghosh *et al.*, 1996). The exact role of phospholipid interaction in Raf-1 activation is currently being researched, but this finding indicates that besides GTP-Ras, PA and phosphatidylserine may facilitate the translocation and stabilization of Raf-1 at the plasma membrane.

1.6.5.1 Regulation of Ras by tyrosine kinase receptors

Elucidation of the molecular mechanisms involved in coupling activated tyrosine kinase receptors to the activation of Ras have been aided largely by studies on protein-protein interactions between intermediates of homologous signalling systems in *Drosophila* and *C. elegans*.

The activation status of Ras is determined by the ratio of bound GTP to GDP which can be regulated either by controlling the rate of nucleotide exchange or by regulating the rate of GTP hydrolysis. Accessory proteins have been described which modulate both of these parameters. A Ras-specific GTPase-activating protein (GAP) promotes inactivation of $p21^{ras}$ by stimulating the intrinsic GTPase activity of Ras (Trahey & McCormick, 1987, Li *et al.*, 1992). Alternatively, a guanine nucleotide exchange factor called mSos, the mammalian equivalent of the *Drosophila* Son-of-Sevenless protein, mediates activation of $p21^{ras}$ by exchanging bound GDP for GTP (Egan *et al.*, 1993, Medema *et al.*, 1993). For a number of agonists, GTP loading on Ras by mSos has been shown to be more important in Ras activation than inhibition of the GTPase turn-off mechanism involving Ras GAP (Medema *et al.*, 1993).

Lacking in SH2 domains, mSos requires SH2 -domain-containing adaptor proteins to link to activated tyrosine kinase receptors. One such connector molecule is growth factor receptor binding protein-2 (Grb2), to which mSos is constitutively bound in the basal state. This interaction is mediated by two Src homology 3 (SH3) domains of Grb2 which bind directly to a proline-rich region in the C-terminal region of mSos (Batzer *et al.*, 1993, Egan *et al.*, 1993, Rozakis-Adcock *et al.*, 1993). In some instances, direct binding of the Grb2 SH2 domain to activated tyrosine kinase receptor may be sufficient in targetting mSos to the plasma membrane where activation of Ras occurs. However, recent evidence indicates that for many agonists such as EGF and insulin in a number of systems, an intermediate signalling molecule called Shc is involved (Rozakis-Adcock *et al.*, 1993). Shc exists as 48, 52, and 65 kDa forms composed of a single SH2 domain, a glycine-rich collagen homology domain (CH) (Pelicci *et al.*, 1992) and a novel phosphotyrosine interaction (PI) domain (Dikic *et al.*, 1995). On binding via its SH2 domains to the tyrosine phosphorylated cytoplasmic domains of activated tyrosine kinase receptors (Pronk *et al.*, 1994), Shc becomes tyrosine phosphorylated on a residue within a consensus binding motif for the Grb2 SH2 domain, resulting in the binding of the Grb2/mSos to form a trimeric complex (Egan *et al.*, 1993, Rozakis-Adcock *et*

al., 1993). Recruitment of Grb2 and mSos to the membrane by receptor-bound Shc is believed to be responsible for Ras activation (Pronk *et al.*, 1994).

1.6.5.2 Regulation of Ras by G-protein coupled receptors

It is now apparent that as well as growth factors acting on receptor tyrosine kinases, the Ras-MAP kinase pathway may represent a general effector route utilized by certain G-protein-coupled receptors, including the thrombin, LPA, m2 muscarinic and α_2-adrenergic receptors (Alblas *et al.*, 1993, van Corven *et al.*, 1993, Winitz *et al.*, 1993). Thrombin has been shown to stimulate $p21^{ras}$ in 1321N1 astrocytoma cells (LaMorte *et al.*, 1993), CCL39 hamster lung fibroblasts (Van Corven *et al.*, 1993) and vascular smooth muscle cells (Irani *et al.*, 1994) as determined by the increase in the proportion of Ras in the GTP-bound state. Furthermore, an obligatory role for Ras protein function in mitogenic signalling by thrombin has been suggested by the findings that expression of a dominant negative Asn^{17} Ha-Ras mutant abolishes thrombin-stimulated gene induction and DNA synthesis in astrocytoma cells (LaMorte *et al.*, 1993) and proliferation of vascular smooth muscle cells (Irani *et al.*, 1994). This inhibition is supported by similar findings obtained by microinjection of an inhibitory antibody to Ras (LaMorte *et al.*, 1993).

While the events linking tyrosine kinase receptors to activation of Ras has been unravelled, much less is known about how Ras, and subsequently other components of the MAP kinase cascade, become activated by G-protein-coupled receptors. In fibroblasts, Ras activation by thrombin and TRAP is abolished by pertussis toxin, indicative of regulation by a heterotrimeric G protein of the G_i subfamily (van Corven *et al.*, 1993). In support of an involvement of this class of G protein, similar effects have been reported following activation of other G_i-linked receptors such as the LPA receptor (van Corven *et al.*, 1993), m2 acetylcholine muscarinic receptor (Winitz *et al.*, 1993) and α_2-adrenergic receptor (Alblas *et al.*, 1993). Furthermore, G_i-mediated regulation of Ras by thrombin is consistent with the PTX sensitivity of thrombin-stimulated signalling events believed to be downstream of Ras such as MAP kinase activation (Meloche *et al.*, 1992, Gupta *et al.*, 1992) (see Section 1.6.1.1).

The primary G-protein subunit responsible for G_i-mediated Ras and/or MAP kinase activation is currently unclear; both α and $\beta\gamma$ subunits may be involved. In certain cell types, the α_i subunit may be important in pertussis toxin-sensitive regulation of the MAP kinase

cascade since expression of a GTPase-deficient $G_{i\alpha}$ subunit (Gip2) induces constitutive activation of MEK and MAP kinase in Rat 1a cells (Gupta *et al.*, 1992, Gardener *et al.* 1993). In other cell types where MAP kinase activity is not enhanced by mutant $G_{\alpha i}$, a role for $\beta\gamma$ subunits in the activation of Ras has been reported. In a similar way that the $\beta\gamma$ subunits of heterotrimeric G proteins are able to activate certain PLC and adenylyl cyclase isoforms (see Section 1.4.2.2), overexpression of $\beta\gamma$ subunits is able to stimulate Ras (Crespo *et al.*, 1994) and MAP kinase (Crespo *et al.*, 1994, Faure *et al.*, 1994) activities in Cos-7 cells. Furthermore, both G_i-dependent Ras and MAP kinase activity stimulated by LPA, another G protein coupled agonist, has been shown to be inhibited by the overexpression of the carboxyl terminal portion of the β-adrenergic receptor kinase 1 (βARK), which sequesters $\beta\gamma$ subunits released by activated G-proteins (Koch *et al.*, 1994). The mechanism by which $\beta\gamma$ heterodimers stimulate Ras is not clear. However, a recent finding has shown that βARK binds free $\beta\gamma$ heterodimers through a region encompassing a plekstrin homology (PH) domain. As the majority of the proteins which regulate Ras activity, such as Ras GAP and mSos also contain PH domains, $\beta\gamma$ may regulate Ras by binding to these PH-containing Ras-regulatory proteins.

Activation of Ras by thrombin and LPA has also been shown to be inhibited by protein tyrosine kinase inhibitors (Van Corven *et al.*, 1994, Hordijk *et al.*, 1994) indicating that additional protein tyrosine kinase intermediates may be required. However, this effect may be agonist and cell type -specific as muscarinic m2 receptor-mediated Ras activation is insensitive to tyrosine kinase inhibition (Winitz *et al.*, 1993). In some cells, it is conceivable that $\beta\gamma$ heterodimers may activate a non-receptor tyrosine kinase of the Src family that resembles an activated growth factor receptor, regulating Ras activation via an adaptor molecule such as the Shc proteins which on tyrosine phosphorylation can complex with Grb2 and Sos (Section 1.6.5.1). In support of this possibility, thrombin has been shown to activate $p60^{src}$ and the family member $p59^{fyn}$ in a pertussis toxin sensitive and insensitive manner (Chen *et al.*, 1994) and stimulate sustained tyrosine phosphorylation of $p46^{Shc}$ and $p52^{Shc}$ as well as formation of Shc-Grb2 complexes (Chen *et al.*, 1996) in hamster lung fibroblasts. Furthermore a Shc mutant unable to complex with Grb2 blocks thrombin-receptor-mediated events downstream of Ras such activation of MAP kinase and cell proliferation (Chen *et al.*, 1996).

1.6.6 Ras-independent activation of MAP kinase

For both activators of receptor tyrosine kinases and G-protein-coupled receptor agonists, activation of MAP kinase has also been shown to occur in a Ras-independent manner, indicating the existence of at least two distinct pathways regulating MAP kinase that can operate in one cell type (de Vries-Smits *et al.*, 1992, Burgering *et al.*, 1993a). In many cases this route appears to be mediated by PKC. It is well established that phorbol esters stimulate MAP kinase activation. Furthermore, depletion of PKC by prolonged pretreatment with phorbol ester or PKC inhibitors has been shown to block to varying extents the activation of MAP kinase following stimulation with a number of agonists (de Vries-Smits *et al.*, 1992, Burgering *et al.*, 1993a, Malarkey *et al.*, 1996). For G-protein coupled receptor agonists that couple to both G_i and G_q, PKC-dependent activation of MAP kinase is likely to occur via a G_q/PLC pathway (reviewed by Blumer & Johnson, 1994) and in many cases may represent the component of the signal that is often PTX-insensitive. In support of this notion, expression of mutationally activated G protein α_q subunits or PLC-β2 modestly activates MAP kinase in COS-7 cells (Faure *et al.*, 1994). PKC is thought to activate the MAP kinase pathway at, or above, the level of Raf-1 since chronic phorbol ester pretreatment prevents agonist-stimulated c-Raf-1 activation. This may occur by direct phosphorylation and activation of Raf-1 by certain PKC isoforms (Sozeri *et al.*, 1992, Kolch *et al.*, 1993, and Section 1.6.4.2). Other authors have proposed, however, that MEKK1 might mediate signals originating from receptors that activate G-proteins and PKC (Lange-Carter *et al.*, 1993).

1.7 THE p70 RIBOSOMAL S6 KINASE /PI 3-KINASE PATHWAY

One of the early obligatory steps in cell growth and proliferation in response to mitogens is the activation and maintenance of high rates of protein synthesis. This process is regulated primarily at the level of translation initiation, a complex process involving several components of the translational machinary, including initiation factors, tRNA and mRNA and the ribosomal subunits. One event recognized for a long time as being important in facilitating the initiation of translation is the multiple phosphorylation of the S6 protein of 40S ribosomal subunits, a region implicated in mRNA binding and thought to reside near the tRNA acceptor site. Phosphorylation at five serine residues in close proximity to each other at the carboxyl terminus of S6 protein correlates with an increased rate of protein synthesis during G1 thought

to be required for entry into S phase (reviewed by Ferrari & Thomas, 1994) and is a highly conserved response of cells on exposure to growth factors and agents which promote cell division.

1.7.1 The p70 ribosomal S6 kinase

Much work has been directed toward identification of the enzyme(s) controlling S6 phosphorylation and elucidating its (their) mode of regulation. Two distinct families of S6 kinases were subsequently identified by molecular cloning, the 90 kDa S6 kinase (designated $p90^{rsk}$ or MAPKAP kinase 1) originally isolated from *Xenopus* oocytes (Jones *et al.*, 1988) and a 70 kDa S6 kinase ($p70^{s6k}$) from rat liver (Kozma *et al.*, 1990, Banergee *et al.*, 1990). Although both of these kinases become activated within minutes after treatment of cells with mitogens, considerable evidence indicates that the activities of $p90^{rsk}$ and $p70^{s6k}$ are regulated by separate pathways; $p90^{rsk}$ is activated on phosphorylation by p42/p44 MAP kinases (Sturgill *et al.*, 1988) through the Ras-Raf pathway (see Section 1.6.1.2) while up until very recently, the upstream components of the signal transduction pathway leading to $p70^{s6k}$ activation were unidentified. While both S6 kinases are able to phosphorylate S6 protein *in vitro*, a number of results have indicated that $p70^{s6k}$ is the major S6 kinase *in vivo*. The $p70^{s6k}$ is highly specific for S6 protein, whereas $p90^{rsk}$ phosphorylates other substrates, and full S6 phosphorylation can occur by $p70^{s6k}$ activation without $p90^{rsk}$ activation (Flotow & Thomas, 1992). Furthermore, rapamycin, an immunosuppressant macrolide antibiotic isolated from *Streptomyces hygroscopius* which blocks serum-stimulated S6 phosphorylation, inhibits activation of $p70^{s6k}$ by mitogenic factors but has no effect on the activity of $p90^{rsk}$ (Calvo *et al.*, 1992, Chung *et al.*, 1992).

Two cDNA clones for $p70^{s6k}$ have been isolated that are derived from a single gene (Grove *et al.*, 1991). While both clones encode for a protein of 502 amino acids, the second clone with an alternative translational start site encompasses a larger open reading frame giving rise to an identical protein with a 23 amino acid extension at its amino terminus (Banerjee *et al.*, 1992). The products of the two clones represent two distinct isoforms of the same kinase with apparent molecular masses of 70 kDa (p70) and 85 kDa (p85) (Banerjee *et al.*, 1990, Grove *et al.* 1991) that are found in different subcellular locations. The $p70^{s6k}$ is found in the cytoplasm while the 23-amino acid amino terminus extension of $p85^{s6k}$ contains a hexa-Arg nuclear targetting sequence that localizes this isoform in the nucleus (Coffer &

Woodgett, 1994, Reinhard *et al.*, 1994). Two lines of evidence have indicated that the function of these kinases during G1 is important for growth factor-induced cell cycle progression. Firstly, inhibition of the $p70^{s6k}$ pathway by treatment of cells with the specific $p70^{s6k}$ inhibitor rapamycin, leads to cell cycle arrest in G1 or a delay of entry into S phase, depending on the cell type (Chung *et al.*, 1992, Price *et al.*, 1992, Terada *et al.*, 1993). Secondly, micro-injection of rat embryo fibroblasts with inhibitory antibodies to either $p70^{s6k}$ or $p85^{s6k}$ prevents serum-induced entry into S phase (Lane *et al.*, 1993, Reinhard *et al.*, 1994).

1.7.2 Activation of $p70^{s6k}$

The mechanism by which $p70^{s6k}$ becomes activated in response to mitogens has not been entirely clarified but involves phosphorylation events on multiple Ser/Thr residues (Ballou *et al.*, 1988). Initial phosphopeptide mapping studies identified Ser^{441}, Ser^{418}, Thr^{421}, and Ser^{424} clustered in a putative autoinhibitory domain in the non-catalytic carboxyl terminal tail as the four major sites that became phosphorylated on growth factor treatment (Ferrari *et al.*, 1992). It was originally envisaged that $p70^{s6k}$ might be maintained in a basal inactive state by this domain and released from inhibitory constraints by phosphorylation. However, this model for activation is not supported by the finding that a truncated recombinant $p70^{s6k}$ in which the carboxyl tail is deleted is not constitutively active (Weng *et al.*, 1995b). Furthermore, this, and other $p70^{s6k}$ mutants in which the four individual sites have been mutated to acidic residues can still be activated by serum (Han *et al.*, 1995, Weng *et al.*, 1995b) indicating that additional mitogen-directed inputs are important for activation. A set of phosphorylation sites distinct from those in the carboxyl-terminal domain has been identified which become dephosphorylated upon rapamycin treatment leading to enzyme inactivation (Ferrari *et al.*, 1993). These have subsequently been identified as Thr^{229}, Thr^{389} and Ser^{404} and found to also be involved in mitogenic activation of $p70^{s6k}$ (Han *et al.*, 1995). Taken together these observations suggest that regulation of $p70^{s6k}$ is likely to be a complex process involving multiple inputs from more than one kinase modifying each set of phosphorylation sites.

1.7.3 Regulation of $p70^{s6k}$

Despite considerable efforts by a number of research groups, the kinases responsible for directly phosphorylating any of the sites on $p70^{s6k}$ required for activation has not been

found. Progress has been made, however, in identifying how mitogens signal to $p70^{s6k}$. Several lines of evidence within the last two years have identified a role for phosphatidylinositol (PI) 3-kinase in the regulation of $p70^{s6k}$ (see Section 1.7.3.5).

1.7.3.1 Phosphatidylinositol (PI) 3-kinase

The structure, regulation and function of phosphatidylinositol (PI) 3-kinase has been extensively reviewed by Downes & Carter (1992) and Fry (1994). This kinase was originally identified as one of two biochemically distinct phosphatidylinositol (PI) kinase activities that associated specifically with several oncogene products and tyrosine phosphorylated proteins from growth factor stimulated cells (Whitman *et al.*, 1987). Labelling studies revealed that this kinase catalyzed the phosphorylation at the D-3 hydroxyl of the inositol ring of PI to form phosphatidylinositol 3-monophosphate (PI-3-P) and was called PI 3-kinase (previously referred to as type I), while the second PI kinase specifically phosphorylated the D-4 position and termed PI 4-kinase (or type II) (Whitman *et al.*, 1988). As well as PI, PI 3-kinase can also utilize phoshatidylinositol-4-monophosphate (PI-4-P) and phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) as substrates *in vitro*, to form phosphatidylinositol-3,4,-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3), respectively (Auger *et al.*, 1989, Carpenter *et al.*, 1990). In most studies conducted in a wide range of intact cells, treatment with hormones and growth factors results in the rapid and sustained increase in PI-3,4,5-P_3 and PI-3,4-P_2, and no significant change in PI-3-P (Auger *et al.*, 1989, Ruderman *et al.*, 1990, Kucera & Rittenhouse, 1990), strongly suggesting that *in vivo*, PI 3-kinase preferentially phosphorylates PI-4,5-P_2 and/or PI-4-P. Acute labelling studies conducted by Stephens and co-workers support a role for an agonist-stimulated PI-4,5-P_2 specific 3-kinase in intact cells although the kinetics for the appearance of PI-3,4-P_2 and PI-3-P indicate that these species result from the stepwise dephosphorylation of PI-3,4,5-P_3 (Hawkins *et al.*, 1992, Stephens *et al.*, 1991).

Purification and molecular cloning of the PI 3-kinase involved in signalling via tyrosine kinase growth factor receptors has revealed that the enzyme is a heterodimer composed of an 85 kDa regulatory subunit (p85), containing two SH2 domains and a single SH3 domain (Escobedo *et al.*, 1991, Skolnik *et al.*, 1991) and a 110 kDa catalytic subunit (p110) (Hiles *et al.*, 1992), which shows homology to the VPS34 gene product (Vps34p), a PI 3-kinase involved in protein sorting in *Saccharomyces cerevisae*. It is now apparent that

multiple mammalian forms of PI 3-kinase isoforms with distinct substrate specificities exist including a variant regulated by G-proteins (described further in Section 1.7.3.2), and are described in greater detail by Carpenter & Cantley (1996).

1.7.3.2 Regulation of PI 3-kinase by G-protein coupled receptors

Most activated tyrosine kinase receptors directly activate PI 3-kinase by recruiting p85/p110 heterodimers to the membrane where they associate via the SH2 domains in p85 with phosphorylated YXXM motifs within the cytoplasmic receptor domain of the receptor itself (Carpenter *et al.*, 1993) or in the case of insulin, the insulin receptor substrate 1 (IRS-1) (Backer *et al.*, 1992) (refer to Section 1.5.1). The ability of G-protein-coupled receptors to regulate PI 3-kinase activity was first indicated by observations that stimulation of platelets with thrombin, or neutrophils with the chemotactic peptide formylated Met-Leu-Phe (fMLP), another agonist which acts on a receptor coupled to G-proteins, results in the rapid accumulation of 3-phosphorylated phosphoinositide species (Traynor-Kaplan *et al.*, 1988, Kucera & Rittenhouse, 1990, Stephens *et al.*, 1991). While these cell types have served as suitable model systems to study PI 3-kinase activation by G-protein-coupled receptors, the mechanisms by which this occurs are still ill-defined relative to the amount of information that has accumulated on PI 3-kinase regulation by receptor tyrosine kinases. In platelets, a role for G-proteins in the regulation of PI 3-kinase has been indicated by the observations that thrombin-stimulation of PI 3-kinase activity is partially inhibited by PTX (King *et al.*, 1991) and that the non-hydrolysable GTP analogue GTPγS activates PI 3-kinase in permeabilized platelets and cytosolic fractions (Kucera & Rittenhouse, 1990, Zhang *et al.*, 1993a). The latter response has been shown to be dependent on the monomeric small molecular weight G protein $p21^{rho}$ since ADP ribosylation of endogenous Rho by the *botulinum* toxin exoenzyme C3 transferase prevents GTPγS-stimulated PI 3-kinase activity, an effect which is reversed by the addition of exogenous recombinant Rho (Zhang *et al.*, 1993a). Cytosolic fractions of platelets and myeloid cells also contain novel PI 3-kinase activities that are stimulated directly by G-protein βγ subunits which might result from an interaction with a p85/p110 heterodimer (Stephens *et al.*, 1993, Thomason *et al.*, 1994). Recently, a PI 3-kinase activated directly *in vitro* by either G-protein α or βγ subunits has been cloned, and termed $p110_{\gamma}$ (Stoyanov *et al.*, 1995). This newly identified PI 3-kinase is a 110 kDa protein that shares the kinase domain of

other mammalian p110 subunits but lacks homology in the N-terminal region responsible for binding to p85 and thus does not interact with the regulatory subunit.

1.7.3.3 Role of PI 3-kinase in mitogenesis and other cellular functions

Early work recognized the correlation between the accumulation of 3-phosphorylated phosphoinositides with the proliferative response to many growth factors, and oncogene products (Whitman *et al.*, 1988, Auger *et al.*, 1989) indicating that PI 3-kinase activity may be important in mitogenesis. Subsequent studies with cells expressing site-directed mutant receptors, or using inhibitory antibodies to the p110 subunit and PI 3-kinase inhibitors, while supporting the initial observations, indicate that the involvement of PI 3-kinase in cell growth is likely to be dependent on both the cell type and stimulus (Coughlin *et al.*, 1989, Roche *et al.*, 1994). The finding that PI 3-kinase activity is evident in terminally differentiated cells such as platelets and neutrophils (see Section 1.7.3.2) has suggested that as well as mitogenesis, PI 3-kinase may have other effects in cells. It is now evident that PI 3-kinase plays a critical role in a number of distinct cellular functions that have been extensively reviewed by Fry, (1994) and Carpenter & Cantley, (1996). These include actin assembly and cytoskeletal reorganization, glucose transport in adipocytes, the respiratory burst in neutrophils, and secretion and vesicle trafficking.

1.7.3.4 Cellular targets for PI 3-kinase

The direct physiological targets of PI 3-kinase have remained elusive. Since no 3-phosphorylated phosphoinositide-specific phospholipase C has been identified to date, it is believed that the phosphoinositide products of PI 3-kinase activity function directly as novel lipid second messengers rather than the precursors of the respective inositol phosphate species. This role is supported by the finding that certain 3-phosphorylated phosphoinositides are involved in the activation of protein kinases that may participate in signal transduction. These include specific calcium-independent PKC family members (Nakanishi *et al.*, 1993, Toker *et al.*, 1994, 1995) and protein kinase B (PKB) the product of the c-*akt* proto-oncogene and cellular homologue of the transforming v-Akt (Burgering & Coffer, 1995, Franke *et al.*, 1995). The role of these effectors of PI 3-kinase activity are discussed in greater detail in Section 4.7.2.

As well as generating potential second messenger molecules by lipid kinase activity, the p110 catalytic subunit of PI 3-kinase has been demonstrated to exhibit intrinsic serine/threonine protein kinase activity (reviewed by Hunter, 1995), although the significance of this activity in signal transduction cascades involving protein phosphorylation remains unclear.

1.7.3.5 Role of PI 3-kinase in agonist-stimulated $p70^{s6k}$ activation

While the direct target of PI 3-kinase is unknown, several recent lines of evidence have indicated that PI 3-kinase lies upstream of protein kinase signalling cascades. Work by Blenis and co-workers was the first to demonstrate a role for PI 3-kinase in the regulation of $p70^{s6k}$ by growth factors acting on receptor tyrosine kinases. Not only did these studies provide clues to the mechanisms involved in $p70^{s6k}$ activation, but also identified a downstream effector for PI 3-kinase that may be a candidate for transducing mitogenic signals by PI 3-kinase, two issues that up until then had remained enigmatic. PDGF receptors containing point mutations at docking sites for specific SH2 domain-containing signalling molecules were employed to determine which effector was important for $p70^{s6k}$ activation. The mutant Y740/Y751 unable to bind PI 3-kinase failed in parallel to activate $p70^{s6k}$ suggesting that PI 3-kinase mediates PDGF receptor signalling to $p70^{s6k}$ (Chung *et al*, 1994). Using a similar approach, however, Ming *et al.*, (1994) reported that the single point mutation at Y751 but not Y740 blocked $p70^{s6k}$, while both mutants suppressed receptor PI 3-kinase association to the same extent, indicating that some other molecule beside PI 3-kinase that binds to residue Y751 is critical for $p70^{s6k}$ activation. Despite this finding, an overwhelming amount of evidence supports an involvement of PI 3-kinase in $p70^{s6k}$. Experiments using PDGF receptor "add back" mutants that abrogate activation of all but one signalling molecule on an individual residue revealed that PI 3-kinase mediates the majority of $p70^{s6k}$ activation (Chung *et al.*, 1994). The findings of genetic studies have been supported by pharmacological approaches using wortmannin and LY294002, two structurally different specific PI 3-kinase inhibitors that have been shown to block $p70^{s6k}$ activation in response to a number of agonists in a variety of cell types (Chung *et al.*, 1994, Cheatham *et al.*, 1994, Monfar *et al.*, 1995). Recently, further strong evidence for a role of PI 3-kinase in $p70^{s6k}$ activation has been provided by Avruch's group demonstrating that expression of a recombinant constitutively active PI 3-kinase causes activation of $p70^{s6k}$ by phosphorylating Thr^{229} (Weng *et al.*, 1995a).

In addition to PI 3-kinase, studies with PDGF receptor mutants have revealed that PLCγ, acting through PKC, plays a lesser but significant role in regulating $p70^{s6k}$. A Y1021 mutant which fails to associate with PLCγ does not fully activate $p70^{s6k}$ while a Y1021 "add back" mutant is sufficient for $p70^{s6k}$ activation on PDGF stimulation (Chung *et al.*, 1994). This finding is consistent with the early data by Thomas and collaborators that showed, using a PKC downregulation protocol or specific PKC inhibitors, that $p70^{s6k}$ activation induced by EGF was largely under the control of PKC (Susa *et al.*, 1992).

Although $p70^{s6k}$ activation in response to agonists that activate tyrosine kinase receptors has been well documented, there is less evidence to implicate $p70^{s6k}$ in signals generated by G-protein-coupled receptor agonists. Thrombin has been shown to activate $p70^{s6k}$ in a PTX-sensitive manner in CCL39 fibroblasts (Kahan *et al.*, 1992). This activity correlates with the majority of S6 kinase activity stimulated by thrombin in these cells (Kahan *et al.*, 1992). Furthermore, $p70^{s6k}$ becomes activated by angiotensin II in vascular smooth muscle cells and cardiac myocytes, and plays a critical role in the hypertrophic response to this mediator (Giasson & Meloche, 1995, Takano *et al.*, 1996). In addition, $p70^{s6k}$ activity is also stimulated in rat 1 cells on activation of endogenously expressed LPA receptors or transfected α_2-adrenergic receptors, which couple exclusively to G_i (Wilson *et al.*, 1996), suggesting that this species of G protein may serve as the link between G-protein-coupled receptors and $p70^{s6k}$. While there is now a wealth of evidence to indicate that PI 3-kinase lies upstream of $p70^{s6k}$ in a signalling cascade induced by a number of tyrosine kinase receptors, and novel paradigms for activation of PI 3-kinase by G-protein-coupled receptors have recently emerged (see Section 1.7.3.2), there is very little information on whether $p70^{s6k}$ activation by G-protein-coupled receptors is mediated by PI 3-kinase. A role for PI 3-kinase has been suggested by the very recent finding that α_2-adrenergic receptor and LPA receptor -mediated activation of $p70^{s6k}$ is blocked by wortmannin (Wilson *et al.*, 1996). However, whether the PI 3-kinase involved represents the same PI 3-kinase regulated by tyrosine kinase receptor ligands or the novel G-protein βγ subunit-regulated isoform (see Section 1.7.3.2), and whether PI 3-kinase-dependent $p70^{s6k}$ activation is universal for other G-protein-coupled receptors such as thrombin, is not known.

1.7.4 Role of FRAP, the mammalian target of rapamycin in $p70^{s6k}$ regulation

Information regarding the regulation of $p70^{s6k}$ has also been gleaned from recent investigations that have shed light on how rapamycin inhibits the activity of the kinase. It appears from *in vitro* studies that rapamycin does not interact directly with $p70^{s6k}$. Instead, the macrolide selectively inhibits a pathway leading to $p70^{s6k}$ activation. The intracellular receptor for rapamycin is one of a family of proteins possessing peptidyl prolyl isomerase activity termed FKBP12 (FK506-binding protein 12 where FK506 is another immunosuppressant structurally similar to rapamycin) (Fruman *et al.*, 1995). This drug-receptor complex then targets a recently purified and molecularly cloned protein called FKB12-rapamycin-associated protein (FRAP) (Brown *et al.*, 1994), alternatively referred to as RAFT1 (rapamycin-FKBP target 1) or mTOR (mammalian TOR), found to be the mammalian equivalent of the yeast gene products Target of Rapamycin (TOR) 1 and 2. Recently, FRAP has been shown to be a rapamycin-sensitive regulator of $p70^{s6k}$ activity *in vivo* (Brown *et al.*, 1995a) although how this occurs is not known. Despite FRAP showing sequence identity to lipid kinases, with highest homology to PI 3-kinase, no convincing demonstration of lipid kinase activity has been reported. Recently, it has been shown that FRAP undergoes rapamycin-sensitive autophosphorylation *in vitro* and that this intrinsic kinase function is necessary for regulating $p70^{s6k}$ *in vivo* (Brown *et al.*, 1995a). This is unlikely to be direct, however, since thus far, FRAP has not been shown to phosphorylate $p70^{s6k}$.

1.7.5 Role of $p70^{s6k}$ in growth-related rapamycin-sensitive responses

In addition to phosphorylation of ribosomal S6 protein, $p70^{s6k}$ has been implicated in regulating many other growth-related events by virtue of the sensitivity of such processes to inhibition by rapamycin. Protein synthesis translation initiation may be prevented by the macrolide at multiple levels. Rapamycin inhibits the selective upregulation of a family of mRNAs characterized by a polypyrimidine tract at their 5' terminus (Jefferies *et al.*, 1994). Members of this family have been shown to encode for proteins essential for cell growth such as ribosomal proteins and elongation factors (Terada *et al.*, 1994). Rapamycin also inhibits phosphorylation of the translational repressor 4E-BPI (PHAS-1) (Lin *et al.*, 1995b, Graves *et al.*, 1995). Phosphorylation of PHAS-1 releases PHAS-1 from a complex with the translation initiation factor eIF-4E, allowing eIF-4E to initiate translation.

In addition to effects on translation, $p70^{s6k}$ may also be important in the mitogenic response at the nuclear level by phosphorylating and activating nuclear proteins involved in transcription. One such target appears to be the transcription factor cAMP-responsive element modulator (CREMτ). $p70^{s6k}$ phosphorylates CREMτ *in vitro* on the same serine residue that is phosphorylated *in vivo* on serum treatment. This modification leads to enhanced CREMτ-mediated transcriptional activation by serum *in vivo*, an effect blocked by rapamycin (de Groot *et al.*, 1994). Other rapamycin-sensitive molecules in the nucleus have been discovered including components of the cell cycle machinary. Rapamycin has also been shown to inhibit the activity of the cdks $p33^{cdk}$ and $p34^{cdc2}$ (Morice *et al.*, 1993) and block the inactivation of the cdk inhibitor $p27^{Kip1}$ which occurs in response to IL-2 in T cells (Nourse *et al.*, 1994). It must be stressed, however, that it is questionable whether $p70^{s6k}$ is directly involved in regulating all of the responses described.

1.8 STRESS-ACTIVATED PROTEIN KINASE PATHWAYS

The last few years have marked the arrival of novel members of what now appears to be a MAP kinase superfamily. Two protein kinases that are highly related to the classical p42/p44 MAP kinase have been identified, and characterized as integral components of parallel protein kinase cascades activated by proinflammatory cytokines and cellular stresses. It has now become apparent that these MAP kinase homologues may play a role in the cellular effects of an increasingly large number of extracellular stimuli including G-protein-coupled receptor agonists (Coso *et al.*, 1995a) including thrombin (Shapiro *et al.*, 1996, Kramer *et al.* 1995b, Saklatvala *et al.*, 1996). The following commentary focuses on the recent developments in the rapidly expanding field of parallel signalling among mammalian MAP kinases, highlighting their specific modes of regulation and their potential function in mitogenic signalling.

1.8.1 c-Jun N-terminal kinases and p38 MAP kinase homologues

An investigation of kinases responsible for phosphorylating the transcription factor c-Jun on Ser^{63} and Ser^{73} within its amino terminal activation domain in cells exposed to UV irradiation identified that this activity was attributable to two protein kinases of 46 and 55 kDa named c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2), respectively (Hibi *et al.*,

1993, Dérijard *et al.*, 1994). The subsequent cloning of JNK1, by screening a human liver cDNA library with degenerate PCR primers against MAP kinase, revealed that this protein kinase shared sequence homology to the p42/p44 MAP kinases (Dérijard *et al.*, 1994). Work conducted concurrently by other investigators to clone a 54 kDa kinase (p54) which was first purified from cycloheximide treated rats, led to the isolation from a rat brain cDNA library of cDNAs encoding a subfamily of MAP kinase homologues which exhibited c-Jun N-terminal kinase activity (Kyriakis *et al.*, 1994) and represented the rat isoform equivalents of human JNKs. The activation of these kinases contrasted with the p42/p44 MAP kinases in that they were generally poorly activated by both growth factors and phorbol ester, yet like JNK activation by UV light (Dérijard *et al.*, 1994), were highly responsive to stress-related stimuli such as heat shock or translation inhibitors, and tumour necrosis factor (TNF)-α, and were thus dubbed stress-activated protein kinases (SAPKs) (Kyriakis *et al.*, 1994). Recent reports have indicated that JNK isoforms appear to be the products of three distinct genes though alternative splice variants of these genes may account for up to 10 identifiable forms with molecular weights of 46 and 55 kDa (Gupta *et al.*, 1996).

In addition to JNKs, a stress-activated MAP kinase homologue has also been identified as the mammalian equivalent to the product of the high osmolarity glycerol responsive 1 (HOG1) gene in *Saccharomyces cerevisiae*, responsible for restoring osmotic gradients across the cell membrane in response to increased external osmolarity (Brewster *et al.*, 1993). Independent investigations have identified important roles for identical or closely related kinases in signal transduction in mammalian cells. Han *et al.* (1993) demonstrated that this kinase, referred to as p38, became phosphorylated on tyrosine and activated in response to hyperosmolarity and bacterial endotoxin in cells of monocytic lineage. Rouse *et al.* (1994) discovered a novel protein kinase recognized by antibodies against the Xenopus Mpk2, a kinase closely related to HOG1, termed reactivating kinase (RK), that led to the activation of MAPKAP kinase-2, a serine kinase responsible for phosphorylating one of a family of small heat shock proteins (Hsp25) in response to chemical stress or heat shock. Freshney *et al.* (1994) purified an IL-1-stimulated kinase from human epidermal carcinoma cells whose biochemical properties closely resembled those of the p38 kinase. Finally, Lee *et al.*, 1994 identified the human homologues of p38 as a target for cytokine synthesis inhibitors termed cytokine-suppressive anti-inflammatory drug (CSAID) binding proteins (CSBP) that are involved in the regulation of IL-1 and TNFα synthesis by endotoxin-stimulated monocytes. A reappraisal of the nomenclature for MAP kinase homologues has included p38 in the SAPK

family on the basis that many of the stress-related stimuli that evoke JNK activation also stimulate p38. Thus, in this report SAPK is used to describe JNK and p38 MAP kinase homologues collectively.

1.8.2 MKK homologues involved in JNK and p38 activation

Although the signalling cascade leading to activation of p42/p44 MAP kinases is relatively well understood, the pathways leading to activation of SAPKs is more obscure. However, as with p42/p44 (see Section 1.6), the SAPK pathways are canonical MAP kinase modules consisting of three protein kinases that act sequentially in a pathway: a MAP kinase homologue (or SAPK/JNK/p38), MAP kinase kinase homologue (or MKK/MEK/SEK/JNKK) and a MAP kinase kinase kinase (or MEKK). Again, like MAP kinase, the activation of the SAPKs relies on their phosphorylation at specific dual phosphorylation motifs, although in this case the sequences are defined by TPY for JNK (Kyriakis *et al.*, 1994, Dérijard *et al.*, 1994, Minden *et al.*, 1994) and TGY for p38 MAP kinase (Han *et al.*, 1994, Rouse *et al.*, 1994). These residues are specifically phosphorylated by MKK/MEK homologues (also termed SAPK kinases (SAPKK)) distinct from MKK/MEKs 1 and 2 that are responsible for the activation of the classical p42/p44 MAP kinase isoforms (Section 1.6.2).

Independent research efforts have isolated mammalian cDNAs which encode for protein kinases distantly related to MEK-1 termed SAPK/ERK kinase-1 or SEK1 in the rat (Sanchez *et al.*, 1994), and MKK4 (Dérijard *et al.*, 1995) or JNK kinase (JNKK) in humans (Lin *et al.*, 1995a). It is now apparent that these kinases represent a common mammalian homologue which serves as a dual function kinase, activating both JNK (Sanchez *et al.*, 1994, Dérijard *et al.*, 1995 Lin *et al.*, 1995a) and p38 MAP kinase (Dérijard *et al.*, 1995) *in vitro*, without affecting p42/p44 MAP kinase isoforms. Another MAP kinase kinase identified in humans and designated MKK3, has been shown to selectively activate p38 MAP kinase (Dérijard *et al.*, 1995). It has become evident that the upstream regulation of SAPKs is more complex than originally envisioned and, dependent on the cell type, likely to involve other dual specificity kinases in addition to MKK3 and MKK4/JNK/SEK-1 characterized initially. Novel JNK-activating activities attributable to kinases immunologically distinct from MKK4 have recently been identified by chromatographic fractionation of osmotically-shocked fibroblasts (Moriguchi *et al.*, 1995) and epidermal carcinoma cells (designated SAPKK-4 and SAPKK-5)

(Meier *et al.*, 1996). Similarly, in the latter cell type, cellular stresses and IL-1 were shown to activate a predominant activator of p38 MAP kinase (termed SAPKK-3) which is different from MKK3 (or SAPKK-2) (Meier *et al.*, 1996). At the same time, independent groups reported the cloning of a novel MEK homologue which like MKK3, exhibits specificity towards p38 MAP kinase *in vitro* or when expressed in cells (Han *et al.*, 1996, Raingeaud *et al.*, 1996, Stein *et al.*, 1996). Subsequent purification and cDNA cloning of SAPKK-3 revealed that this kinase was identical to MKK6 (Cuenda *et al.*, 1996).

1.8.3 Activation of MKK homologues by MEKKs

Activation of the relevant MKK/MEK/SAPKK activity requires its phosphorylation by an upstream kinase analogous to the Raf kinase isoforms that function upstream in the classical p42/p44 MAP kinase pathway. The novel serine/threonine kinase MEKK1, which was originally identified as a kinase able to phosphorylate MEK-1 *in vitro* (Lange-Carter *et al.*, 1993) (see Section 1.6.3.2), has been subsequently shown to be a more efficient activator of MKK/MEK4/SEK-1/JNKK in *in vitro* and co-transfection studies (Minden *et al.*, 1994, Yan *et al.*, 1994). In this role, MEKK1, but not Raf-1 was also found to participate in both growth factor and TNF-stimulated activation of JNK (Minden *et al.*, 1994). Therefore, MEKK1 serves to couple signals arising from exposure to various stimuli to c-jun phosphorylation by a sequential cascade defined by MEKK1, MKK4 and JNK. It is likely, however, that under specific conditions, variants of this pathway exist at the level of MEKK since additional MEKK isoforms involved in the activation of JNK have recently been identified (Blank *et al.*, 1996).

Less is known regarding the identity of the equivalent MEKK-like protein upstream of MKK3/6 in the p38 pathway. One candidate for this role is TAK-1, isolated as a transforming growth factor β (TGF-β)-activated kinase (hence TAK-1) related to MEKK (Yamaguchi *et al.*, 1995). TAK-1 has been shown to directly phosphorylate and activate both MKK6 and MKK3 *in vitro* (Moriguchi *et al.*, 1996). Thus, activation of p38 MAP may occur via a linear kinase pathway involving TAK-1, MKK3/6 and p38 MAP kinase.

1.8.4 Role of low molecular weight G-proteins and p21-activated kinases (PAKs) in JNK and p38 activation regulation

By virtue of the role that $p21^{ras}$ plays in the upstream events initiating activation of the Raf/MEK/MAP kinase pathway by growth factors (reviewed by Malarkey *et al.*, 1995a), much interest has centred on the role of low molecular weight guanosine 5'-triphosphate (GTP)-binding proteins, in the stress related pathways. This includes not only $p21^{ras}$ itself but also members the Rho superfamily; Rac and Cdc42 upstream of the JNK and p38 cascades. Evidence now supports a role for Rac and cdc42 lying upstream of JNK and p38 MAP kinase cascades. Constitutively active forms of both monomeric G-proteins, have been shown to stimulate the activation of JNK1 (Coso *et al.*, 1995b, Minden *et al.*, 1995, Olson *et al.*, 1995) and p38 (Bagrodia *et al.*, 1995, Zhang *et al.*, 1995, Minden *et al.*, 1995) but not p42/p44 MAP kinase, indicating a level of specificity for the stress pathways. Furthermore, these signalling molecules are likely to serve as critical intermediates as dominant negative Rac or Cdc42 have been reported to inhibit JNK and p38 MAP kinase activity in response to IL-1 (Bagrodia *et al.*, 1995), EGF (Coso *et al.*, 1995b, Minden *et al.*, 1995) muscarinic receptor activation and heterotrimeric G-protein $\beta\gamma$ subunit complexes (Coso *et al.*, 1996).

Some evidence also supports a role for $p21^{ras}$ in the regulation of SAPK activation. Expression of constitutively active Ras, Ha-Ras or D12Ras, as with Rac/Cdc42, results in increased activity of JNK (Minden *et al.*, 1994, Olson *et al.*, 1995), while JNK activation in response to EGF is abrogated under conditions of expression of dominant negative Ras (N17) (Minden *et al.*, 1994). How the role of Ras in JNK activation relates to that of Rac/Cdc42 is not entirely clear, but functional inter-relationships between these small GTPases may occur as has been demonstrated in some systems where Ras can activate Rac-1 (Ridley *et al.*, 1992) and conversely, Rac can play a role in Ras-mediated transformation (Qui *et al.*, 1995). Therefore, Ras activation may precede Rac in the initiation of the SAPK cascade and may well be a point at which the p42/p44 MAP kinase and SAPK pathways bifurcate.

Although a recent study has now positioned Rac upstream of MEKK1 in the JNK cascade in a manner analogous to Ras (Minden *et al.*, 1995), this interaction does not appear to be direct as an additional component of the pathway has been identified. Evidence has accumulated in support of an involvement of members of a family of serine/threonine protein kinases called p21-activated kinases (PAKs) that are the mammalian homologues of STE20 in yeast, as intermediates in the SAPK pathway. Three related enzymes termed PAK1 (αPAK),

PAK2 (γPAK) and PAK3 (βPAK) (Manser *et al.*, 1994, Martin *et al.*, 1995, Bagrodia *et al.*, 1995) have been shown to be substrates for, and become activated by, binding to Cdc42 and Rac *in vitro*. A role for PAK in the activation of JNK and p38 has been indicated by the finding that constitutively active PAK or PAK1/2 overexpression activates JNK/SAPKα (Bagrodia *et al.*, 1995, Frost *et al.*, 1996) and p38 (Zhang *et al.*, 1995) in a number of cell types. Furthermore, Rac-activated JNK1 is inhibited by the expression of the PAK1 N-terminal regulatory domain (Minden *et al.*, 1995), and activation of JNK and p38 by IL-1, Rac and Cdc42 is inhibited by dominant-negative catalytically inactive PAK (Zhang *et al.*, 1995). PAK may also be involved in the activation of both JNK and p38 by growth factor and G-protein coupled receptors as these stimuli have been shown to activate PAKs as well (Knaus *et al.*, 1995). Whether PAKs activate the upstream components regulating the stress-activated signalling cascades such as MEKK is not yet known. However, GTPγS-dependent association of PAK and MEKK1 in Cdc42 complexes has been demonstrated, and in yeast, the PAK equivalent STE20 activates the MEKK1 homologue STE11 (Herskowitz, 1995).

1.8.5 Potential role of JNK and p38 activation in cell growth

Information regarding the cellular targets and functional significance of JNK and p38 activation are only just beginning to come to light. With regard to thrombin, p38 activation by this agonist has recently been shown to play a key role in platelet aggregation and may regulate $cPLA_2$ in this cell type (Kramer *et al.*, 1995a, Saklatvala *et al.*, 1996). Despite a number of studies that have linked JNK and p38 activation to apoptosis or cell cycle arrest (Xia *et al.*, 1995, Verheij *et al.*, 1996) there is increasing evidence to support a role for SAPKs in positive regulation of cell proliferation. Thrombin stimulation of JNK in airway smooth muscle cells correlates with DNA synthesis (Shapiro *et al.*, 1996), indicating that this kinase may regulate the mitogenic effect of this agonists, although data to directly support this is lacking. Perhaps the most persuasive piece of evidence to indicate that SAPKs may be involved in growth regulation is their ability to activate transcription factors which regulate the expression of various genes including immediate early genes that are induced rapidly on mitogenic stimulation (see Section 1.3.2). JNK binds to and phosphorylates the N-termini of c-Jun and activating transcription factor-2 (ATF2) (Gupta *et al.*, 1995, Van Dam *et al.*, 1995), a member of the cAMP-responsive element family of transcription factors. Phosphorylation of these proteins potentiate the ability to assemble AP-1 complexes of

homodimers, or a variety of heterodimers between either these proteins or with Fos proteins, thereby facilitating the transcription of various genes containing TRE elements in their promotors, including c-*jun* itself (reviewed by Karin, 1995). JNK, like p42/p44 MAP kinase (see Section 1.6.1.2) is also capable of phosphorylation and activation of Elk-1/p62^{TCF} suggesting that it may be involved in mediating c-*fos* induction under certain circumstances. The p38 MAP kinase also phosphorylates and activates ATF2 and Elk-1/p62^{TCF} *in vitro* and in transfected cells (Raingeaud *et al.*, 1996), although additional transcriptional events associated with activation of the p38 pathway may be mediated by the downstream effector MAPKAP kinase-2. While AP-1 activity is a characteristic of cell stimulation with mitogens, including thrombin (see Section 1.3.2), a link between transcriptional events regulated by JNK and p38 and cell division has not been established. Despite this, activation of JNK by carbachol correlates with AP-1 activity and c-*jun* mRNA expression in NIH 3T3 cells (Coso *et al.*, 1995a) suggesting that JNK may be a likely candidate to be an integral part of the mitogenic signalling pathway utilized by G protein coupled receptors.

1.9 AIMS

Characterization of the cellular signalling events that result as a consequence of cell activation by thrombin is an important issue from both biological and clinical perspectives. From a biological view, this will further our understanding of the processes by which thrombin mediates cellular effects including proliferation. From a clinical view, this will provide a model for the pathways that operate in response to thrombin *in vivo* under normal or pathological conditions, such as during the pathogenesis of vascular states characterized by blood vessel wall cell hyplerplasia. In addition, intracellular targets may be identified for the development of novel pharmaceuticals to alleviate such diseases.

The review of the literature indicates that while thrombin causes the proliferation of a number of cell types, the mechanisms by which this is achieved is far from resolved. The importance of protein phosphorylation events in mitogenesis has been made apparent from studies on growth factor signal transduction, yet the level at which these pathways are shared by thrombin and other G protein coupled receptor agonists, has not been fully established. Recent evidence has indicated that the p42/p44 MAP kinases plays a central role in thrombin-stimulated cell cycle re-entry, although the role of the upstream regulators of this pathway in the regulation of MAP kinase by this agonist has not been clarified in a single cell type.

Furthermore, there is very little information on the involvement of the PI 3-kinase/$p70^{s6k}$ signalling pathway or the SAPKs in thrombin-stimulated mitogenesis.

Currently defined signalling phenomena associated with stimulation of cell growth have been exposed by studies which have routinely used fibroblast cell lines. For thrombin, most of the signalling systems involved in mitogenesis have been characterized in one cell type, the hamster lung fibroblast line CCL39. These are not definitive models as the cells have been immortalized and therefore their growth regulatory mechanisms have been fundamentally altered. This may be problematic particularly when examining pathways involved in proliferation. Caution must therefore be taken in predicting how cells will behave physiologically by extrapolating from signalling events characterized in cell lines.

The aim of this thesis was to define more clearly the protein kinase signal transduction pathways involved in mitogenic signalling by thrombin. These investigations have been carried out in fibroblasts derived from primary cultures obtained from bovine pulmonary arteries, which should be a more predictive model of mammalian cells under physiological or pathophysiological conditions.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 General Reagents

All materials and reagents were of the highest commercial purity available and were supplied either by Sigma Chemical Co. (Poole, Dorset, UK) or BDH Chemicals Ltd (Poole, Dorset, UK) unless otherwise stated.

Amersham International Plc, Aylesbury, Buckinghamshire, UK.

ECL detection reagents, BIOTRAK™ MAP kinase assay kit.

Bio-Rad Laboratories, Hertfordshire, UK.

Bio-Rad DC™ Protein Assay kit.

Calbiochem, Nottingham, UK.

Pertussis toxin (PTX), Pansorbin™ and forskolin.

Costar, Buckinghamshire, UK.

nitrocellulose membranes.

Flowgen Instruments Ltd, Sittingbourne, Kent, UK.

DYNAZYME II™ DNA polymerase.

Gibco Life Technologies Ltd, Paisley, Scotland.

Cell culture plasticware, media, antibiotics and sera, SUPERSCRIPT™ II first strand cDNA kit.

PE-Applied Biosystems Division, Warrington, UK.

ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit.

Qiagen Ltd, Dorking, Surrey, UK

QUIAQUICK SPIN™ PCR purification kit.

Upstate Biotechnology Inc., Buckinghamshire, UK.

40S ribosomal S6 substrate peptide RRRLSSLRA, cAMP-dependent protein kinase (PKA) inhibitor peptide (TYADFIASGRTGRRNAI).

Wallac UK, Milton Keynes, UK.

Optiphase Hi-Safe™ scintillant

Whatman, Kent, UK.

P-81 phosphocellulose paper, 3MM paper.

2.1.2 Antibodies

Affiniti Research Products Ltd, Exeter, UK.

rabbit polyclonal anti-phosphotyrosine antibody; anti-p85α PI 3-kinase, mouse monoclonal anti-MEK-1/2; mouse monoclonal anti-MAP kinase (MK12) antibody; mouse monoclonal anti-Raf-1 antibody, mouse monoclonal anti-PKCα antibody; mouse monoclonal anti-PKCε antibody, mouse monoclonal anti-FAK antibody.

Amersham International Plc, Aylesbury, Buckinghamshire, UK.

horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody, HRP-conjugated donkey anti-rabbit IgG antibody.

Santa Cruz Biotechnology Inc. CA, USA.

rabbit polyclonal anti-$p70^{s6k}$ antibody, rabbit polyclonal anti-c-Raf-1 antibody, mouse monoclonal anti-phosphotyrosine antibody (PY20).

The PKC ζ antibody was kindly donated by P. J. Parker (Imperial Cancer Research Fund, Lincoln's Inn, Fields, London, UK).

2.1.3 Other materials

PAR 2-activating peptide SLIGRL was synthesized by Genosys Europe (Cambridge, UK) and was >95% pure by chromatographic and mass spectral analysis.

Plasmids containing histidine-tagged wild type and kinase defective (K52R) MAP kinase and kinase-inactive MEK-1 (MEK-B) were generous gifts from G. L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado, USA).

The plasmid containing the cDNA encoding GST-tagged N-terminal amino acids 5-89 of c-Jun (GST-c-Jun_{5-89}) was donated by J. R. Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada).

The GST-tagged full length MAPKAP Kinase-2 vector was a gift from Christopher J. Marshall (Institute of Cancer Research, Chester Beatty Laboratories, London, UK).

The plasmid containing mouse PAR-2 cDNA was kindly supplied by J. Sundelin (Division of Molecular Neurobiology, The Wallenburg Laboratory, Lund University, Sweden).

Rapamycin, PD098059 and SB203580 were generous gifts from Professor P. Cohen (MRC Protein Phosphorylation Unit, University of Dundee, UK).

The hexapeptide LSIGRL was kindly donated by Wadie F. Bahou (Stony Brook Health Sciences Center, State University of New York, Stony Brook, New York).

Sheep mast cell proteinase (SMCP) was provided by Alan D. Pemberton (Department of Veterinary Clinical Studies, University of Edinburgh, UK).

Custom-designed oligonucleotide primers were synthesized in the Molecular Biology Laboratory, University of Strathclyde, Glasgow, UK.

2.1.4 Radiochemicals

New England Nuclear, Hertfordshire, UK.

[γ-^{32}P]adenosine triphosphate (3000 Ci/mmol), [methyl-^{3}H]thymidine (38Ci/mmol)

2.2 CELL CULTURE

2.2.1 Bovine pulmonary arterial (BPA) fibroblast primary culture

Fibroblast cultures were isolated from pulmonary arteries of adult cows by a primary explant procedure essentially described by Freshney (1983). Pulmonary arteries were removed under sterile conditions from bovine lungs freshly delivered from an abattoir. Sections of artery located towards the apex of the lobe, approximately 20 mm in length and 3.8-4.0 mm in diameter, were cut longitudinally and opened into flat sheets. Parenchymal tissue was removed from the adventitial surface and the endothelial surface removed by gentle abrasion. Arteries were dissected into portions of approximately 1 mm^2 and planted evenly on the bottom surface of 25 cm^2 tissue culture flasks containing 1 ml of DMEM supplemented with 20% newborn calf serum (NCS), 5 μg/ml amphotericin B (Fungazone), 400 U/ml penicillin and 400 μg/ml streptomycin. Explants were cultivated at 37^{o}C in a humidified atmosphere of 95% air, 5% CO_2 and left for 5 days before the media was replaced. Media was changed thereafter on alternate days. After approximately 2 weeks, when over 50% of the surface area of the culture flask was covered by outgrowths of cells from adherent explants, cells were passaged by trypsinization as described in section 2.2.3 and returned initially to fresh 25 cm^2 culture flasks. On the second passage, cells were transferred to a 80 cm^2 flask and maintained as described in Section 2.2.3.

2.2.2 Rat aortic (RA) smooth muscle cell primary culture

Primary cultures of rat aortic smooth muscle cells were established following a protocol described by Brock *et al.* (1985). For each preparation, two thoracic aortae were excised from male Sprague-Dawley rats (180-200 g) under sterile conditions and rinsed in DMEM containing 250 U/ml penicillin, 250 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Fungazone) 2 mM glutamine. The aortae were incubated in DMEM containing 1 mg/ml collagenase type II from *Clostridium histolyticum* (EC 3.4.24.3) in a water bath for 10 minutes at 37°C with periodic shaking. The tissues were removed and pinned onto a silgard dish. Unwanted tissue was dissected and the tissue rinsed thoroughly outside and intralumenally through a 1 ml pipette tip to remove endothelial cells. The aortae were chopped finely using a razor blade and RA smooth muscle cells dissociated enzymatically from the elastic laminae by incubating the tissue in DMEM containing 1 mg/ml collagenase type II and 0.1 mg/ml pancreatic elastase (EC 3.4.21.36) for 3 h at 37°C with agitation. Cells were collected by centrifugation at 200 x g for 5 minutes. The media was aspirated and the pellet of cells rinsed twice with serum-free media by centrifugation. RA smooth muscle cells were resuspended in DMEM supplemented with 20 % (v/v) fetal calf serum (FCS), 250 IU/ml penicillin, 250 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Fungazone) and initially grown in 25 cm^2 cell culture flasks at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Media was replaced after approximately 2-3 days when cells had adhered to the culture flask surface, and on alternate days thereafter until areas of confluent cells appeared. Cells were passaged by trypsinization (as described in Section 2.2.3) and returned initially to fresh 25 cm^2 culture flasks. On the second passage, cells were transferred to a 80 cm^2 flask and maintained as described in Section 2.2.3.

2.2.3 Subculturing of BPA fibroblasts and RA smooth muscle cells by enzymatic dissociation

At approximately 90% confluency, both BPA fibroblasts and RA smooth muscle cells were subcultured by enzymatic dissociation with trypsin. Media was aspirated and cells rinsed in sterile phosphate-buffered saline (PBS) (150 mM NaCl, 5.4 mM KCl, 10 mM Na_2PO_4, 1.5 mM KH_2PO_4) containing 0.02 % (w/v) EDTA. Cells were incubated with trypsin (0.5 % w/v) diluted in PBS and 0.2% (w/v) EDTA at 37°C to remove cell monolayers from the culture

flask surface. This process was monitored carefully and the trypsin solution immediately aspirated at the first sign of cell detachment. Flasks were tapped to expedite removal of cells. When completely detached, cells were washed with DMEM containing 10 % (v/v) FCS and supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin and dispersed by pipetting repeatedly over the surface of the monolayer. Cell suspensions of BPA fibroblasts and RA smooth muscle cells were diluted (1:3) and transferred to fresh 80 cm^2 culture flasks. Both RA smooth muscle cells and BPA fibroblasts were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and media was replaced on alternate days. Cultures were examined daily under an Olympus inverted phase-contrast microscope at 100 x magnification.

2.2.4 Preparation of cells for experiments

Subcultures of BPA fibroblasts and RA smooth muscle cells at passage levels 4-10 were utilized routinely for experiments. Cells dissociated by trypsinization (Section 2.2.3) were seeded onto appropriate culture surfaces. When approximately 90% confluent, cells were rendered quiescent in serum-free DMEM for 48 h prior to treatment.

2.3 DETECTION AND ANALYSIS OF PROTEINS

2.3.1 Preparation of samples for SDS-PAGE and immunoblotting

Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS) (150 mM NaCl, 5.4 mM KCl, 10 mM Na_2PO_4, 1.5 mM KH_2PO_4) and lysed by adding 0.5 ml of hot (85°C) Laemmli sodium dodecyl sulphate (SDS) sample buffer (63 mM Tris HCl pH 6.8, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% glycerol, 2% SDS and 0.007% (w/v) bromophenol blue to which was added 50 mM dithiothreitol (DTT) immediately before use). Cells were scraped from wells on ice and lysates drawn repeatedly through a 16 gauge needle attached to a 1 ml syringe to shear chromosomal DNA. Samples were transferred to a microfuge tube, boiled for 5 minutes to denature proteins and stored at -20°C until required for SDS-polyacrylamide gel electrophoresis.

2.3.2 SDS-polyacrylamide gel electrophoresis of proteins

The resolving gel was prepared containing an appropriate amount of acrylamide:N, N'-methylenebis-acrylamide (37.5:1), 0.375 M Tris pH 8.8, 0.1% (w/v) SDS and 0.05% (w/v) ammonium persulfate. Polymerization was initiated by the addition of 0.05% (v/v) N, N, N, N', N'-tetramethylethylenediamine (TEMED), the acrylamide solution poured between two glass plates assembled in a vertical slab configuration according to the manufacturers instructions, and overlayed with 100 µl 0.1 % (w/v) SDS. A stacking gel was prepared of the following composition: 5% (w/v) acrylamide, 125 mM Tris HCl pH6.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulphate, 0.1 % (v/v) TEMED from and poured directly onto the surface of the polymerized resolving gel. A teflon comb was immediately inserted into the stacking gel solution. After polymerization was complete, the comb was removed and the gel assembly mounted in a Hoeffer Series SE600™ or BioRad Mini-Protean II™ electrophoresis tank and both upper and lower reservoirs filled with electrophoresis buffer (24.8 mM Tris, 191.8 mM glycine, 0.1% SDS). Aliquots of samples containing equal amounts of protein (approximately 20 µg for large gels and 5 µg for mini-gels) were loaded into the wells of the stacking gel. An electrical current with a potential difference of 60 V was passed between the electrodes of the electrophoresis apparatus provided by a Hoeffer P250/2.5 amp DC power supply. Pre-stained SDS protein markers of known molecular weights were run concurrently in order to identify the polypeptide of interest.

2.3.3 Fixing and drying gels

Gels containing proteins labelled with [^{32}P] were fixed in 20 % (v/v) methanol/10 % (v/v) acetic acid for 30 min and rinsed in distilled water. The gel was sandwiched between cellophane sheets in a drying frame and dried under vacuum for 60 min at 80°C by a Hoeffer Eazy-Breeze™ gel dryer. The radiolabelled protein was detected by autoradiography. The dried gel was exposed to Kodak X-OMAT LS X-ray film for 4-24 h at -20°C in a spring-loaded metal cassette and developed by a KODAK M35-M X-OMAT processor.

2.3.4 Western blotting

Separated proteins on polyacrylamide gels were transferred to nitrocellulose filters by electrophoretic blotting (Towbin *et al.*, 1979). The gel was firmly and evenly pressed against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between Whatmann 3MM paper and two porous pads. A glass pipette was used as a roller to squeeze out trapped air bubbles. The cassette was immersed in a Hoeffer TE Series Transfor™ or BioRad Mini Trans-Blot™ electrophoresis tank containing blotting buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol) with the nitrocellulose filter facing the anode. An electric current of 1.0 A was applied for 5-6 h for large gels or 250 mAmp for 2 h for mini-gels by a Hoeffer P250/2.5 amp DC power supply, during which time the electrophoresis tank was cooled.

2.3.5 Immunological detection of proteins on nitrocellulose

Nitrocellulose membranes on which proteins were immobilized following SDS-PAGE and Western blotting were rinsed in a buffer containing 150 mM NaCl, 20 mM Tris base pH 7.4 and 0.2% (v/v) Tween 20 (NaTT buffer). The residual binding capacity of the filter was saturated by incubating the membrane in NaTT buffer containing 3% BSA for 3 h at room temperature with gentle agitation on a platform shaker. The blocking buffer was decanted and blots incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA. On the following day membranes were washed in NaTT every 15 minutes for 90 minutes with gentle agitation. Blots were incubated for a further 2 h at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to 1:10000 in NaTT buffer containing 0.2% BSA. After six additional washes in NaTT as described before, immunoreactive protein bands were detected by ECL chemiluminescence. Membranes were blotted on a paper towel and exposed to ECL reagent for 90 seconds. The membranes were blotted again, mounted in an exposure cassette and covered with Saranwrap™ cling film. Blots were exposed to Kodak X-OMAT LS film for 1-10 minutes and developed by a KODAK M35-M X-OMAT processor.

2.3.6 Reprobing nitrocellulose membranes

In instances where the same membrane was probed with more than one antiserum, primary and secondary antibodies were stripped from the nitrocellulose by incubating in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris HCl pH 6.7 for 30 minutes at 50°C with agitation. Blots were rinsed with NaTT buffer four times over 40 minutes before the immunodetection protocol was repeated (Section 2.3.5).

2.3.7 Assay of protein concentration

Quantification of protein concentration was determined using a Bio-Rad™ DC Protein Assay Kit based on a colorimetric assay devised by Lowry *et al.*, (1951). Protein samples were prepared in appropriate lysis buffer or in 1% nonadet NP-40 PBS solution. For each assay performed, a standard curve was prepared using five dilutions of BSA as a protein standard diluted in the same buffer as the sample over the concentration range 0.025-0.4 mg/ml protein. Appropriate dilutions of both samples and standards were made in H_2O to a volume of 200 µl. To aliquots of standards and samples was added 100 µl of reagent A' (an alkaline copper tartrate solution supplemented with 5% SDS) and vortexed. To this mixture was added 800 µls reagent B (a dilute Folin Reagent) and immediately vortexed. Samples were left for 15 minutes for colour to develop as a result of reduction of Folin reagent by copper-protein complex. Colour development was quantified by absorbance spectroscopy at 750 nm within 1 h. Estimates of protein content in samples were made from the standard curve.

2.4 *IN VITRO* KINASE ASSAYS

For all *in vitro* kinase assays performed, preliminary experiments were undertaken to optimize the conditions of each reaction so that the concentration of substrates were in excess with respect to the concentration of enzyme present in order that the incorporation of [^{32}P]phosphate into the respective substrates at the specified temperature was linear for the duration of the kinase reaction.

2.4.1 MAP kinase activity assay

In vitro MAP kinase activity was assessed using a BIOTRAK™ MAP kinase assay kit to measure the incorporation of radiolabelled phosphate into a specific MAP kinase substrate peptide (KRELVEPLT^{669}PAGEAPNALLR) derived from a portion of the epidermal growth factor receptor (EGFR$^{661-680}$).

After treatment, cells in 6-well plates were rinsed in PBS and solubilized in lysis buffer (150 mM NaCl, 10 mM Tris pH7.5, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40 to which 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin were added immediately before use). Plates were placed on ice for 30 minutes before cells were scraped and lysates transferred to microfuge tubes. Lysates were clarified by removing insoluble material and unlysed cells by centrifugation at 13000 x g for 5 min at 4°C. Equal quantities (approximately 5 μg protein) of supernatent were transferred to fresh tubes to which was added 600 μM EGFR$^{661-680}$. The reactions were initiated by addition of [γ-^{32}P]ATP (50 μM, 1 μCi) in 75 mM HEPES buffer (pH 7.4) containing 1.2 mM $MgCl_2$, and incubated at 37°C for 15 min. Reactions were stopped by the addition of 300 mM H_3PO_4 and aliquots (25 μl) were spotted onto 3 x 3 cm P-81 phosphocellulose paper. Filters were washed for 5 minutes twice with 75 mM H_3PO_4, twice with distilled water and transferred to 6 ml plastic scintillation vials to which 3 mls Wallac Optiphase Hi-Safe™ scintillant was added. Radioactive phosphopeptide bound to the filter was quantified by liquid scintillation counting in a Wallac scintillation counter. In every assay, blanks were prepared from a sample of kinase buffer reaction mixture in the absence of lysate.

2.4.2 MEK-1/2 activity assay

MEK activity in immunoprecipitates prepared with an antibody which recognizes shared epitopes on both MEK-1 and MEK-2 (MEK-1/2) was measured by a coupled *in vitro* assay utilizing purified recombinant wild type MAP kinase and EGFR$^{661-680}$ as sequential substrates.

2.4.2.1 Immunoprecipitation of MEK-1/2

MEK-1/2 was immunoprecipitated by incubating equal quantities (approximately 150 μg) of precleared lysates prepared in an identical manner to the MAP kinase assay with anti MEK-1/2 antibody (2 μg/ml). The contents of the tube were mixed gently by placing tubes on a rotating wheel (20 rpm) for 2 h at 4°C. MEK-bound antibody complexes were coupled to Pansorbin™ (2 μg) for a further 2 h at 4°C with mixing. Immunoprecipitates were collected by centrifugation at 12, 000 x g for 30 seconds at 4°C in a microfuge. The supernatent was removed by aspiration and the immunecomplex resuspended in lysis buffer and vortexed to wash and displace proteins non-specifically adsorbed to the Pansorbin™ while leaving the antibody-MEK complexes intact. The immunoprecipitates were washed in a similar manner in lysis buffer without detergents.

2.4.2.2 *In vitro* MEK-1/2 activity assay

MEK-1/2 immunoprecipitates were washed twice in kinase buffer (50 mM HEPES pH 7.2, 0.5 mM EDTA, 0.1 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 5 mM $MgCl_2$). Immunocomplexes were resuspended in 30 μl kinase buffer containing 1 μg recombinant wild-type MAP kinase and 200 μM $EGFR^{661-680}$ peptide. The reactions were started on addition of [γ-^{32}P]ATP (50 μM, 2 μCi) and incubated at 30°C for 15 minutes. Reactions were terminated on ice and to an aliquot of each sample was added 300 μM H_3PO_4. [^{32}P]Phosphorylation of $EGFR^{661-680}$ by recombinant MAP kinase activated by MEK-1/2 was quantified as described for the MAP kinase activity assay (Section 2.4.1). The remainder of each sample was reconstituted in 4x Laemmli SDS sample buffer and resolved by 10% SDS-PAGE. Gels were dried under vacuum and incorporation of [^{32}P] into MAP kinase detected by autoradiography as described in Section 2.3.3.

2.4.3 c-Raf-1 kinase assay

C-Raf-1 kinase activity was assessed in anti- c-Raf-1 immunoprecipitates by measuring radiolabelled phosphate incorporation into a recombinant modified form of MEK-1 that is catalytically inactive(MEK-B) (Lange-Carter *et al.*, 1993).

2.4.3.1 Immunoprecipitation of c-Raf-1

C-Raf-1 was immunoprecipitated as described for MEK-1/2 in section 2.4.2.1 except that the lysis buffer was supplemented with 40 mM β-glycerophosphate and 10% glycerol and precleared lysates containing approximately 800 μg protein incubated with rabbit polyclonal anti-c-Raf-1 antibody (2 μg/ml).

2.4.3.2 *In vitro* c-Raf-1 kinase activity assay

C-Raf-1 immunoprecipitates were resuspended in a kinase buffer containing 100 mM NaCl, 10 mM PIPES, 10 mM $MnCl_2$, 10μM aprotinin and 1 μg recombinant MEK-B. The reaction was started by the addition of [γ-^{32}P]ATP (25 μM, 10 μCi), incubated at 30°C for 15 minutes and stopped by with 4x Laemmli SDS sample buffer. Samples were resolved by SDS-PAGE and the extent of [^{32}P] incorporation into MEK-B assessed by autoradiography as described in Section 2.3.3.

2.4.4 p70^{s6k} activity assay

p70^{s6k} activity was assessed in p70^{s6k} immunoprecipitates by measuring incorporation of [^{32}P] into the substrate peptide RRRLSSLRA corresponding to amino acids 231-239 of human 40S ribosomal subunit protein S6 (S6$^{231-239}$) (Heinze *et al.*, 1988) as described previously by Calvo *et al.*, (1994).

2.4.4.1 Immunoprecipitation of p70^{s6k}

Following treatment, cell monolayers in 9 cm culture dishes were rinsed twice with ice-cold PBS and solubilized in 0.5 ml of lysis buffer (50 mM Tris pH 8.0, 120 mM NaCl, 20 mM NaF, 3 mM EGTA, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM p-nitro phenylphosphate, 1 mM benzamidine, 0.1 mM PMSF and 1% v/v NP-40) for 30 min at 4°C. Insoluble material was cleared from lysates by centrifugation (13000 xg for 5 min at 4°C). Immunoprecipitates were prepared by incubating equal quantities of the supernatant (approximately 800 μg) with 1 μg of polyclonal anti-p70^{s6k} antibody for 2 h at 4°C with mixing and with 2 μg Pansorbin™ for a further 2 h. Immunocomplexes were washed twice in lysis buffer and twice in the same

buffer without detergents. To confirm the recovery of $p70^{s6k}$ in immunoprecipitates and analyze mobility shifts, samples were reconstituted in Laemmli SDS sample buffer, resolved by SDS-PAGE on 7.5% polyacrylamide mini-gels and immunoblotted for $p70^{s6k}$ as described above.

2.4.4.2 *In vitro* $p70^{s6k}$ activity assay

Immunoprecipitates were washed twice in kinase buffer (20 mM HEPES, pH 7.4 containing 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 3 mM EGTA, 0.2 mM Na_3VO_4 and 2 mM dithiothreitol) and resuspended in 25 µl kinase buffer containing 100 µM $S6^{231-239}$ and 10 µM cAMP-dependent protein kinase (PKA) inhibitor peptide (TYADFIASGRTGRRNAI). Reactions were started on addition of [γ-^{32}P]ATP (1 µCi 10 µM), incubated for 20 min at 30°C and terminated by the addition of 10 µl of 300 mM H_3PO_4. Radiolabelled phosphopeptide bound to the filter was quantified by ion exchange chromatography as described for the MAP kinase activity assay in Section 2.4.1. In every assay, blanks were prepared from a sample of kinase buffer reaction mixture in the absence of immunopurified $p70^{s6k}$. Protein kinase activity is expressed as fold stimulation over control of [^{32}P]phosphate incorporated into ribosomal S6 peptide.

2.4.5 Solid phase c-Jun N-terminal kinase (JNK) activity assay

C-Jun N-terminal kinase activity was measured in affinity precipitates of c-Jun bound to a recombinant truncated N-terminus of c-Jun (c-Jun_{5-89}) substrate immobilized on glutathione (GSH)-sepharose beads (Dai *et al.*, 1995). Stimulated cells were lysed in solubilization buffer (20 mM HEPES pH7.7, 50 mM NaCl, 0.1 mM EDTA, 0.2 mM PMSF, 0.5 mg/ml leupeptin, 0.5 mg/ml aprotinin, 1% (v/v) Triton X-100). Aliquots (150 µg) of solubilized cell extracts, clarified by centrifugation, were added to 1 µg of GST-c-Jun_{5-89}/GSH-sepharose beads and mixed for 3 h at 4°C. The sepharose beads were collected by centrifugation and washed three times in solubilization buffer and once in kinase buffer (25 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT). The beads were resuspended in 25 µl of kinase buffer and the reaction started by the addition of [γ-^{32}P]ATP (20 µM, 2 µCi) and incubated for 30 min at 30°C. The reaction was terminated by adding 10 µl 4x Laemmli SDS sample buffer. Samples were boiled for 5

minutes and proteins resolved by SDS-PAGE (as described in Section 2.3.2) on 11 % polyacrylamide gels. Gels were dried and incorporation of [^{32}P] into GST-c-Jun$_{5-89}$ detected by autoradiography (Section 2.3.3).

2.4.6 Solid phase p38/reactivating kinase activity assay

The activity of p38 MAP kinase was assayed *in vitro* by a procedure based on Bogoyevitch *et al.* (1996) and essentially as detailed for the c-Jun kinase assay (Section 2.4.5) except that cell extracts were prepared in a solubilization buffer containing 20 mM Tris-HCL pH7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 20 mM NaF, 2.5 mM β-glycerophosphate, 0.2 mM PMSF, 0.5 mg/ml leupeptin and 0.5 mg/ml aprotinin and p38 affinity purified by incubating lysates with GST-MAPKAP kinase-2 immobilized on GSH-sepharose. The kinase reaction was carried out in the same buffer as that used for the c-Jun activity assay at pH 7.5.

2.4.7 Measurement of *in vitro* phosphatidylinositol kinase activity

Phosphatidylinositol kinase activity was measured in anti-phosphotyrosine immunoprecipitates as described by Ruderman *et al.* (1990) or anti-FAK immunoprecipitates.

2.4.7.1 Immunoprecipitation of tyrosine phosphorylated proteins

Cells in 50 mm^2 culture dishes were stimulated with agonists, rinsed in PBS and lysed in ice-cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 200 µM Na_3VO_4, 10 mM $Na_4P_2O_7$ and 100 mM NaF). Tyrosine phosphorylated proteins were immunopurified from cell lysates with 1 µg anti-phosphotyrosine antibody coupled to 2 µg Pansorbin™ as described previously. Recovery of tyrosine phosphorylated p85 was determined by SDS-PAGE and p85 immunoblotting (as described in Sections 2.3.2., 2.3.4 & 2.3.5).

2.4.7.2 Immunoprecipitation of Focal Adhesion Kinase ($p125^{FAK}$)

After treatment, cells in 6-well plates were washed twice with 0.5 ml PBS and solubilized in a lysis buffer containing 50 mM HEPES, 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 2mM EGTA, 10 mM $Na_2P_2O_7$, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.5 mM PMSF, 0.5 mM benzamidine, 1 % Triton X-100, 0.25 % (w/v) deoxycholate. Aliquots of supernatent containing approximately 100 μg protein were clarified by centrifugation and incubated with 2 μg of anti- FAK antibody for 2 h at 4°C with gentle mixing. A bridge for binding the mouse monoclonal anti-FAK antibody to protein A agarose was prepared by mixing 10 mg protein A agarose with 20 μg rabbit anti-mouse IgG for 3 hours at 4°C. The antibody-Protein A agarose complex was washed once in lysis buffer and incubated with the lysate/anti-FAK mixture for a further 2 h. Immunocomplexes were collected by centrifugation and washed as described for immunoprecipitation of other proteins.

2.4.7.3 Phosphatidylinositol kinase activity assay

Immunocomplexes were resuspended in 50 μl assay buffer (100 mM HEPES pH 7.4, 200 mM NaCl and 1 mM EGTA) to which was added 20 μl of a sonicated equimolar (0.2 mg/ml) mixture of phosphatidylinositol and phosphatidylserine. To start the reaction, [γ-^{32}P]ATP (50 μM, 10 μCi) was added and the mixture incubated for 30 min at 30°C. Reactions were terminated by the addition of 600 μl of chloroform/methanol/11.6 M HCl (3:4:8, v/v/v). After mixing vigorously and centrifugation to separate the phases, the organic layer containing phospholipid products was removed. This phase was washed a further two times by phase separation with 500 μl of chloroform/methanol/11.6 M HCl/0.1 M HCl (2:1:2:2.5, v/v/v/v) to remove contaminating water soluble materials. Phosphoinositides were concentrated *in vacuo* in a Howe Gyrovap GT™ vacuum drier, dissolved in 25 μl chloroform/methanol (1:1, v/v) and resolved by thin-layer chromatography on Silica Gel-60 plates in a solvent tank containing methanol/chloroform/ammonia solution/water (11:7:2:2.5). Radiolabelled phosphatidylinositol phosphate was detected by autoradiography (as outlined in Section 2.3.3.) and scraped from dried plates for quantification by liquid scintillation counting.

2.5 MEASUREMENT OF DNA SYNTHESIS

DNA synthesis was measured by the incorporation of [^{3}H]thymidine into DNA of replicating cells. Preliminary studies were conducted to find the optimum period for DNA synthesis by examining the time course for agonist-stimulated [^{3}H]thymidine incorporation using pulse labelling. Quiescent cells in triplicate wells of 24-well plates were stimulated with agonists for different times between 12 and 48 h at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and pulsed with 0.1 μCi/ml [methyl-^{3}H]thymidine 4 h before the end of each incubation. The maximal rate of [^{3}H]thymidine incorporation was found to be at approximately 20 h for all agonists tested and in all subsequent studies, [methyl-^{3}H]thymidine (0.1 μCi/ml) was added for the final 4 h of 24 h agonist exposure. After 24 h, cells were washed twice with 0.5 ml of ice-cold PBS and fixed by washing three times with 0.5 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) and twice with 0.5 ml of ice-cold ethanol. Cells were solubilized in 0.5 ml of 0.3 M NaOH and left for over 30 minutes. The content of each well was transferred to 5 ml scintillation vials and incorporation of [^{3}H]thymidine into TCA-insoluble fractions, representing thymidine localized in the nucleus, determined by liquid scintillation counting. In some instances, the acid-soluble fraction indicative of thymidine uptake into the cytosol was counted. In all cases, however, this pool was found under the parameters used to be negligible compared to the acid-insoluble fraction.

2.6 DETECTION AND ANALYSIS OF NUCLEIC ACID

2.6.1 Isolation of total RNA from cells and tissue

Disposable sterile plasticware was used throughout and as with all solutions used, treated with 0.1% diethyl pyrocarbonate (DEPC) and sterilized by autoclaving prior to use. Approximately 200 mg of fresh tissue was transferred to a chilled sterile tube containing 2 ml of GTC denaturing solution (1 M sodium citrate pH7, 10 % sarcosyl, 4 M guanidium isothiocyanate, 0.7% (v/v) β-mercaptoethanol). Tissues were chopped finely and disrupted using a teflon homogenizer or fine-gauge needle attached to a 5 ml syringe. Aliquots of samples (500 μl) were transferred to sterile Eppendorf tubes to which was added 2M sodium acetate pH4 (50 μl) to precipitate nuclear material, and mixed by vortexing. Water-saturated phenol (500 μl) was added followed by chloroform/isoamyl alcohol (49:1) (100 μl) and the

mixture vortexed vigorously for 15 seconds to form an emulsion. Samples were left on ice for 15 minutes before centrifugation at 14,000g for 20 minutes to separate organic and aqueous phases. Samples were allowed to warm to room temperature slightly and the upper aqueous phase was carefully transferred to a fresh tube avoiding the interphase. To precipitate RNA, 800 µl of isopropanol was added to this solution, mixed well, and left for at least 1 h at -20°C. The RNA was recovered by centrifugation at 14,000g for 10 min at 4°C and the supernatent removed and discarded. The pellet was washed twice by precipitation with 75 % ethanol (200 µl) and collected by centrifugation. The nucleic acid was allowed to dry at room temperature until appearing translucent, resuspended in DEPC-treated H_2O or TE buffer (10 mM Tris HCl pH7.4, 1 mM EDTA) and stored at -70°C.

2.6.2 Reverse-transcription of poly(A) mRNA

Messenger RNA (mRNA) containing the Poly(A) tract at the 3' terminus, served as templates for the synthesis of first strand cDNA by recombinant DNA-dependent RNA polymerase (reverse transcriptase) derived from Moloney Murine Leukemia Virus using oligo$(dT)_{12\text{-}18}$ primer (SUPERSCRIPT™ II). Approximately 1-5 µg of total RNA extracted in Section 2.6.1 was added to 10 µg oligo $(dT)_{12\text{-}18}$ and heated to 70°C for 10 minutes and chilled quickly on ice. The contents of the tube were collected by brief centrifugation and made up to a 20 µl reaction volume containing first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.01 mM DTT and 0.5 mM dNTP mix (0.05 mM each of dATP, dGTP, dCTP, and dTTP at neutral pH). The contents of the tube were mixed gently and incubated at 42°C for 2 min. SUPERSCRIPT™ II RNAse H- reverse transcriptase (200 units), was added to the reaction mixture, the contents mixed again by pipetting gently up and down, and incubated for 90 min at 42°C. The reverse transcriptase was inactivated by heating the reaction to 70°C for 15 min.

2.6.3 Polymerase chain reaction (PCR) amplification of PAR-2 cDNA

First strand cDNA or mouse PAR-2 cDNA were used as templates for amplification by PCR using a recombinant thermostable DNA polymerase from *Thermus brockianus* (DYNAZYME™ II). A reaction mixture was added to 2 µl template cDNA (from Section 2.6.2) containing the following components at the indicated concentrations in a final volume of

100 µl in a GeneAmp thin-walled reaction tube: 0.1 µM each of forward and reverse PAR-2 primers (see Section 2.6.4 for primer design), 0.2 mM dNTP mix, PCR buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100 and DYNAZYME™ II DNA polymerase (2.5 U). The contents of the tube were mixed gently and overlayed with 100 µl of mineral oil to prevent evaporation of the sample during thermal cycling. The reaction was heated at 94°C for 10 min to denature the cDNA. Automated amplification was performed by a Perkin Elmer 480 thermal cycler and allowed to proceed for 35 cycles according to the conditions for PCR detection of PAR-2 described by Al-Ani *et al.* (1995) using the same primers outlined in Section 2.6.4. Each cycle began with a 1 min denaturation period at 94°C, followed by a 1 min annealing time at 55°C and a primer extension period of 1 min at 72°C. When the cycles had finished, samples were quenched at 4°C and transferred to -20°C for storage.

2.6.4 Design of PAR-2 primers

Primers were synthesized by an Applied Biosystem 392 DNA/RNA Synthesizer based on the published PAR-2 nucleotide sequence accessed from GenBank™ data base (Z48043) using Gene Runner™ Version 3.04 by Hastings Software. Primers designed were checked using NCBI Blast to find nearly identical sequences. The PAR-2 primers for PCR were as follows: PAR-2 forward primers, (PF1) 5' CAC CAC CTG TCA CGA TGT GCT 3' and (PF2) 5' TGG CTG CTG GGA GGA GGT ATC AC 3'; and PAR-2 reverse primers, (PR1) 5' CCC GGG CTC AGT AGG AGG TTT TAA CAC 3' and (PR2) 5' TGC CAT GTA GGT GGT AGG AGA TC 3'. The forward primers were targeted to sequences encoding amino acid residues within the second extracellular loop of the mouse PAR-2 receptor (PF1) and towards the N-terminus from the putative trypsin cleavage site on the extracellular domain (PF2). The reverse primers were directed against a sequence encoding the mouse PAR-2 C-terminus (PR1) and residues located within the first extracellular loop of the mouse PAR-2 receptor (PR2). Primer pairs PF1/PR1 (Al-Ani *et al.*, 1995) and PF2/PR2 were used to screen cDNA from tissues and cells. PF2/PR2 encompasses a 710 bp region of mouse PAR-2 spanning a 310 bp intron sequence. The detection of a 400 bp PCR product using this primer pair can confirm the absence of intron-derived cDNA in the RT product. Signals yielded by the PAR-2 primer pairs were normalized to the PCR product generated by an intron-spanning actin primer pair (Watson *et al.*, 1992): actin forward primer (AF1) 5' CGT GGG CCG CCC

TAG GCA CCA 3'; actin reverse primer (AR1) 5' TTG GCC TTA GGG TTC AGG GGG 3'.

2.6.5 Spectroscopic quantitation of DNA and RNA

The concentration and purity of nucleic acid was quantified by spectroscopic measurement of the absorbance of UV irradiation at wavelengths of 260 nm and 280 nm. An OD_{260} of 1 corresponded to approximately 50 μg/ml for double stranded DNA or 40 μg/ml for RNA while OD_{260}/OD_{280} values of 1.8 and 2.0 indicated that DNA and RNA samples, respectively, were pure.

2.6.6 Separation of PCR products by agarose gel electrophoresis

PCR products were separated by 2% agarose gel electrophoresis. An appropriate amount of powdered agarose was added to a measured quantity of TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0). The agarose was dissolved by heating the slurry in a microwave for 2 min on full power. The mass of the solution lost by evaporation was replenished with water. The solution was allowed to cool to 60°C and the fluorescent dye ethidium bromide was added to give a final concentration of 0.5 μg/ml. The agarose solution was immediately poured into the gel casting apparatus in a horizontal slab configuration with loading combs in place and left to set for at least 30 min at room temperature. The reservoir of the electrophoresis apparatus was filled with enough electrophoresis buffer (1x TBE) to submerge the gel. Aliquots (15 μl) of cDNA samples were mixed with 2 μl gel loading buffer (0.5% (w/v) xylene cyanol, 0.5% (w/v) bromophenol blue in Tris-Borate EDTA buffer) and loaded into the wells of the gel. A mixture of DNA markers of known sizes was also loaded to estimate the size of the DNA fragment of interest. An electrical current of a potential difference of 70 V was applied across the gel at room temperature for approximately 2 h by a Kodak Biomax MBP300 power supply. The gel was examined under ultraviolet irradiation and the transmitted light photographed by a Polaroid DS34 instant camera using an exposure of 2 seconds at an aperture of F22.

2.6.7 Automated DNA sequencing

The nucleotide sequence of cDNA was determined by a method based on Sanger (1977) involving chain-terminating reactions using fluorescent dye-labelled dideoxynucleotides (ABI PRISM™ kit). The PCR product (from Section 2.6.3) was purified using a Qiagen QUIAQUICK SPIN™ PCR purification kit. The cycle sequencing reaction was performed on 30 ng of purified PCR product in a final volume of 20 µl containing Ready Reaction Premix (ABI PRISM™)(Tris HCl pH 9.0, $MgCl_2$ and AmpliTaq DNA Polymerase FS) and 3.2 pmol of either one of the primers used in Section 2.6.4. The dNTP mix included deoxyinosine triphosphate (dITP) in place of dGTP to minimize band compression during separation of extension products by SDS-PAGE, and fluorescent dye-labelled chain-terminating dideoxynucleotides (A-dye terminator, C-dye terminator, G-dye terminator and T-dye terminator) lacking the hydroxyl residue at the 3' position of deoxyribose. Thermal cycling was performed under the same conditions outlined in Section 2.6.3. Unincorporated dye-labelled terminators were removed by precipitating nucleic acid with ethanol and washing the pellet with 75 % ethanol (as described in section 2.6.1). The extension products of the sequencing reaction, a mixture of fluorescent fragments of DNA that differ in length by a single nucleotide and terminate at positions occupied by every base in the template strand, were resuspended in a gel-loading buffer (de-ionized formamide, 50 mM EDTA pH 8.0), loaded into a single lane of a denaturing polyacrylamide sequencing gel and resolved by electrophoresis. The order of nucleotides was determined by real-time detection by an Applied Biosystem 373A DNA sequencer and data was automatically processed by GENESCAN software.

2.7 DATA ANALYSIS

The values on the abcissa of log concentration response curves are arithmetic values. Data are expressed as mean±s.e.m. for three or more separate observations. The statistical significance of differences between mean values from control and treated groups were determined by Student's *t* tests, unpaired and paired where applicable, using Minitab software. Two tailed probability values of less than 0.05 ($P < 0.05$) were considered to be significant.

CHAPTER 3

CHARACTERIZATION OF AGONIST-STIMULATED ACTIVATION OF THE MITOGEN ACTIVATED PROTEIN KINASE CASCADE IN BOVINE PULMONARY ARTERY FIBROBLASTS

3.1 INTRODUCTION

The mitogen-activated protein (MAP) kinase cascade is thought to play a key role in the propagation of signals both by growth factors which activate protein tyrosine kinase receptors and by G-protein-coupled receptor agonists (reviewed by Malarkey *et al.*, 1995a). Considerable evidence supports a role for the p42 and p44 MAP kinases in the transmission of proliferative signals. MAP kinases are rapidly activated after treatment of cells with agents that behave as mitogens. Furthermore, a number of events regulated by MAP kinase are believed to be important for mitogenesis. These include the phosphorylation and activation of the 90 kDa ribosomal S6 protein kinase ($p90^{S6k}$) (Sturgill *et al.*, 1988, Wood *et al.*, 1992), cytosolic phospholipase A_2 (Lin *et al.*, 1993, Nemenoff *et al.*, 1993) the nuclear transcription factor $p60^{TCF}$/ELK-1 (Gille *et al.*, 1992, Marais *et al.*, 1993) leading to the activation of processes regulating c-Fos expression (Kortenjann *et al.*, 1994) and stimulation of glucose uptake (Merrall *et al.*, 1993). An obligatory role for p42 and p44 MAP kinases in growth factor-stimulated mitogenic responses *in vivo* has been demonstrated in fibroblasts and smooth muscle cells using p42 and p44 MAP kinase oligonucleotide antisense (Pagès *et al.*, 1993, Sale *et al.*, 1995, Robinson *et al.*, 1996) and a p44 MAP kinase-deficient mutants (Pagès *et al.*, 1993). The mitogenic response to growth factors is also inhibited by MKP-1, a MAP kinase-specific phosphatase (Brondello *et al.*, 1995). Furthermore, a kinase-defective MAP kinase mutant has been shown to prevent v-Raf -dependent transformation in fibroblasts (Kortenjann *et al.*, 1994).

Extensive work, primarily investigating signalling processes mediated by tyrosine kinase encoded receptors, has elucidated the sequential events leading to activation of the p42 and p44 MAP kinases (reviewed in Section 1.6). Activation of p42 and p44 MAP kinases requires phosphorylation on both tyrosine and threonine residues within a specific TEY motif (Anderson *et al.*, 1990, Payne *et al.*, 1991) brought about by the dual specificity kinases of the MEK family (Matsuda *et al.*, 1992, Nakielny *et al.*, 1992). MEK activation, in turn, is regulated by serine phosphorylation catalysed by MEK-activating kinases including members of the Raf family of proto-oncogene products (Kyriakis *et al.*, 1992, Reuter *et al.*, 1995) of which c-Raf-1 is the most well characterised. Similar strategies using transfected dominant-negative and constitutively active mutants have identified roles for the different upstream components of this signalling pathway in growth. For example, permanent activation of the p42/p44 MAP kinase pathway upon expression of constitutively activated forms of MEK and

Raf-1 caused by site-directed mutagenesis induces mitogenesis and transformation in fibroblasts (Storm *et al.*, 1990, Cuadrado *et al.*, 1993, Cowley *et al.*, 1994, Mansour *et al.*, 1994). In addition, pharmacological intervention with the specific MEK inhibitor PD098059 has indicated an obligatory involvement of MEK in regulating growth-factor-induced DNA synthesis in fibroblasts (Dudley *et al.*, 1995).

Evidence in the literature supports a role for MAP kinase in the mitogenic effect of thrombin. MAP kinase has been shown to be activated in response to thrombin in CCL39 hamster lung fibroblasts (Kahan *et al.*, 1992, Meloche *et al.*, 1993, Vouret-Craviari *et al.*, 1993b) and vascular smooth muscle cells (Rao *et al.*, 1995, Molloy *et al.*, 1996) in which thrombin also evokes a mitogenic response. Furthermore, in fibroblasts, persistent activation of MAP kinase during G1 progression appears to be essential for triggering entry into the S phase of the cell cycle (Meloche *et al.*, 1992, Vouret-Craviari *et al.*, 1993b) and correlates with a rapid translocation to the nucleus (Chen *et al.*, 1992, Lenormand *et al.*, 1993). Most notably, use of both antisense and kinase-deficient mutants has revealed that p44 MAP kinase is required for thrombin-stimulated fibroblast proliferation (Pagès *et al.*, 1993).

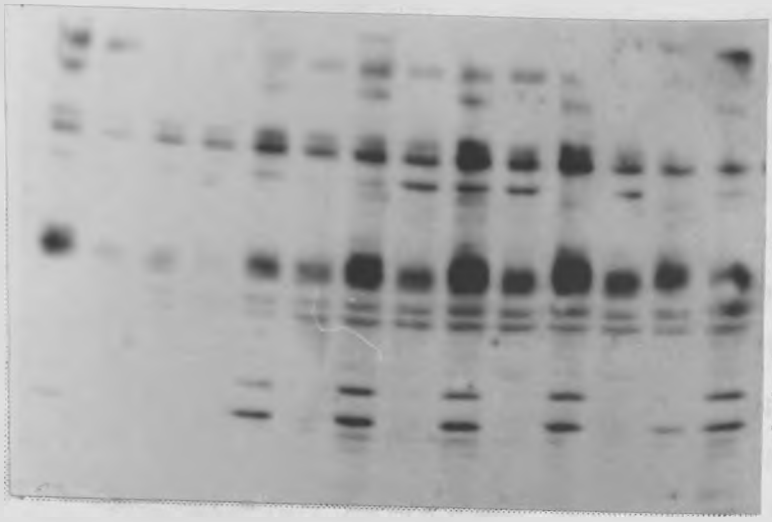
Most of the present information regarding MAP kinase activation by thrombin has been gained from studies characterizing responses in cultured cell lines, most notably fibroblasts. Very few investigations have addressed whether analogous responses are evoked in a physiologically relevant cell type. In addition, many of these studies have examined MAP kinase in isolation and not the activation of direct upstream regulators of this signalling cascade in parallel. The aims of this chapter are twofold; firstly, to characterize the activation of p42 and p44 MAP kinases to thrombin and a range of other agonists in BPA fibroblasts and consider some of the aspects of the control of these signalling molecules including regulation by MEK and Raf-1, and secondly, to determine the role that this pathway plays in mitogenesis.

3.2 Agonist-stimulated tyrosine phosphorylation and activation of p42 and p44 MAP kinase in BPA fibroblasts

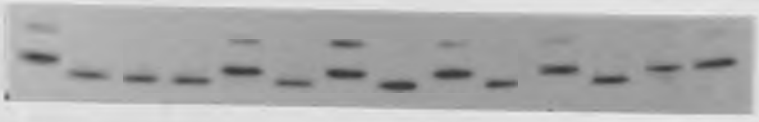
Thrombin (300 nM) evoked a time and concentration -dependent tyrosine phosphorylation of a set of proteins in serum-deprived BPA fibroblasts as assessed by anti-phosphotyrosine immunoblotting of total cell lysates (Fig. 3.1.1A). Two phosphotyrosyl protein bands migrating in the 40-45 kDa molecular weight range co-migrated with

immunoreactive bands detected when phosphotyrosine immunoblots were stripped and re-probed with a monoclonal antibody raised against the p42 and p44 MAP kinase isoenzymes as described in Section 2.3.6 (Fig. 3.1.1B). The tyrosine phosphorylation by thrombin of the proteins described correlated with an apparent decrease in the electrophoretic mobility of both p42 and p44 MAP kinases such that on immunoblotting with anti-MAP kinase antibody, each immunoreactive band was shifted to a higher apparent molecular weight (Fig. 3.1.1B). This slower migration of MAP kinases is believed to be a consequence of increased phosphorylation, presumably reflecting an alteration in the SDS binding and/or conformation of these proteins, and is further evidence to support the likelihood that the bands identified by phosphotyrosine immunoblotting represent the two tyrosine phosphorylated MAP kinase isoforms. Phosphorylation on specific tyrosine and threonine residues of MAP kinases is required for catalytic activation (Anderson *et al.*, 1990, Payne *et al.*, 1991). To confirm that the apparent phosphorylation of MAP kinases was accompanied by kinase activation, the activity of MAP kinase in response to thrombin was assessed by measuring the ability of MAP kinase to phosphorylate a specific substrate peptide $EGFR^{661-680}$ *in vitro* (Fig. 3.1.1C & Table 3.1). Stimulation of BPA fibroblasts with thrombin resulted in an increase in MAP kinase activity with kinetics which were comparable to the thrombin-stimulated tyrosine phosphorylation and decrease in electrophoretic mobility of the 42 and 44 kDa proteins. The onset of MAP kinase activation in response to thrombin often varied in a number of observations from different fibroblast cultures explaining why in the immunoblot shown, a full shift in MAP kinase electrophoretic mobility is evident at 5 minutes (Fig. 3.1.1B) while kinase activity remained low (Fig. 3.1.1C). In most cases, MAP kinase activity increased slowly after 2 minutes to reach a maximum between 15 and 30 minutes (approximately 3.5 fold) before slowly declining, although still apparent at 2 hours (Fig. 3.1.1). Basal MAP kinase activity in BPA fibroblasts tended to be variable, and in these experiments were slightly elevated, thereby reducing the apparent fold stimulation in response to thrombin. To determine the concentration-dependency of MAP kinase activation by thrombin, the intensity of phosphotyrosyl protein bands of apparent molecular weight of 42 and 44 kDa were quantified by densitometry and expressed as percentage of maximal phosphorylation (Fig. 3.1.2C). The threshold concentration of thrombin was around 3 nM (0.06 U/ml), the half-maximally effective concentration at approximately 130 nM (2.61 U/ml) and maximum effects observed at 300 nM (6.03 U/ml) thrombin (Fig. 3.1.2).

A. pTYR



B. MAPK



	S	C	T	C	T	C	T	C	T	C	T	C	T	P
min		2	2	5	5	15	15	30	30	60	60	120	120	

C.

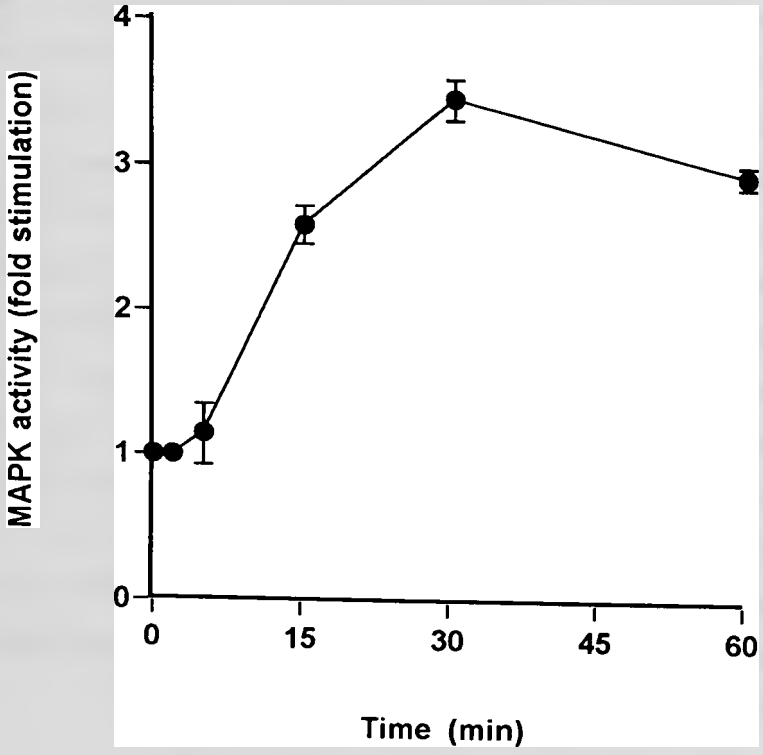


Figure 3.1.1 Time course for thrombin-stimulated MAP kinase activation in BPA fibroblasts. Cells deprived of serum for 48 hours were incubated with thrombin (300 nM) (T) or vehicle (C) for the times indicated, or PDGF (30 ng/ml) (P) or NCS (10%) (S) for 10 minutes. Lysates were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (**A**) and blots reprobed for MAP kinase (**B**), or assayed for *in vitro* MAP kinase activity (**C**) as described in Sections 2.3 & 2.4.1. Bands corresponding to the p42 and p44 MAP kinase isoforms and the mobilities of molecular weight markers in kDa are indicated on the right of immunoblots, each of which is typical of four replicative experiments. Kinase activity is expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one assay performed in duplicate and is representative of three separate experiments (control: 187.3±37.1 pmol Pi min^{-1} mg^{-1} protein).

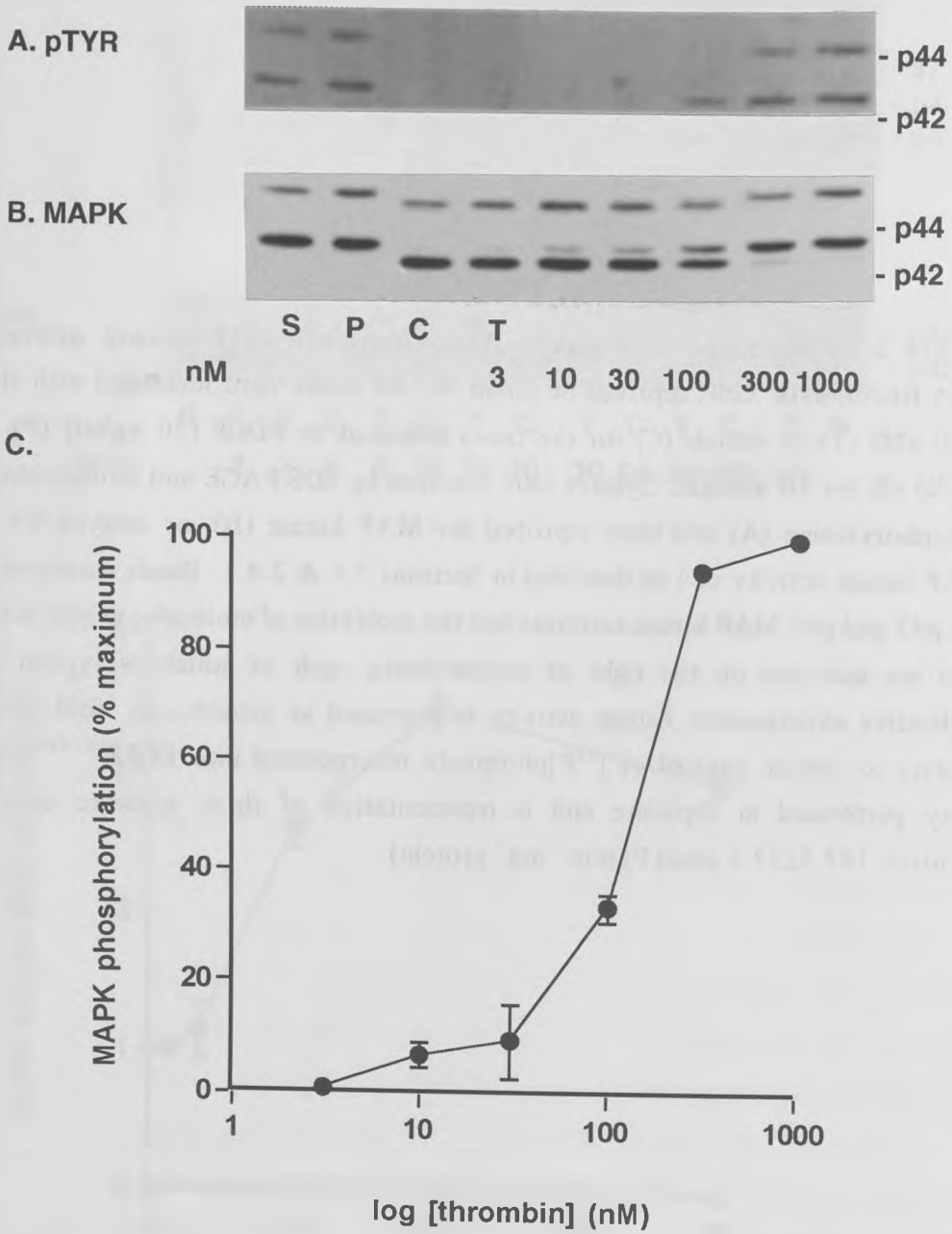

A. pTYR
- p44
- p42
B. MAPK
- p44
- p42
S P C T
nM 3 10 30 100 300 1000
C.
MAPK phosphorylation (% maximum)
100
80
60
40
20
0
1
10
100
1000
log [thrombin] (nM)

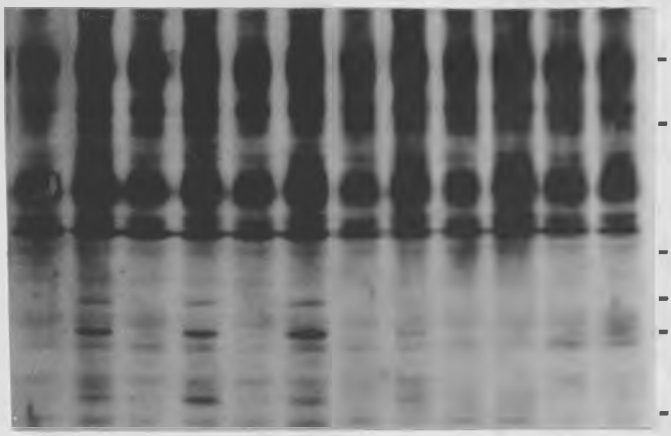
Figure 3.1.2 Concentration-dependency of thrombin-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were incubated with thrombin (T) at the concentrations indicated or vehicle (C) for 20 minutes, or PDGF (30 ng/ml) (P) or NCS (10%) (S) for 10 minutes. Lysates were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (**A**) and blots reprobed for MAP kinase (**B**) as outlined in Section 2.3. Bands corresponding to the p42 and p44 MAP kinase isoforms are indicated on the right of immunoblots, each of which is representative of four replicative experiments. The extent of tyrosine phosphorylation of MAP kinase was quantified by densitometric analysis of a typical phosphotyrosine immunoblot (**C**) and is expressed as percentage of maximal phosphorylation of the p42 isoform evoked by thrombin (1 μM).

The growth factor PDGF-BB also stimulated the phosphorylation of multiple proteins on tyrosyl residues, including species which migrate at the same apparent molecular weight as the p42 and p44 MAP kinases (Fig. 3.2.1A). Activation of MAP kinases was monitored by the appearance of electrophoretic mobility shifts associated with the two isoforms (Fig. 3.2.1B) which corresponded to the temporal increase in MAP kinase activity following PDGF treatment (Fig. 3.2.1C). Increased MAP kinase activity in response to PDGF (30 ng/ml) was detectable as early as 2 minutes, and reached a peak at approximately 10 minutes causing approximately 6 fold increase in activity (Table 3.1) and thereafter declined to pre-stimulation levels by 2 h (Fig. 3.2.1). In the phosphotyrosine immunoblot depicted, the tyrosine phosphorylation of p42 and p44 MAP kinases at PDGF exposure times greater than 15 minutes, when kinase activity was declining, was beyond the limits of immunodetection with the phosphotyrosine antibody used. Activation of MAP kinase by PDGF in BPAFs was also concentration-dependent requiring a threshold concentration of 3 ng/ml (0.12 nM), maximal at 100 ng/ml (4.07 nM) with an EC_{50} value of approximately 15 ng/ml, as assessed by densitometry of phosphotyrosine immunoblots (Fig. 3.2.2.).

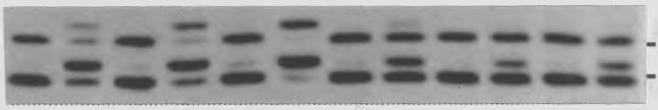
The synthetic protein kinase C activator phorbol 12-myristate 13-acetate (PMA) appeared to activate MAP kinase weakly compared to PDGF and serum, as judged by the density of the immunoreactive phosphotyrosyl bands likely to be the tyrosine phosphorylated MAP kinase isoforms, and the incomplete shift in MAP kinase electrophoretic mobility that was often observed following treatment with this agent (Fig. 3.3). Furthermore, this effect of phorbol ester was found to be variable between experiments. Activation of MAP kinase by PMA (100 nM) was maximal at 5 minutes, declined thereafter before falling to control levels by 2 hours (Fig. 3.3A). The threshold and maximally effective concentration of PMA for evoking this response was 10 nM and 300 nM respectively (Fig. 3.3B). Comparison of the efficacy of PMA to stimulate *in vitro* MAP kinase activity with thrombin and PDGF confirmed that at a maximally-effective concentration and exposure time, the phorbol ester was the weakest in this regard (3.3 fold), followed by thrombin (4.4 fold), while PDGF was the strongest (5.9 fold) and found to be as effective as serum (Table 3.1)

The bioactive serum phospholipid lysophosphatidic acid (LPA) (1-acyl-*sn*-glycerol-3-phosphate) at a concentration (10 μM) shown to stimulate MAP kinase in Cos-1 and EAhy926 endothelial cells (Howe & Marshall, 1993, McLees *et al.*, 1995) caused only a very weak and transient MAP kinase activation as assessed by mobility shifts, reaching a maximum at 5 minutes yet undetectable at 15 minutes and longer stimulation durations (not

A. pTYR



B. MAPK



	C	P	C	P	C	P	C	P	C	P	C	P
min	2	2	5	5	15	15	30	30	60	60	120	120

C.

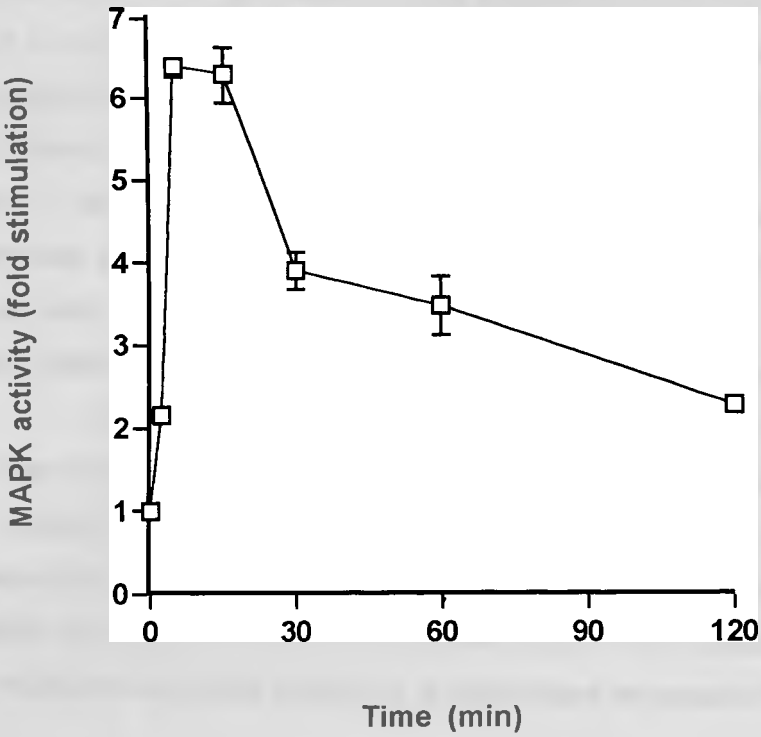


Figure 3.2.1 Time course for PDGF-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were incubated with PDGF-BB (30 ng/ml) (P) or vehicle (C) for the times indicated. Lysates were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (**A**) and blots reprobed for MAP kinase (**B**), or assayed for *in vitro* MAP kinase activity (**C**) as described in Sections 2.3 & 2.4.1. Bands corresponding to the p42 and p44 MAP kinase isoforms and the mobilities of molecular weight markers in kDa are indicated on the right of immunoblots, each of which is typical of three replicative experiments. Kinase activity is expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one assay performed in duplicate and is representative of three separate experiments (control: 43.4±1.3 pmol Pi min^{-1} mg^{-1} protein).

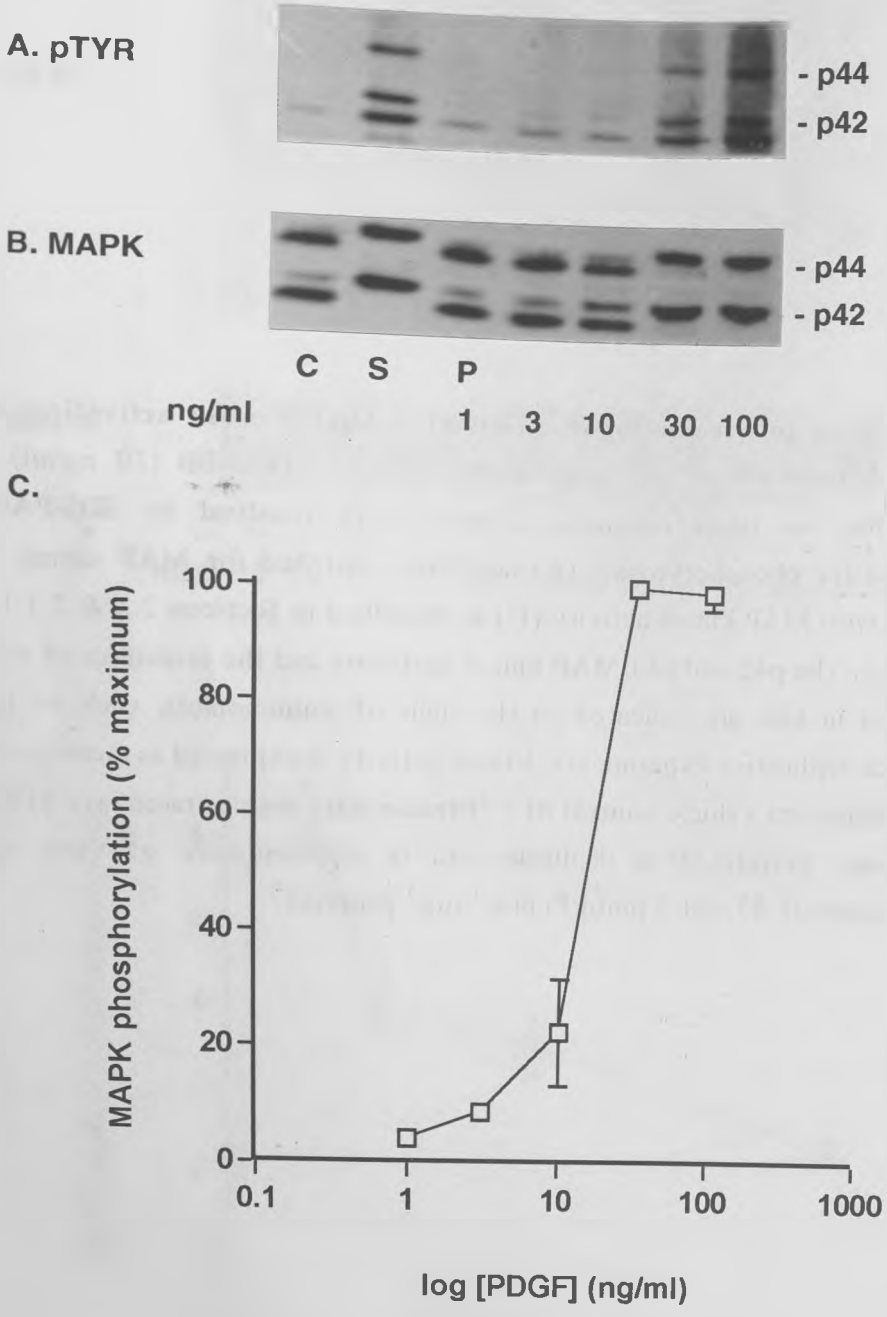
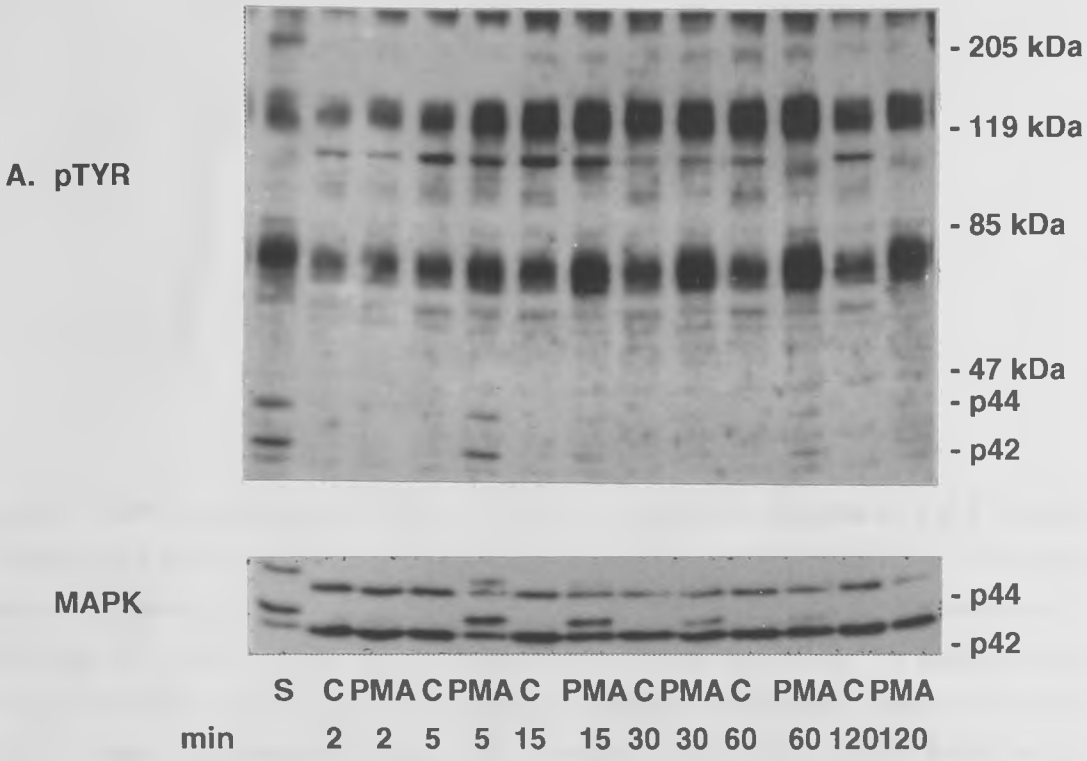

A. pTYR
- p44
- p42
B. MAPK
- p44
- p42
C
S
P
ng/ml
1
3
10
30
100
C.
MAPK phosphorylation (% maximum)
100
80
60
40
20
0
0.1
1
10
100
1000
log [PDGF] (ng/ml)

Figure 3.2.2 Concentration-dependency of PDGF-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were incubated with PDGF-BB (P) at the concentrations indicated, vehicle (C) or NCS (10%) (S) for 10 minutes. Lysates were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (**A**) and blots reprobed for MAP kinase (**B**) as outlined in Section 2.3. Bands corresponding to the p42 and p44 MAP kinase isoforms are indicated on the right of immunoblots, each of which is representative of three replicative experiments. The extent of tyrosine phosphorylation of MAP kinase was quantified by densitometric analysis of a typical phosphotyrosine immunoblot (**C**) and is expressed as percentage of maximal phosphorylation of the p42 isoform evoked by PDGF (30 ng/ml).


A. pTYR
- 205 kDa
- 119 kDa
- 85 kDa
- 47 kDa
- p44
- p42
MAPK
- p44
- p42
S C PMA C PMA C PMA C PMA C PMA C PMA
min 2 2 5 5 15 15 30 30 60 60 120 120

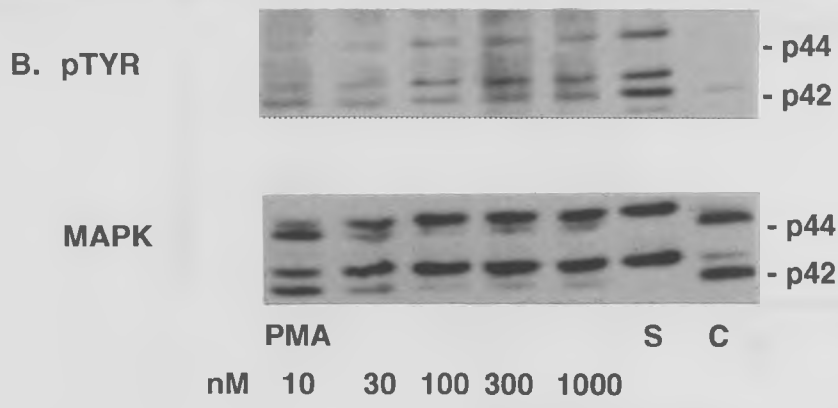

B. pTYR
- p44
- p42
MAPK
- p44
- p42
PMA S C
nM 10 30 100 300 1000

Figure 3.3 Phorbol 12-myristate 13-acetate (PMA)-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were incubated with PMA (100 nM) or vehicle (C) for the times indicated (**A**), PMA for 5 minutes at the concentrations indicated (**B**) or NCS (10%) (S) for 10 minutes. Lysates were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (upper panels) and blots reprobed for MAP kinase (lower panels), as described in Section 2.3. Bands corresponding to the p42 and p44 MAP kinase isoforms and mobilities of molecular weight markers in kDa are indicated on the right of immunoblots, each of which is representative of 3-5 replicative experiments.

Treatment	MAPK activity mean±s.e.m. fold stimulation
C	1.0
T	4.37±0.56
P	5.87±0.10
PMA	3.27±0.39
ET	1.27±0.17
S	5.75±1.19

Table 3.1 Agonist-stimulated MAP kinase activity in BPA fibroblasts. Serum-deprived cells were incubated with thrombin (300 nM for 20 minutes) (T), PDGF (30 ng/ml for 10 minutes) (P), PMA (100 nM for 5 minutes), endothelin-1 (100 nM for 5 minutes) (ET-1), NCS (10% for 10 minutes) (S) or vehicle (C). Lysates were assayed for *in vitro* MAP kinase activity as described in Section 2.4.1. Kinase activity is expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from three separate experiments (control: 19.9±8.0 pmol Pi min^{-1} mg^{-1} protein).

shown). Treatment of cells with endothelin-1 (ET-1) (10-300 nM) at any exposure time up to 2 hours did not cause any apparent slowing in the migration of p42 or p44 MAP kinases following SDS-PAGE electrophoresis. Furthermore, the effect of ET-1 on MAP kinase activity was negligible at a concentration (100 nM) and exposure time (5 minutes) that has been found in this laboratory to be maximally-effective at stimulating MAP kinase activity in rat aortic vascular smooth muscle cells (Malarkey *et al.*, 1996) (Table 3.1).

3.3.1 Thrombin and PDGF-stimulated MEK-1/2 activation in BPA fibroblasts

Activation of MAP kinase occurs on phosphorylation of two key amino acid residues brought about by the dual specificity kinase MEK (Crews *et al.*, 1992, Nakielny *et al.*, 1992, Seger *et al.*, 1992). The activity of MEK-1 and MEK-2 was investigated in parallel with *in vitro* MAP kinase activity in immunoprecipitates prepared with an antibody raised against an epitope motif common to both MEK-1 and MEK-2 (MEK-1/2). Western blotting analysis revealed the presence of a single band migrating at an apparent molecular weight of approximately 45 kDa in MEK-1/2 immunoprecipitates but not supernatents resolved by SDS-PAGE (Fig. 3.4.1A). Equivalent amounts of MEK-1/2 were recovered in immunoprecipitates from the lysates of cells treated with either vehicle, thrombin or PDGF at the exposure times found to cause maximal thrombin or PDGF -stimulated MEK-1/2 activity (see below) confirming that the recovery of MEK-1/2 was not affected by agonist stimulation (Fig. 3.4.1). The two MEK isoforms were not resolved under the gel conditions used since they differ by only 1 kDa. However, stripping and reprobing blots with specific monoclonal antisera to MEK-1 and MEK-2 revealed that anti- MEK-1/2 immunoprecipitates contained both MEK-1 and MEK-2 (Fig. 3.4.1).

Both thrombin and PDGF stimulated MEK-1/2 activity in a time-dependent manner, assessed by the incorporation of [^{32}P] into purified recombinant wild type MAP kinase (Fig. 3.4.2A). Phosphorylation of MAP kinase by MEK-1/2 resulted in a corresponding increase in MAP kinase catalytic activity as measured by the sequential phosphorylation of EGFR$^{661-680}$ peptide in a coupled kinase assay (Fig. 3.4.2B). To assess the degree of phosphorylation of wild type MAP kinase attributable to MAP kinase autokinase activity that has been reported previously (Seger *et al.*, 1991, Her *et al.*, 1993), a recombinant kinase-defective MAP kinase mutant (K52R) was compared as a substrate. This mutant contains a lysine to arginine substitution which prevents ATP binding and autophosphorylation at the concentrations of

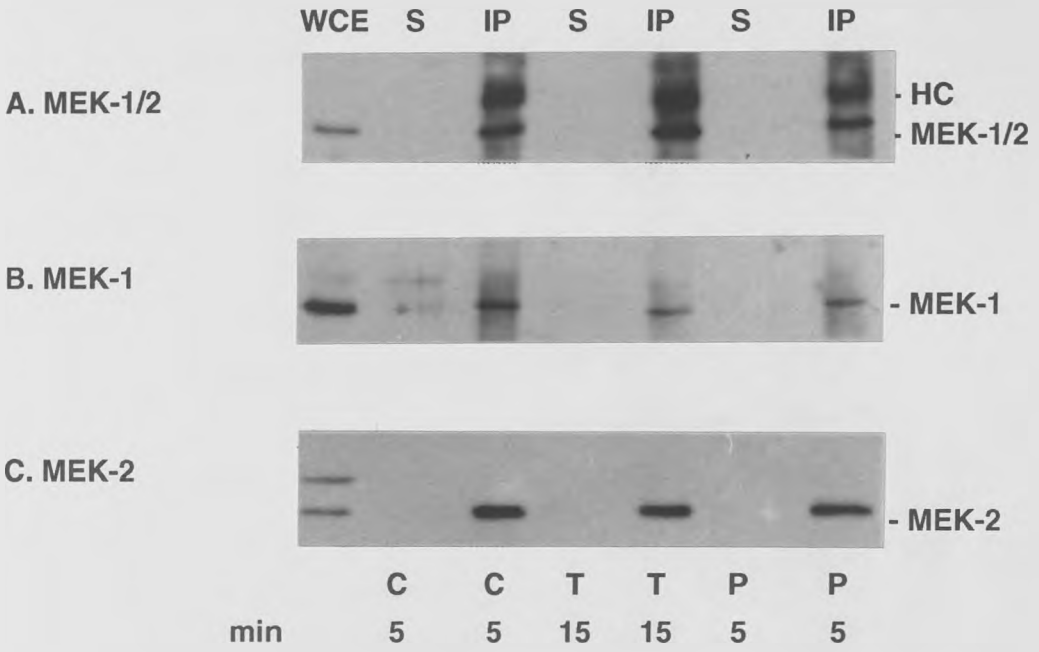


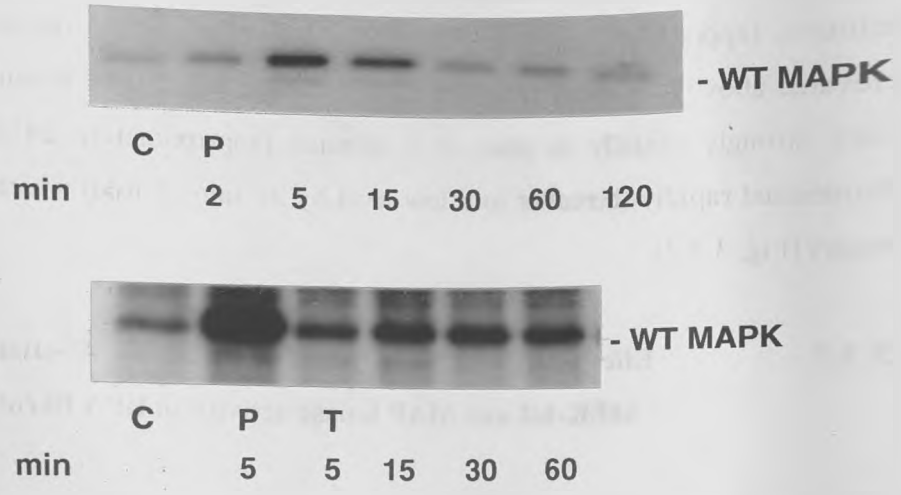
Figure 3.4.1 Immunoprecipitation of MEK-1 and MEK-2 in BPA fibroblasts. Cells were exposed to thrombin (300 nM) (T), PDGF (30 ng/ml) (P) or vehicle (C) for the indicated times. Immunoprecipitates of MEK-1/2 prepared from cell lysates (IP), supernatents (S) or whole cell extracts (WCE) were immunoblotted for MEK-1/2 (**A**) and blots reprobed for MEK-1 (**B**) and MEK-2 (**C**) as described in Sections 2.3 & 2.4.2.1. Bands corresponding to MEK-1 and MEK-2 and the immunoglobulin G heavy chain (HC) are shown on the right of immunoblots, each of which is a representative example from two independent experiments.

ATP used in the MEK assay (Her *et al.*, 1993). Although K52R was phosphorylated to a lesser extent than wild type MAP kinase at an equal concentration and in the presence of the same specific radioactivity and concentration of immunopurified MEK, the relative fold increase in phosphorylation evoked by each agonist was comparable for either substrate (not shown). On exposure to thrombin (300 nM), MEK-1/2 activity increased gradually up to 15 minutes, (approximately 3 fold increase) after which, activity declined slowly yet was still elevated above basal levels at 2 hours. In comparison, PDGF stimulated MEK-1/2 activity very strongly initially to peak at 5 minutes (approximately 24 fold increase). Activity diminished rapidly thereafter to a low level by 30 min (2 fold) which was sustained up to 2 hours (Fig. 3.4.2).

3.3.2 Effect of PD 098059 on thrombin and PDGF -stimulated MEK-1/2 and MAP kinase activity in BPA fibroblasts

The involvement of MEK-1 in the activation of MAP kinase *in vivo* was investigated using PD098059 ([2-(2'-amino-3'-methoxyphenyl)-oxanapthalen-4-one]), a compound that acts both *in vitro* and *in vivo* as a highly specific synthetic inhibitor of MEK-1 (Alessi *et al.*, 1995, Dudley *et al.*, 1995). At a concentration (50 μM) reported to cause near-maximal inhibition of PDGF-stimulated MEK activity in Swiss 3T3 cells (Alessi *et al.*, 1995), PD098059 was found to completely inhibit the activation of MEK-1/2 in response to thrombin in BPA fibroblasts and suppress by 90 % the MEK-1/2 activity evoked by PDGF exposure for 5 minutes (Fig. 3.4.3A). The effect of the MEK inhibitor was reflected at the level of MAP kinase activation where PD098059 caused similar magnitudes of inhibition of MAP kinase activities evoked by thrombin and PDGF (Fig. 3.4.3B). In addition to inhibiting MAP kinase activity, pretreatment of BPA fibroblasts with PD098059 (50 μM) prevented the retardation of electrophoretic mobility of p42 and p44 MAP kinase isoforms in response to thrombin and PDGF (Fig. 3.4.3B). As the efficacy of PD098059 to inhibit MEK may depend on the strength of activation of MEK (Alessi *et al.*, 1995), the effect of PD098059 on the MEK and MAP kinase signals after 30 minutes stimulation with PDGF was assessed. At this point a low level of MEK activity (Fig. 3.4.2) is associated with the sustained phase of MAP kinase activation (Fig. 3.2.1). The level of MEK-1/2 activity after 30 minutes exposure to PDGF was abolished by PD098059 (Fig. 3.4.3A) while surprisingly, the MAP kinase activity response was less sensitive to inhibition than at 5 minutes (66 %).

A.


- WT MAPK
C P
min 2 5 15 30 60 120
- WT MAPK
C P T
min 5 5 15 30 60

B.

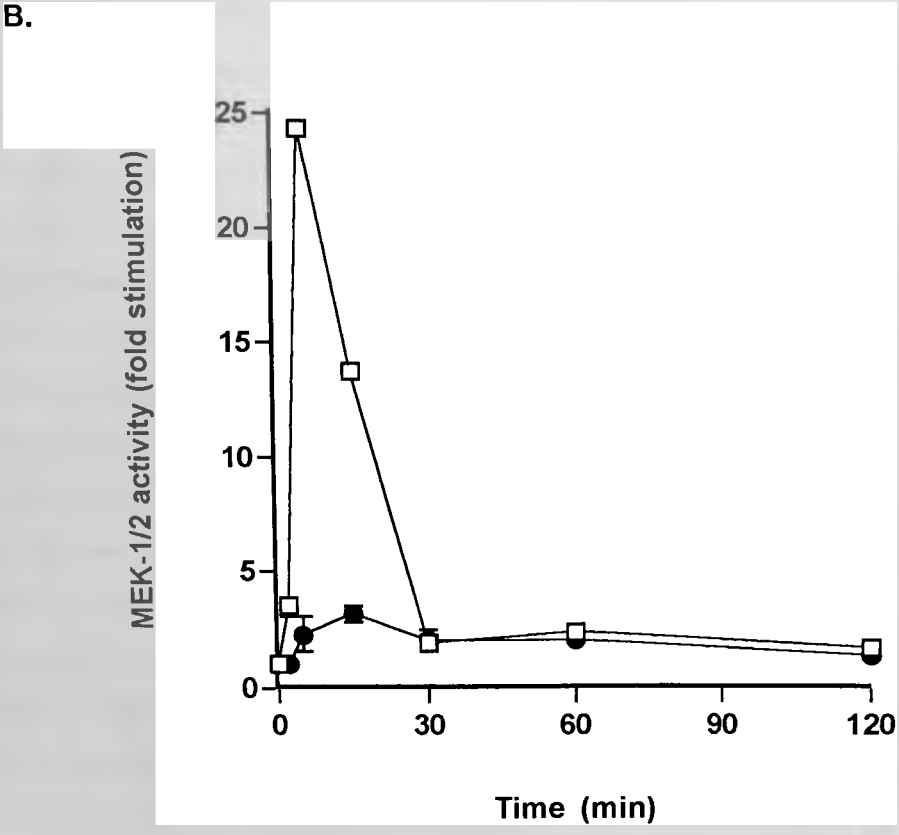

MEK-1/2 activity (fold stimulation)
25
20
15
10
5
0
0
30
60
90
120
Time (min)

Figure 3.4.2 MEK-1/2 activation by thrombin and PDGF in BPA fibroblasts. Serum-deprived cells were exposed to thrombin (300 nM) (T), PDGF (30 ng/ml) (P) or vehicle (C) for the indicated times. Immunoprecipitates of MEK-1/2 were prepared from cell lysates and MEK activity measured using a coupled kinase assay utilizing recombinant wild type MAP kinase (WT MAPK) and $EGFR^{661-680}$ as sequential substrates as outlined in Section 2.4.2. [^{32}P]Phosphorylation of WT MAPK, resolved by SDS-PAGE, was detected by autoradiography (**A**). Thrombin (●) and PDGF (□) -stimulated MEK-1/2 activity (**B**) is expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into $EGFR^{661-680}$ from two separate experiments (control: 0.28±0.02 pmol Pi min^{-1} mg^{-1} protein (T) and 0.15±0.06 pmol Pi min^{-1} mg^{-1} protein (P).

Figure 3.4.3 Effect of PD09859 on thrombin and PDGF -stimulated MEK-1/2 and MAP kinase activity in BPA fibroblasts. Serum-deprived cells were pretreated with vehicle (- or open bars) or PD098059 (50 μM) (+ or hatched bars) for 30 minutes prior to exposure to thrombin (300 nM for 15 minutes) (T), PDGF (30 ng/ml for 5 and 30 minutes) (P5 and P30) or vehicle (15 minutes) (C). **A)** Immunoprecipitates of MEK-1/2 were prepared from cell lysates and *in vitro* MEK activity measured using a coupled kinase assay utilizing recombinant wild type MAP kinase (WT MAPK) and $EGFR^{661-680}$ as sequential substrates as outlined in Section 2.4.2. [^{32}P]Phosphorylation of WT MAPK, resolved by SDS-PAGE, was detected by autoradiography (upper panel). **B)** Lysates were assayed for *in vitro* MAP kinase activity or as described in Section 2.4.1. Kinase activities are expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into $EGFR^{661-680}$ from four separate experiments (control: 0.18±0.03 pmol Pi min^{-1} mg^{-1} protein (MEK); 44.36±4.98 pmol Pi min^{-1} mg^{-1} protein (MAPK)). * denotes significant effect of PD098059 treatment at $p < 0.05$ level using Student's paired t test.

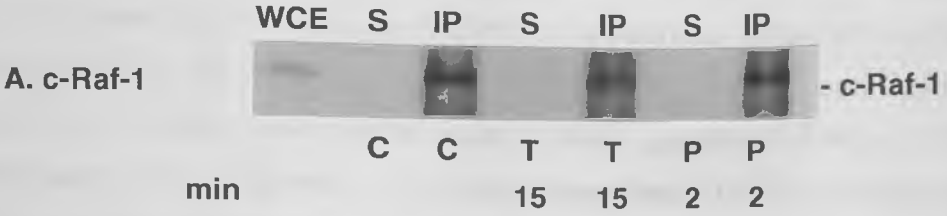
3.4 Thrombin and PDGF-stimulated c-Raf-1 activation in BPA fibroblasts

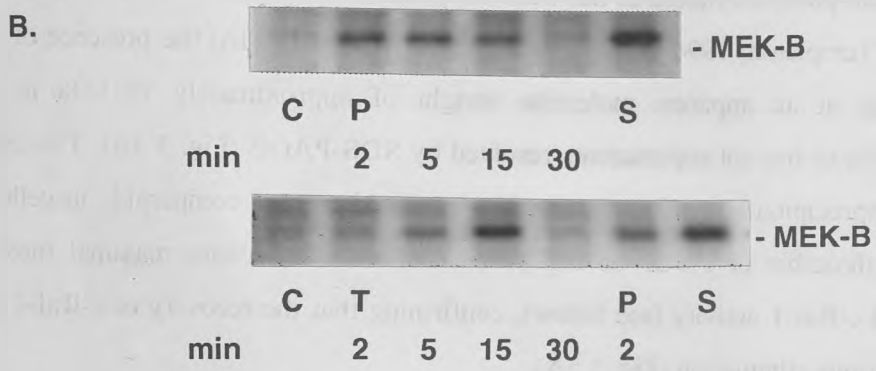
The serine-threonine kinase c-Raf-1 has been shown to phosphorylate and activate MEK both *in vitro* and *in vivo* (Dent *et al.*, 1992, Howe *et al.*, 1992, Kyriakis *et al.*, 1992). To investigate whether c-Raf-1 kinase was involved in the regulation of MEK by thrombin and PDGF in BPA fibroblasts, c-Raf-1 kinase activity was assayed by the ability of immunoprecipitated c-Raf-1 from thrombin and PDGF -stimulated cells to phosphorylate a recombinant kinase-inactive MEK-1 (MEK-B) *in vitro* as described in Section 2.4.3. Because this substrate does not exhibit catalytic activity itself, the phosphorylation observed was not due to MEK autophosphorylation as has been observed for wild type MEK-1 (Gardner *et al.*, 1993, Yan & Templeton, 1994). Western blotting analysis revealed the presence of a single band migrating at an apparent molecular weight of approximately 75 kDa in c-Raf-1 immunoprecipitates but not supernatents resolved by SDS-PAGE (Fig. 3.5A). The level of c-Raf-1 immunoprecipitated, as assessed by immunoblotting, was comparable in cells treated with vehicle, thrombin or PDGF at exposure times found to cause maximal thrombin or PDGF-evoked c-Raf-1 activity (see below), confirming that the recovery of c-Raf-1 was not affected by agonist stimulation (Fig. 3.5A).

Exposure of cells to thrombin (300 nM) evoked a gradual increase in c-Raf-1 kinase activity that reached a maximum at 15 minutes, causing approximately an 18 fold increase over activity in vehicle-treated cells, and returned to basal levels at 30 minutes (Fig. 3.5.B & C). The magnitude of the peak c-Raf-1 signal in response to thrombin was approximately equivalent to that evoked by FCS (10%) after 5 minutes. In contrast, activation of c-Raf-1 by PDGF was more rapid, peaking as early 2 minutes (approximately 14 fold increase) and declining thereafter to return to basal levels at 30 minutes.

3.5.1 Agonist-stimulated DNA synthesis in BPA fibroblasts

The mitogenic effect of agents in quiescent BPA fibroblasts was assessed by measuring the incorporation of [^{3}H]thymidine as a marker for DNA synthesis which occurs during re-entry into the S phase of the cell cycle. Treatment of fibroblasts that had been deprived of serum for 48 hours with increasing concentrations of thrombin induced a concentration-dependent increase in the incorporation of [^{3}H]thymidine. Thrombin-stimulated [^{3}H] thymidine incorporation was apparent at 3 nM and increased with concentrations up to


A. c-Raf-1
WCE
S
IP
S
IP
S
IP
- c-Raf-1
C
C
T
T
P
P
min
15
15
2
2


B.
- MEK-B
C
P
S
min
2
5
15
30
- MEK-B
C
T
P
S
min
2
5
15
30
2

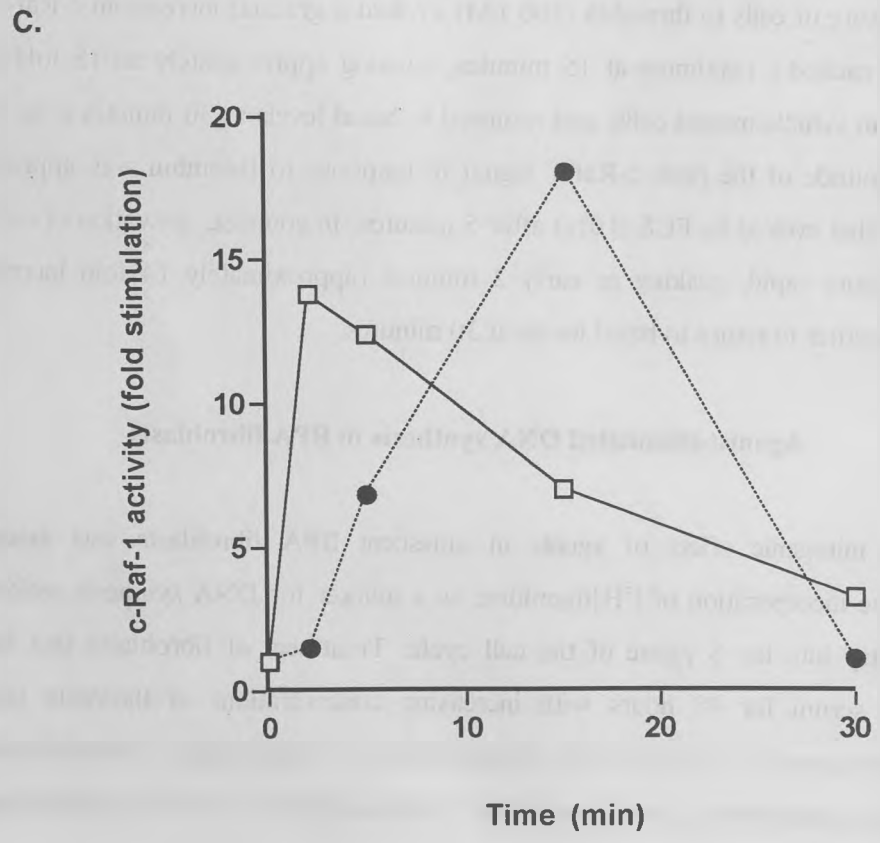

C.
c-Raf-1 activity (fold stimulation)
20
15
10
5
0
0
10
20
30
Time (min)

Figure 3.5 C-Raf-1 kinase activation by thrombin and PDGF in BPA fibroblasts. Serum-deprived cells were stimulated with thrombin (300 nM) (T) or PDGF (30 ng/ml) (P) for the indicated times, or vehicle (C) or NCS (10%) (S) for 5 minutes. Immunoprecipitates of c-Raf-1 prepared from cell lysates (IP), supernatents (S) or whole cell extracts (WCE) were immunoblotted for c-Raf-1 (**A**) or assayed for *in vitro* c-Raf-1 kinase activity as outlined in Sections 2.3 & 2.4.3. Incorporation of [^{32}P]phosphate into recombinant kinase-inactive MEK-1 (MEK-B), resolved by SDS-PAGE, was detected by autoradiography (**B**). The extent of MEK-B phosphorylation in c-Raf-1 immunoprecipitates from cells treated with thrombin (●) or PDGF (□) was quantified by densitometry (**C**) and is expressed as fold stimulation relative to control for a single experiment.

300 nM (~ 25 fold increase) (Fig. 3.6), above which cell monolayers became detached from the plate within 30 minutes. For this reason, the concentration of thrombin required to evoke a half-maximal response could not be assessed accurately. At 300 nM, the mitogenic effect of thrombin was found to be equal to that evoked by a maximally-effective concentration of PDGF-BB (100 ng/ml), and markedly stronger than the response induced by 10 % NCS (~ 16 fold increase in [^{3}H] thymidine incorporation) (Table 3.2) although PDGF was the most potent mitogen of all the stimuli tested (Fig. 3.6). PDGF-stimulated [^{3}H]thymidine incorporation was evident at a concentration of PDGF as low as 3 ng/ml (0.12 nM) (Fig. 3.6) with an EC_{50} of approximately 20 ng/ml (0.8 nM) (Fig. 3.6). In comparison with thrombin and PDGF, the mitogenic effect of PMA was relatively weak. The profile for the effect of PMA concentration on DNA synthesis was bell-shaped (Fig. 3.6). Incorporation of [^{3}H]thymidine increased incrementally with concentrations of phorbol ester above 1 nM to reach a maximum at 30 nM (~ 8 fold increase) with an EC_{50} value of approximately 4 nM. At concentrations higher than 30 nM, the response to PMA became progressively smaller (Fig. 3.6). In contrast to the agonists described above, stimulation of cells for 24 hours with LPA (300 nM-100 μM), which acts as a potent mitogen for Rat-1 fibroblasts (Van Corven *et al.*, 1993), endothelin-1 (3-300 nM), or angiotensin II (10 nM -3 μM) had no effect on the incorporation of [^{3}H]thymidine in BPA fibroblasts (Table 3.2). Similarly, bradykinin (10 nM-10 μM) was not found to exhibit any mitogenic activity (not shown).

3.5.2 The effect of PD098059 on thrombin and PDGF -stimulated DNA synthesis in BPA fibroblasts

The potential role of the MAP kinase cascade in the mitogenic effect of thrombin and PDGF in BPA fibroblasts was evaluated using PD098059. Quiescent cells were treated with the MEK inhibitor for 30 minutes prior to treatment with thrombin (300 nM) or PDGF (30 ng/ml). PD098059 was found to cause a dose-dependent inhibition of [^{3}H]thymidine incorporation evoked by both thrombin and PDGF with an IC_{50} value of approximately 5 μM for both stimuli (Fig. 3.7), and similar to that found for PD098059 inhibition of PDGF-stimulated [^{3}H]thymidine incorporation in BALB 3T3 mouse fibroblasts (Dudley *et al.*, 1995), and c-Raf-1 activation of MEK-1 *in vitro* (Alessi *et al.*, 1995).

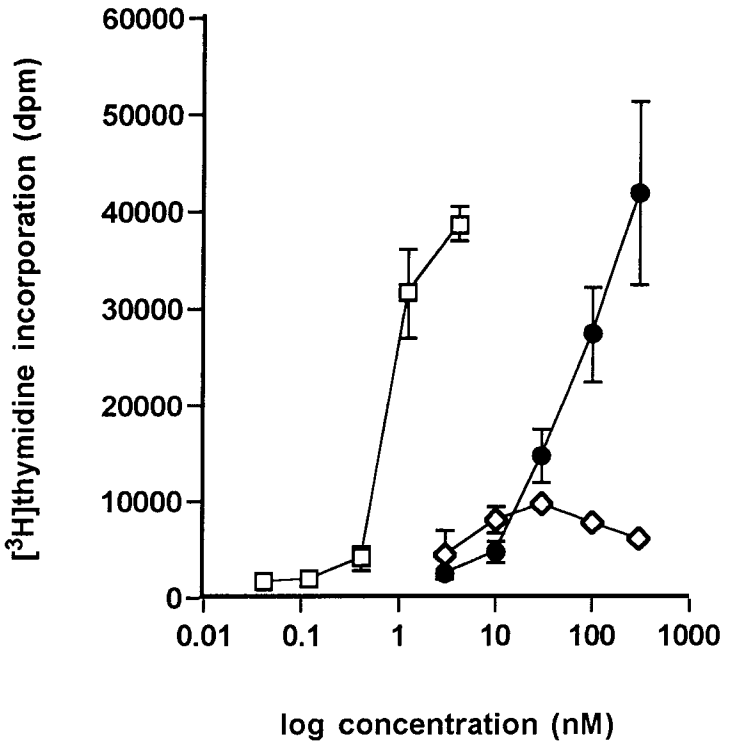


Figure 3.6 Agonist-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Serum-deprived cells were exposed for 24 hours to thrombin (●), PDGF (□) or PMA (◇) at the concentrations indicated. Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNAreplicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. dpm for 2-4 independent experiments each performed in triplicate.

Treatment	[^{3}H]thymidine incorporation mean±s.e.m. dpm
C	1426±328
T	39860±8219
P	39617±8739
PMA	10098±2425
ET	1529±198
LPA	2177±572
AII	1676±156
S	25880±8866

Table 3.2 Agonist-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Serum-deprived cells were exposed for 24 hours to thrombin (300 nM) (T), PDGF (30 ng/ml) (P), PMA (100 nM), endothelin-1 (100 nM) (ET-1), lysophosphatidic acid (10 μM) (LPA) angiotensin II (100 nM) (AII), NCS (10%) (S) or vehicle (C). Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNA replicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. dpm for 3 independent experiments each performed in triplicate.

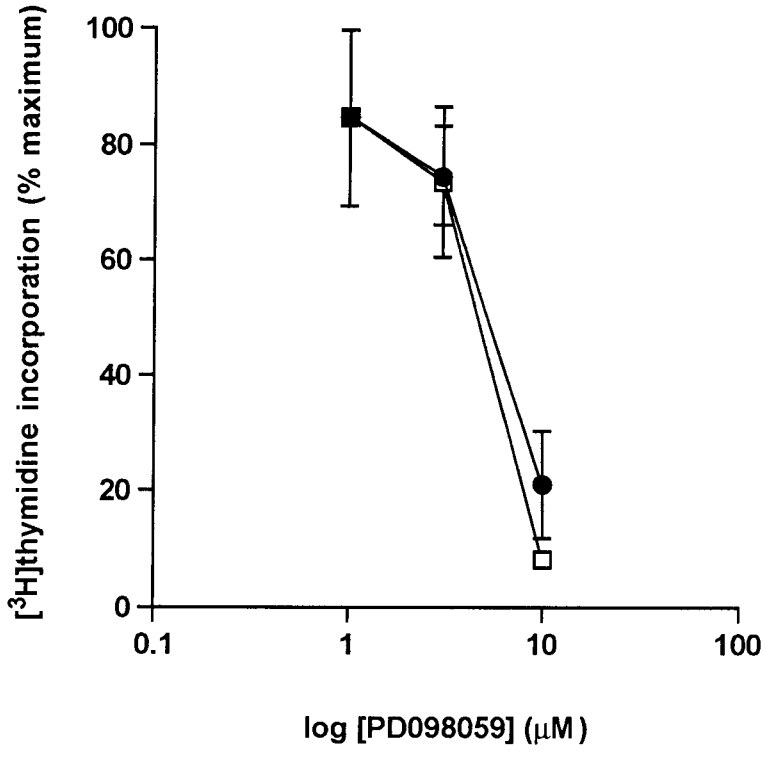


Figure 3.7 Effect of PD09859 on thrombin and PDGF -stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Cells were pretreated with vehicle or PD098059 at the indicated concentrations for 30 minutes prior to exposure to thrombin (300 nM) (●) or PDGF (30 ng/ml) (□) for 24 hours. Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNA replicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. percentage of maximal thrombin or PDGF -stimulated [^{3}H]thymidine incorporation in the absence of PD098059 from 3 independent experiments each performed in triplicate.

3.6 The effect of PKC downregulation on agonist-stimulated MAP kinase activation and DNA synthesis in BPA fibroblasts

For a number of G-protein coupled receptor agonists, an involvement of protein kinase C (PKC) in MAP kinase activation has been implicated (Burgering *et al.*, 1993a, Malarkey *et al.*, 1996). The possibility that thrombin or PDGF induced MAP kinase activation and/or DNA synthesis was mediated by a PKC-dependent pathway was investigated using a PKC downregulation protocol. A consequence of long term exposure of cells to high concentrations of phorbol ester is the marked decrease in phorbol ester binding, PKC activity and cellular expression believed to be due to depletion of cellular PKC (Rodriquez-Pena & Rozengurt, 1984, Blackshear *et al.*, 1985) caused by an increased rate of degradation of the polypeptide (Young *et al.*, 1987). Protocols which differ in the concentration and exposure time to phorbol ester have been described which selectively deplete specific mammalian PKC isoforms which vary in their susceptibility to downregulation (Malarkey *et al.*, 1996). Initially, the conditions for depletion of all cellular PMA-sensitive PKC in BPA fibroblasts, as inferred by the complete loss of responsiveness to acute PMA challenge, were determined. These studies revealed that cells required prior exposure to 1 μM PMA for 48 hours in order to prevent PMA -stimulated MAP kinase activation and DNA synthesis (see below). Confirmation of the depletion of cellular PMA-sensitive PKC was confirmed by Western blotting with monoclonal antibodies raised against specific epitopes within the individual PKC isoforms. Immunoblotting analysis of equivalent amounts of protein from whole cell lysates revealed that untreated BPA fibroblasts in culture expressed PKC α, ε and ζ isotypes, as judged by the appearance of single immunoreactive bands each migrating in the 80-90 kDa molecular weight range (Fig. 3.8A). The β isoform was not easily detected (not shown). Pretreatment of fibroblasts with 1 μM PMA for 48 hours completely downregulated immunodetectable PKC α and ε isoforms (Fig. 3.8A). Expression of the atypical ζ isoform was unaffected by preincubation of cells with PMA and is consistent with the inability of this isoform to bind or be activated by phorbol esters (Nakanishi & Exton, 1992). Serum-deprived, PKC-depleted BPA fibroblasts were then exposed to PMA, thrombin or PDGF and the extent of MAP kinase activation assessed by gel mobility shifts. As outlined previously, MAP kinase was not activated by PMA (100 nM) in cells which had received prior long-term treatment of phorbol ester. In comparison, PMA pretreatment did not affect the ability of thrombin to activate MAP kinase but marginally inhibited (approximately 30 % as assessed by

densitometry of phosphotyrosine immunoblots) PDGF-stimulated MAP kinase activation (Fig. 3.8B). Western blotting confirmed that the PKC downregulation regimen did not affect the level of immunoreactive MAP kinase after 48 hours (Fig. 3.8B).

The effect of phorbol ester-induced PKC depletion on the mitogenic effect of thrombin and PDGF was investigated. Pretreatment of cells with 1 μM PMA for 48 hours prevented the incorporation of [^{3}H]thymidine on a subsequent exposure to PMA (100 nM). Under these conditions where BPA fibroblasts were non-responsive to PMA, [^{3}H]thymidine incorporation evoked by thrombin (100 nM) was unaffected while the mitogenic response to PDGF (30 ng/ml) was attenuated by approximately 30 % (Fig. 3.10.A).

Attempts were made to confirm the findings of the PKC depletion strategy on the contribution of PKC to agonist-stimulated MAP kinase activation and DNA synthesis using Ro-318220 and calphostin C, compounds which have been reported to be specific competitive PKC inhibitors (Davis *et al.*, 1989). These experiments yielded inconclusive results, however, as at concentrations (10 μM) reported to be required for PKC inhibition (Malarkey *et al.*, 1996), Ro-318220 and calphostin C affected cell viability, causing large changes in morphology, cell rounding and detachment from the plate within 24 hours.

3.7 The effect of pertussis-toxin (PTX) on thrombin -stimulated MAP kinase activation and DNA synthesis

Heterotrimeric G-proteins of the G_i family have been implicated in the activation of MAP kinase by thrombin in fibroblasts (Kahan *et al.*, 1992) and DNA synthesis by the sensitivity of this response to inhibition to *Bordetela pertussis* toxin (PTX). This toxin mediates the ADP ribosylation of the α subunits of G_i and G_o proteins only when these are in the heterotrimeric GDP-bound conformation associated with their βγ subunits, thus locking them in an inactive state (Ui, 1984). The effects of PTX on thrombin-induced MAP kinase activation and DNA synthesis in BPA fibroblasts were examined by pretreating cells with 100 ng/ml pertussis toxin (PTX) for 18 hours which has previously been demonstrated to inhibit LPA-induced protein tyrosine phosphorylation and MAP kinase activation in Rat-1 fibroblasts (Hordijk *et al.*, 1994, Koch *et al.*, 1994). This regimen had no effect on thrombin-stimulated tyrosine phosphorylation as well as the mobility shift of p42 and p44 MAP kinases at either 20 or 60 min exposure times. As expected, PTX did not modify the mobility gel shift of p42/p44 MAP kinase induced by PDGF (Fig. 3.9) or PMA (not shown). The PTX

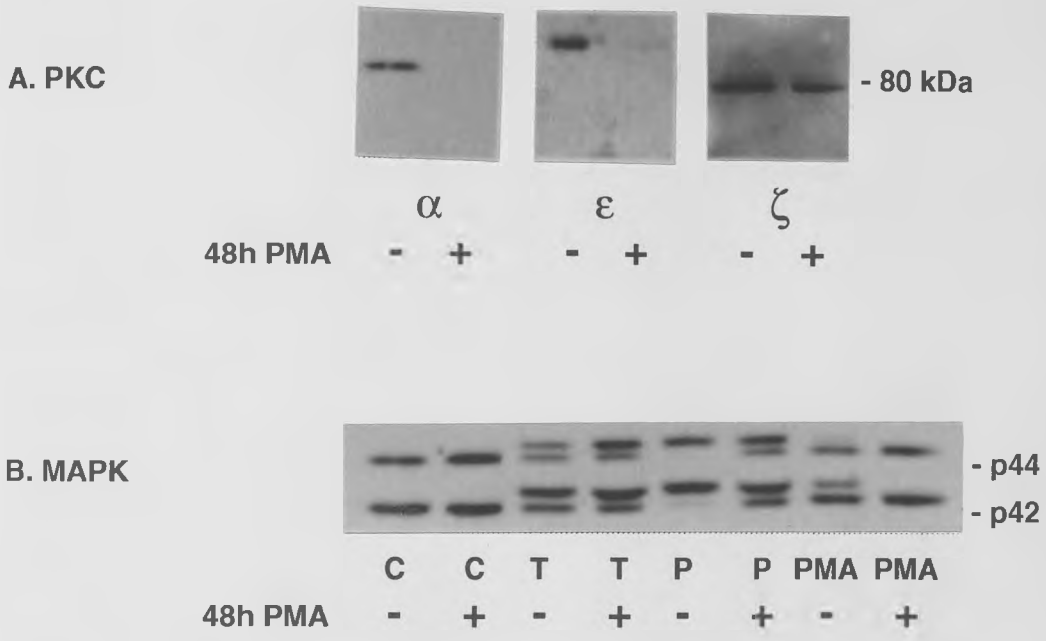


Figure 3.8 Effect of PMA pretreatment on agonist-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were pretreated with vehicle (-) or 1 μM PMA (+) for 48 hours prior to exposure to thrombin (300 nM for 20 minutes) (T), PDGF (30 ng/ml for 10 minutes) (P), PMA (100 nM for 5 minutes) or vehicle (10 minutes) (C). Cell lysates were immunoblotted for PKC α, ε and ζ isoforms (**A**) or MAP kinase (**B**) as described in Section 2.3. The mobility of the 80 kDa marker and bands corresponding to the p42 and p44 MAP kinase isoforms are shown on the right of the respective panels. Each blot is a representative example from at least three independent experiments.

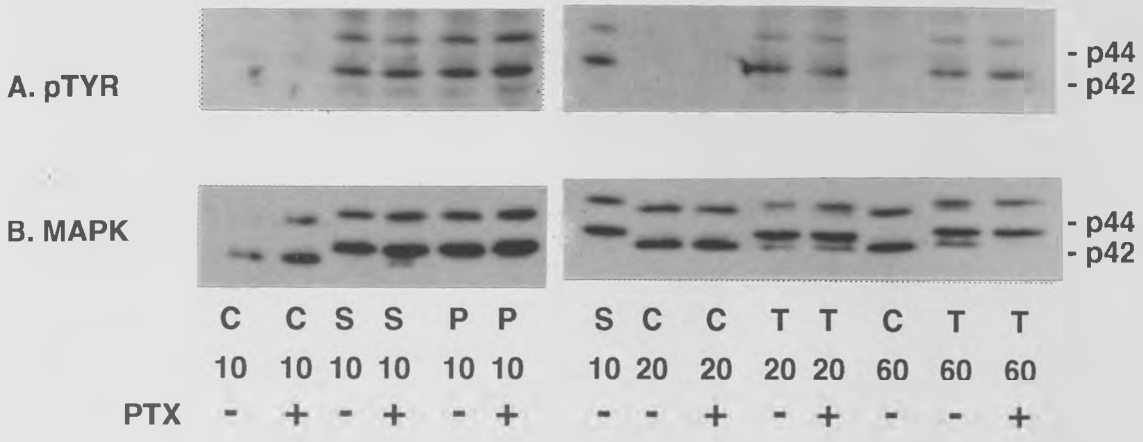


Figure 3.9 Effect of PTX on agonist-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived cells were pretreated with vehicle (-) or PTX (100 ng/ml) (+) for 18 hours prior to exposure to thrombin (300 nM) (T), PDGF (30 ng/ml) (P), NCS (10%) (S) or vehicle (C) for the times indicated. Whole cell lysates were immunoblotted for phosphotyrosine (**A**) and blots reprobed for MAP kinase (**B**), as described in Section 2.3. Bands corresponding to the p42 and p44 MAP kinase isoforms are shown on the right of the immunoblots, each of which is a representative example of three independent experiments.

pretreatment protocol was effective at causing approximately 40% inhibition of thrombin-stimulated [^{3}H]thymidine incorporation but did not affect the response to either PDGF or PMA (Fig. 3.10B).

3.8 The effect of hirudin on thombin-stimulated MAP kinase activation and DNA synthesis

The specificity of thrombin-induced responses in BPA fibroblasts, was checked using the selective thrombin inhibitor hirudin. Treatment of thrombin with hirudin for 5 minutes prior to addition to cells caused a concentration-dependent inhibition of thrombin-stimulated MAP kinase activation as assessed by mobility shifts (Fig. 3.11). Inhibition was complete at 1 μM hirudin. This concentration of hirudin was also found to markedly inhibit thrombin-stimulated [^{3}H]thymidine incorporation (approximately 90 %) (Table 3.3). The 14-mer synthetic peptide thrombin receptor-activating peptide (TRAP-14) SFLLRNPNDKYEPF, corresponding to the new N-terminal portion from the human thrombin receptor, was unable to stimulate the activation of MAP kinase in BPAFs, at a concentration (30 μM) that was found to cause strong activation of MAP kinase in rat aortic smooth muscle cells (Fig. 3.11B) and shown to be active in other bovine cells (Lum *et al.*, 1993). TRAP-14 also had a negligible effect on [^{3}H]thymidine incorporation (Table 3.3).

3.9 DISCUSSION

3.9.1 Agonist-stimulated activation of components of the MAP kinase cascade

To ascertain whether the MAP kinase pathway plays a role in the mitogenic response of thrombin and other mediators in BPA fibroblasts, the ability of these agents to induce the activation of p42 and p44 MAP kinases activation was examined. The findings of this investigation provide very strong evidence that of the agents screened, thrombin, the classical growth factor PDGF, the tumour-promoting phorbol ester PMA and serum stimulate the activation of p42 and p44 MAP kinases in a time and concentration manner on the basis of the following criteria: 1) Treatment of fibroblast cultures with these agonists cause a prominent tyrosine phosphorylation of two proteins of apparent molecular weight of approximately 40-45 kDa that comigrate with p42 and p44 MAP kinases isoenzymes following SDS-PAGE.

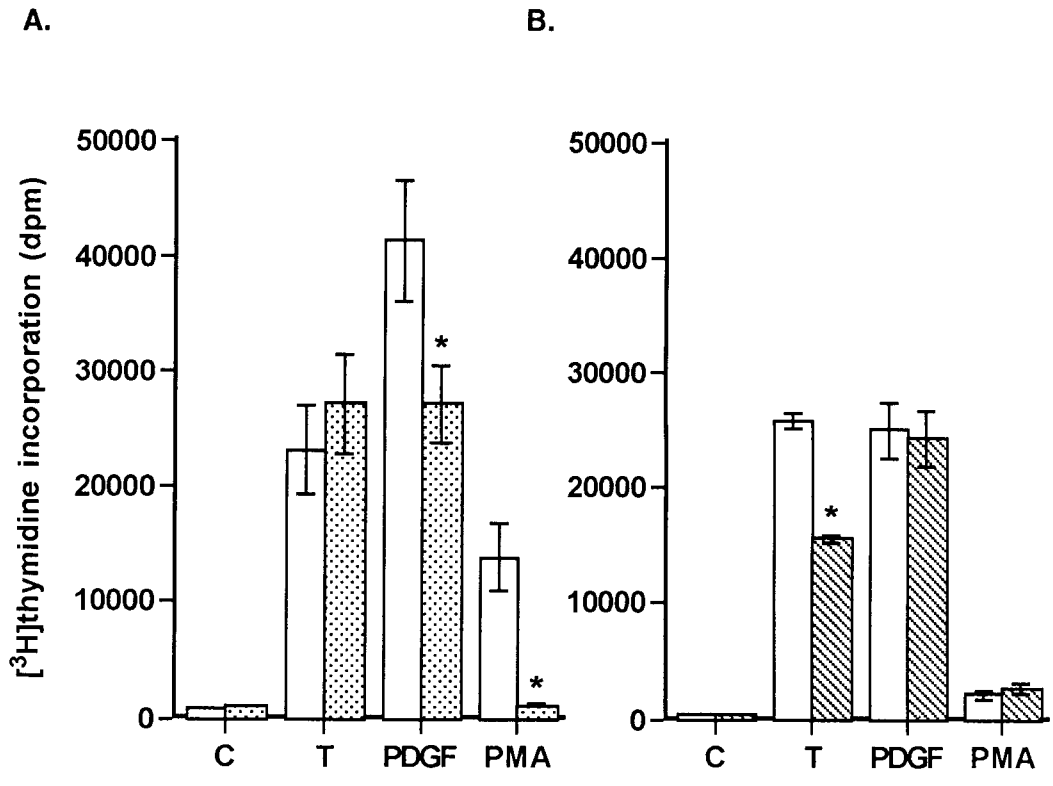


Figure 3.10 Effect of PMA pretreatment and PTX on agonist-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Cells were pretreated with vehicle (open bars) or **A)** 1 μM PMA for 48 hours (shaded bars) or **B)** PTX (100 ng/ml) for 18 hours (hatched bars) prior to incubation with thrombin (300 nM) (T), PDGF (30 ng/ml), PMA (100 nM) or vehicle (C) for 24 hours. Incorporation of [^{3}H]thymidine during the final 4 hours of agonist treatment was measured as described in Section 2.5. Data is expressed as mean±s.e.m. dpm for five independent experiments with each treatment performed in triplicate.* denotes significant effect of PMA pretreatment or PTX at $p < 0.05$ level using Student's paired t test.

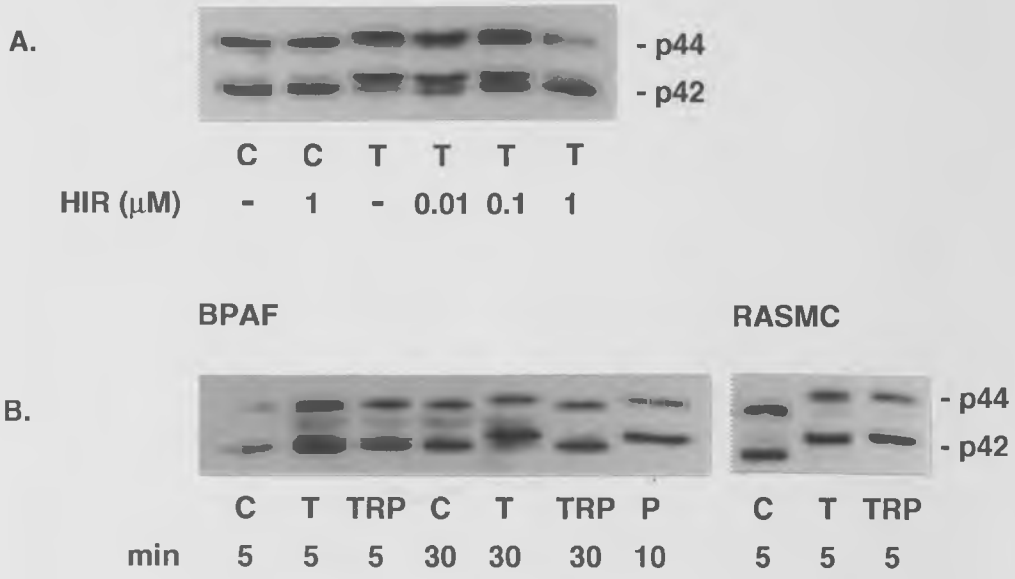


Figure 3.11 Effect of hirudin on agonist-stimulated MAP kinase activation in BPA fibroblasts. Serum-deprived BPA fibroblasts (BPAF) were exposed to **A)** thrombin (300 nM for 20 minutes) (T) or vehicle (C) that had been treated with (+) or without (-) hirudin (HIR) at the indicated concentrations for 10 minutes at room temperature prior to application to cells; **B)** thrombin (300 nM) (T), thrombin receptor-activating peptide (100 μM) (TRP) or vehicle (C) for the times indicated. The effect of these agonists was also investigated in serum-deprived RA smooth muscle cells (RASMC) where the concentration of thrombin used was 30 nM (right panel). Whole cell lysates were immunoblotted for MAP kinase as described in Section 2.3. Bands corresponding to the p42 and p44 MAP kinase isoforms are shown on the right of the immunoblots, each of which is a representative example of two independent experiments.

Treatment	[^{3}H]thymidine incorporation mean±s.e.m. dpm
C	315±38
H	317±17
T	21196±1162
T+H	2822±156 *
TRP	436±40

Table 3.3 Effect of hirudin on thrombin-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Serum-deprived cells were incubated for 24 hours with thrombin (300 nM) (T) that had been treated with or without hirudin (1 μM) (H) for 10 minutes at room temperature prior to application to cells, thrombin receptor-activating peptide (100 μM) (TRP) or vehicle (C). Incorporation of [^{3}H]thymidine during the final 4 hours of agonist treatment was measured as described in Section 2.5. Data is expressed as mean±s.e.m. dpm for 3 independent experiments each performed in triplicate.* denotes significant effect of hirudin at $p < 0.05$ level using Student's paired t test.

Phosphorylation on specific tyrosine and threonine residues is believed to be critical event in the activation of these enzymes (Anderson *et al.*, 1990) and a number of studies have demonstrated that p42 and p44 MAP kinase undergo rapid tyrosine and threonine phosphorylation in response to many polypeptide growth factors as well as G protein-coupled agonists, accompanied by an increase in their intrinsic kinase activities (Boulton *et al.*, 1991, Molloy *et al.*, 1996); 2) Agonist treatment resulted in a marked decrease in electrophoretic mobility of p42 and p44 MAP kinases believed to be due to increased phosphorylation; 3) Agonists stimulated an increase in *in vitro* phosphotransferase activity against the peptide KRELVEPLT^{669}PAGEAPNALLR corresponding to a portion of the EGF receptor (EGFR$^{661-680}$) that has been modified to contain a single Thr^{669} phosphorylation site and believed to be a MAP kinase specific substrate (Takishima *et al.*, 1991); 4) The temporal patterns for the effects described above in response to each stimulus correlated well. To corroborate these observations, attempts were made to measure kinase activity in MAP kinase immunocomplexes, but efforts to immunoprecipitate MAP kinase from the lysates of agonist-stimulated cells using more than one commercially available MAP kinase antibody were unsuccessful. In spite of this, taking the evidence together, these results strongly indicate that the p42 and p44 MAP kinases become activated on exposure of BPA fibroblasts to thrombin, PDGF, PMA and serum.

While similar effects of PDGF and PMA in fibroblasts and other cell types have been well documented in the literature (l'Allemain *et al.*, 1991b, Malarkey *et al.*, 1996), certain characteristics of thrombin-evoked responses in BPA fibroblasts were unusual. Firstly, the concentration range of thrombin required was found to be approximately 30 fold greater than that reported by other authors to cause an equivalent effect in another fibroblast type (Pagès *et al.*, 1993, Vouret Craviari *et al.*, 1993). While the much higher specific activity of thrombin used in these reports (3200 NIH units/mg) compared to the present study (600 NIH units/mg) may partly explain the discrepancy, the concentration of thrombin required to be maximally effective at stimulating MAP kinase activation in BPA fibroblasts (300 nM) was still 5 fold higher than that in rat aortic smooth muscle cells (Fig. 3.11) in this laboratory (Malarkey *et al.*, 1996). Secondly, the kinetics for activation of MAP kinase by thrombin appears to be distinctly different to that reported in other cell systems. In contrast to the cells used here, hamster lung fibroblasts and other cell types including vascular smooth muscle cells and astrocytes exhibit a biphasic activation of MAP kinase in response to thrombin (Kahan *et al.*, 1992, Bhat *et al.*, 1995, Rao *et al.*, 1995, Molloy *et al.*, 1996). In these cases, the serine-

protease stimulated both an initial rapid increase peaking around 5 minutes, which is followed by a persistent activation lasting for several hours. A monophasic activation of MAP kinase comprising of only the initial transient peak at 5 minutes has also been observed in rat aortic smooth muscle cells (Malarkey *et al.*, 1996). This initial peak in kinase activity, occurring within minutes, was not observed in BPA fibroblasts where the onset of thrombin-stimulated MAP kinase activation was delayed until after 5 minutes. The reasons for the difference in the kinetics of MAP kinase activation following thrombin exposure are not entirely clear, and are discussed further elsewhere but may reflect the ability to activate more than one subtype of thrombin receptor in different tissue types or the contribution of differential signal transducers at different time points. In support of the latter possibility, PKC and calcium may be responsible for the first peak of the thrombin-stimulated biphasic activation of MAP kinase in CCL339 fibroblasts while sensitivity to inhibition by PTX distinguishes the sustained phase of kinase activity (Kahan *et al.*, 1992, Meloche *et al.*, 1992). Other studies have provided evidence to suggest that at least two distinct pathways, one Ca^{2+} dependent and one Ca^{2+} independent/cAMP responsive are involved in MAP kinase activation by thrombin in CHO cells (Hirano *et al.*, 1996).

To characterize the MAP kinase pathway further, the effect of thrombin and PDGF on the activity of MEK, the upstream activator of MAP kinase was examined. The two highly homologous mammalian MEK isoforms, MEK-1 and MEK-2 are dual specificity kinases shown to phosphorylate and activate p42 and p44 MAP kinases *in vitro* (Crews *et al.*, 1992, Nakielny *et al.*, 1992, Seger *et al.*, 1992, Zheng & Guan, 1993a, 1993b). In BPA fibroblasts, both thrombin and PDGF were found to stimulate MEK-1/2 activity (Fig. 3.4.2) with kinetics which paralleled the initial rise in MAP kinase activation (Figs. 3.1.1 & 3.2.1). However, the MEK signals were more transient than the respective MAP kinase activities observed for each agonist and fell to a low level of activity at 30 minutes, at which point the MAP kinase signal was maintained. The most striking observation was that while thrombin evoked a modest increase in MEK-1/2 activity (2 fold over basal levels), the magnitude of MEK-1/2 activation in response to PDGF was approximately 15 times greater yet this difference was not reflected in the strengths of the respective MAP kinase signals where PDGF-stimulated MAP kinase activity was only three times as strong as that by thrombin (Figs. 3.4.2 & 3.4.3).

The simplest interpretation why MEK-1/2 signal in response to thrombin was weak relative to PDGF is that thrombin may employ a different signalling molecule to activate MAP kinase. However, this is unlikely since to date, MEK-1 and MEK-2 are the only known

enzymes capable of phosphorylating and activating ERK1 and ERK2 (Zheng & Guan, 1993b). Furthermore, MEK-1 has been reported to be a common MAP kinase activator in Rat 1a and NIH 3T3 cells in response to both thrombin, where MEK activity of comparable magnitude was seen, and EGF (Gardner *et al.*, 1993), another growth factor, which like PDGF, signals through a tyrosine kinase-encoded receptor. Although a novel mammalian MAP kinase activator most closely related to MEK-1 and MEK-2 has recently been isolated from the rat and designated MEK-5 (English *et al.*, 1995), this kinase was unable to activate ERK1 or ERK2. While the possibility that these MAP kinases may be substrates for a novel uncharacterized MAP kinase kinase activated in response to thrombin in this cell system cannot be excluded, strong evidence to support a role for MEK-1 in the activation of MAP kinase by thrombin and PDGF *in vivo* was inferred by the inhibition of MAP kinase activity and of the decrease in electrophoretic mobility of MAP kinase by PD098059. This compound has been shown to be a highly specific synthetic inhibitor of MEK-1 by binding at a site which prevents the phosphorylation and activation of MEK-1 by activating enzymes such as Raf or MEK kinase (Alessi *et al.*, 1995) and does not significantly affect the activities of 18 other protein kinases (Alessi *et al*, 1995, Dudley *et al.*, 1995). This finding suggests that the low level of MEK-1/2 activity evoked by thrombin accounts entirely for the MAP kinase signal. For PDGF, however, the much stronger MEK signal relative to thrombin may not be manifested by a corresponding level of MAP kinase activation as MAP kinase may be maximally activated by PDGF in the cell. The potential of the PDGF-generated MEK signal is only realized *in vitro* where, under the assay conditions used, MAP kinase is a substrate in excess. Thus, in the intact cell, there may be a large signalling reserve at the level of MEK that is evident when signal transduction via MAP kinase becomes saturated easily in response to agents such as PDGF that activate MEK strongly. Other investigators have suggested that considerable amplification occurs in the MAP kinase cascade at the level of MEK such that a very small activation of MEK is sufficient to produce significant activation of MAP kinase (Alessi *et al.*, 1995, Plevin *et al.*, 1996). This may provide a plausible explanation why at later times after exposure to PDGF, MAP kinase activity remained elevated while the MEK-1/2 activity had fallen to just above unstimulated levels (2 fold) and comparable to that evoked by thrombin (Fig. 3.4.2). To test whether this late phase of PDGF-stimulated MAP kinase activation can be supported by the residual MEK-1/2 activity, the effect of PD098059 on MEK and MAP kinase signals 30 minutes after PDGF addition was examined. Surprisingly, at this time point, while the MEK signal was abolished as expected, the MAP kinase response

was even less sensitive to inhibition than at 5 minutes when PDGF-stimulated MEK activity was in comparison very much stronger. One reason for this may be that the sustained latter phase of MAP kinase activation by PDGF may be mediated preferentially by MEK-2 which is only weakly inhibited by PD098059 (Alessi *et al*, 1995). Alternatively, PD098059 may block a negative feedback loop which functions to suppress further activation of MAP kinase at later times, as PD098059 has been shown to prevent the inactivation of the MEK-1/2 activator c-Raf-1 in response to PDGF (Alessi *et al.*, 1995). However, for either of these explanations, MEK-1/2 activity would be expected to be preserved but was found to be completely abolished at 30 minutes by PD098059 (Fig. 3.4.2). Thus it appears that the sustained phase of PDGF-stimulated MAP kinase activity may be independent of regulation by MEK-1/2. The mechanism by which this occurs is not apparent but may involve a novel MAP kinase activator. In support of this possibility, other work in this laboratory has implicated another pathway in the activation of MAP kinase by PDGF in rat aortic smooth muscle cells which is MEK-1/2 independent (K. Malarkey, personal communication).

A variety of reports in different systems have suggested that c-Raf-1 is a direct activator of MEK-1 both *in vitro* and *in vivo* (Dent *et al.*, 1992, Howe *et al.*, 1992, Kyriakis *et al.*, 1992). The role of c-Raf-1 in the activation of MEK in response to thrombin and PDGF in BPA fibroblasts was investigated to evaluate whether the differential magnitudes of MEK-1/2 activity observed could be detected at the level of signals upstream of MEK activation. Thrombin and PDGF were found to activate c-Raf-1 kinase in BPA fibroblasts as assessed by the phosphotransferase activity of immunopurified Raf-1 kinase against a kinase-inactive MEK substrate *in vitro*. The temporal pattern observed for c-Raf-1 activity evoked by both thrombin and PDGF was similar but for PDGF, rose more rapidly than the activity of endogenous MEK-1/2 (Figs. 3.4.2 & 3.5), consistent with the possibility that c-Raf-1 may be the physiological activator of MEK in agonist-stimulated BPA fibroblasts. Surprisingly, however, thrombin was found to stimulate c-Raf-1 activity to a level comparable to FCS (10%), and greater than PDGF, although as described previously, the magnitude of MEK-1/2 activity was very much smaller for thrombin than PDGF. While it is difficult to interpret the relative strengths of sequential signals without performing assays for the kinases simultaneously, one explanation why c-Raf-1 kinase activity in response to thrombin was not manifested at the level of MEK activation may be that the extent of phosphorylation of kinase-inactive MEK by c-Raf-1 *in vitro* may not correlate with a comparable increase in catalytic activity if wild type MEK had been used as a substrate. While this possibility could be

addressed by a coupled kinase assay utilizing both wild type MEK and MAP kinase, it is unlikely since c-Raf-1 from PDGF-treated BPA fibroblasts was found to phosphorylate MEK *in vitro* to an almost equivalent degree as thrombin (Fig. 3.5) and yet was accompanied by much greater extractable MEK-1/2 activity than thrombin (Fig. 3.4.2).

Another explanation why thrombin-stimulated c-Raf-1 kinase activity is not reflected by MEK-1/2 is that *in vivo*, MEK activity could be negatively regulated by phosphatases or other kinases. Little is known about the processes responsible for inactivating MEK. Although phosphoprotein phosphatase 2A can inactivate MEK *in vitro* (Gómez & Cohen, 1991) there is no direct evidence to support this phosphatase as a physiological MEK inactivator. Furthermore, the effect of a phosphatase would have to be a specific component of signalling events evoked by thrombin and not PDGF. Similarly, while MEK-1 has been shown to be inhibited following phosphorylation on specific threonine residues by growth-factor-stimulated MAP kinase (Brunnet *et al.*, 1994) it is difficult so envisage how thrombin could affect this feedback loop to suppress MEK activation more effectively than PDGF especially as PDGF stimulates MAP kinase more strongly.

A more plausible explanation for the discrepancy in the strength of agonist-stimulated c-Raf-1 and MEK-1/2 activities may be that while c-Raf-1 becomes activated by thrombin and PDGF in BPA fibroblasts it may not be involved in the activation of MEK *in vivo*. Regulation of downstream components of the MAP kinase cascade by a Raf-1-independent pathway is supported by a number of findings in the literature. In 3T3 L1 cells, while insulin can activate Raf-1 in a Ras-dependent manner, this is dissociated from MAP kinase activation suggesting that Raf-1 may be a component of a separate parallel signalling pathway emerging from Ras (Porras *et al.* 1993). Similar dissociation between Raf-1 activation and MAP kinase activation has also been shown in PC12 cells in response to nerve growth factor (Wood *et al.*, 1992). Other studies using dominant negative Raf-1 mutants and c-Raf-1 antisense expression have also questioned the role of c-Raf-1 in agonist-stimulated activation of MAP kinase (Kizaka-Kondoh & Okayama, 1993, Chao *et al.*, 1994) although in these studies, the level of Raf activation was not addressed. Thus, it is possible that in BPA fibroblasts, the strong activation of c-Raf-1 by thrombin and PDGF may not represent the MEK-activating stimulus and that an alternative pathway involving an additional kinase may be involved. Other MEK activators have been described besides Raf-1 including MEK kinase (MEKK), a mammalian homologue of the yeast gene STE11, which has been shown to phosphorylate and activate MEK-1 (Lange-Carter, 1993). This MEK activator was isolated and cloned following the

discovery that, contrary to the present study, thrombin did not measurably activate Raf-1 in Rat 1a and NIH3T3 fibroblasts when endogenous MEK and MAP kinase were clearly activated (Gardner *et al.*, 1993). It was initially suggested that MEKK may be recruited as a MEK activator by agonists acting through heterotrimeric G proteins. However, other studies have demonstrated that thrombin can activate c-Raf-1 in NIH3T3 and EC4A1 fibroblasts, albeit weakly (Kyriakis *et al.*, 1993, Reuter *et al.*, 1995). Furthermore, the role of MEKK in the MAP kinase cascade is less clear than is the role of c-Raf-1 and the physiological function of this protein may be to regulate equivalent MEKs of the stress-activated protein kinase cascade (Minden *et al.*, 1994, Yan *et al.*, 1994) (see Chapter 6). Of the other members of the Raf family, A-Raf and B-Raf are less well characterized as MEK activators and exhibit tissue-specific expression (Storm *et al.*, 1990). Nevertheless, recent evidence has shown that B-Raf and an unidentified 40-50 kDa MEK activator are major MEK-activators in response to PDGF in NIH3T3 fibroblasts (Reuter *et al.*, 1995) and A-Raf phosphorylates and activate MEK-1, but not MEK-2 in Hela cells (Wu *et al.*, 1996). Thus, while these results demonstrate that thrombin and PDGF -activated c-Raf-1 kinase is capable of phosphorylating MEK, it is not obvious whether this activity accounts exclusively for the activation of MEK by these agonists *in vivo*, whether other MEK activators are involved or whether this response is physiologically relevant to MEK activation at all.

3.9.2 Role of PKC and PTX-sensitive G-proteins in thrombin and PDGF -stimulated MAP kinase activation

Details of events upstream of the MEK activator in the activation of the MAP kinase cascade are beginning to emerge and are reviewed in Section 1.6.4.1. For both tyrosine kinase-encoded receptors and G-protein-coupled receptors such as thrombin, activation of MAP kinase has been shown to occur through both Ras-dependent and Ras-independent pathways (Burgering *et al.*, 1993a, van Corven *et al.*, 1993, Hordijk *et al.*, 1994). A number of studies have implicated PKC in the Ras-independent pathway (Medema *et al.*, 1991, de Vries Smit *et al.*, 1992) which may involve the direct phosphorylation and activation of c-Raf-1 by certain PKC isoforms (Sozeri *et al.*, 1992, Kolch *et al.*, 1993).

Evidence that MAP kinase can be regulated by PKC is based on findings showing that short-term treatment with phorbol esters, which mimic the effects of diacylglycerol in the activation of PKC *in vitro*, induces early activation of MAP kinase (L'Allemain *et al.*, 1991b,

Kahan *et al.*, 1992). Using PKC inhibitors and PKC dowregulation protocols, a number of studies have also implicated a role for PKC in the regulation of MAP kinase by a variety of agonists in different cell types including thrombin in CCL339 fibroblasts (Kahan *et al.*, 1993), angiotensin II and PDGF in rat aortic smooth muscle cells (Malarkey *et al.*, 1996) and growth hormone in preadipocytes (Anderson, 1992). The observation that MAP kinase was activated by acute exposure of BPA fibroblasts to PMA prompted an investigation to address whether PKC was involved in the activation of MAP kinase by thrombin and PDGF. Receptors for both of these agonists have been shown to couple to different isoforms of phospholipase C (PLC) (Brass *et al.*, 1986, Kondo *et al.*, 1996) and a tentative role for PKC can be predicted by the findings that in fibroblasts, both thrombin and PDGF generate on hydrolysis of membrane phosphatidylinositol 4,5 bisphosphate, inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG), the endogenous activator of the majority of PKC isoforms (Habenicht *et al.*, 1981, Paris & Pouysségur, 1986, Ha & Exton, 1993).

The possible role of PKC in the regulation of MAP kinase in response to thrombin and PDGF was clarified using a PKC downregulation strategy. This is a well established technique which, by prolonged exposure to high concentrations of active phorbol ester, renders cells effectively deficient in classical PKC isoforms (Rodriguez-Pena & Rozengurt, 1984, Blackshear *et al.*, 1985). The effect of this regimen on PMA-sensitive PKC was validated by the complete loss of responsiveness to phorbol ester, and the loss of immunodetectable PKC α and ε isoforms in cell lysates.

In contrast to the findings of Kahan *et al.* (1993), PMA-sensitive PKC isoforms do not appear to play a role in the regulation of MAP kinase by thrombin in BPA fibroblasts as thrombin-stimulated MAP kinase activation was found to be unaffected by PKC downregulation. For PDGF, however, MAP kinase activation was attenuated by approximately 30 % in cells in which PKC α and ε was depleted suggesting a modest proportion of PDGF-stimulated MAP kinase activation is regulated by a PKC-dependent component. The major PKC-independent route to MAP kinase activation by PDGF may be mediated by ras-dependent activation of Raf-1 via adaptor proteins such as Grb2. The contribution of PKC to the activation of MAP kinase by a particular stimuli may depend on the cell type. In Swiss 3T3 fibroblasts, as for BPA fibroblasts described here, MAP kinase activation by PDGF is activated by both PKC -dependent and independent routes (l'Allemain *et al.*, 1991b, Burgering *et al.*, 1993a) while in Rat-1 fibroblasts, is exclusively PKC-independent (Burgering *et al.*, 1993a). The findings of the present study cannot rule out the

possible involvement of PMA-insensitive isoforms of PKC in MAP kinase activation. Indeed, a surprising recent finding by Moscat and co-workers showed that PKC ζ may also function as a MEK activator (Berra *et al.*, 1995), further outlining the complexity of this signalling system.

In support of the finding that in BPA fibroblasts PKC does not play a major role in the activation of MAP kinase by thrombin or PDGF was the observation that in comparison to these agonists, PMA caused a weaker activation of p42 and p44 MAP kinases which was often found to be variable between experiments. Furthermore, the temporal effect of PMA on MAP kinase activation did not resemble the kinetics for activation by thrombin. Indirect evidence to further negate an involvement for a PLC-PKC pathway in the regulation of MAP kinase by thrombin is indicated by the observation that thrombin has not been found to appreciably stimulate IP_3 generation in BPA fibroblasts (D. Welsh, personal communication). In contrast, endothelin, which activates its own G-protein-coupled receptor to evoke second messenger events in common with thrombin, was shown to be a stimulant for phophoinositide hydrolysis (D. Welsh, personal communication) but did not phosphorylate or activate MAP kinase. Thus, while a pathway involving PMA-sensitive PKC isoforms can phosphorylate and activate MAP kinase in BPA fibroblasts, and accounts for a minor proportion of MAP kinase activation in response to PDGF, a receptor-mediated PLC-PKC element does not appear to be the route by which thrombin activates MAP kinase, implying that at least one other pathway is involved.

For some G-protein-coupled agonists in certain cell types, there is no apparent role for the phospholipase C-PKC cascade in activation of MAP kinase. In a number of these systems, a PKC-independent route that is PTX-sensitive has been described, implicating a role for the G_i/G_o subfamily of G proteins. For example, PTX inhibits the activation of MAP kinase by thrombin in fibroblasts (Gupta *et al.*, 1992, Meloche *et al.*, 1992) and by other G-protein-coupled agonists including LPA in fibroblasts and endothelial cells (Howe & Marshall, 1993, Hordijk *et al.*, 1994, McLees *et al.*, 1995) and endothelin-1 in smooth muscle cells (Malarkey *et al.*, 1995b). The signalling pathway by which G_i-protein-coupled receptors activate MAP kinase remains poorly understood but may involve the activation of Ras (van Corven *et al.*, 1993, Hordijk *et al.*, 1994) mediated by βγ subunits (Crespo *et al.*, 1994, Koch *et al.*, 1994, Hawes *et al,* 1995) and possibly a non-receptor tyrosine kinase (van Corven *et al.*, 1993, Hordijk *et al.*, 1994). Other reports, however, have implicated a role for the G_i α subunit as it

has been found that a GTPase-deficient $G_{\alpha i2}$ mutant referred to as the *gip*2 oncogene product stimulates MAP kinase in Rat 1a fibroblasts (Gupta *et al.*, 1992).

In this study, thrombin-stimulated activation of MAP kinase was found to be insensitive to PTX and therefore did not appear to involve G_i/G_o. The activation of MAP kinase by PDGF was unaffected by PTX as might by expected for an agonist that operates via the tyrosine kinase receptor pathway. While the ability of PTX to catalyse the ADP-ribosylation of G_i/G_o α subunits in BPA fibroblasts was not tested, this regimen has been used by other authors to effectively inhibit signalling events in other cell types (Hordijk *et al.*, 1994, Koch *et al.*, 1994), and was found to attenuate thrombin-stimulated DNA synthesis in BPA fibroblasts (see Section 3.9.3). However, as for PKC, the role of PTX -sensitive G proteins in relaying responses to thrombin and other G-protein-coupled receptor agonists in a range of cells has been shown to be variable. In agreement with the findings here, thrombin-stimulated MAP kinase activation in astrocytes is insensitive to PTX (Bhat *et al.*, 1995). This may reflect that signalling via a PTX-sensitive route may require the cell-selective expression of the G_{i2}-coupled effectors (Gupta *et al.*, 1992) and again demonstrates that there may be multiple cell-specific mechanisms for the regulation of MAP kinases.

3.9.3 The role of MAP kinase in agonist-stimulated DNA synthesis

A role for MAP kinase in the transmission of proliferative signals in BPA fibroblasts was supported by the finding that thrombin, PDGF and PMA, which all evoked a sustained activation of MAP kinase lasting up to 60 minutes, stimulated [^{3}H]thymidine incorporation. Conversely, endothelin-1 and LPA that either had no effect, or caused a very weak and transient activation of MAP kinase, failed to induce DNA synthesis. The duration of MAP kinase activation is believed to determine different cellular responses in a particular cell type (Marshall, 1995). In fibroblasts, the sustained activation of MAP kinase correlates with mitogenesis (Meloche *et al.*, 1992, Vouret-Craviari *et al.*, 1993b). This long lasting phase of MAP kinase activation by agonists such as thrombin is associated with the translocation of both p42 and p44 MAP kinases to the nucleus whereas transient activation of MAP kinase does not lead to nuclear translocation (Chen *et al.*, 1992, Lenormand *et al.*, 1993). Nuclear accumulation of active MAP kinase may be important for the activation of MAP kinase-responsive nuclear transcription factors (Alvarez *et al.*, 1991, Gille *et al.*, 1992) leading to the expression of new genes required to initiate mitogenesis.

An obligatory involvement of MEK and, consequently, of the MAP kinase cascade in promoting DNA synthesis was demonstrated by the ability of PD098059 to inhibit both thrombin and PDGF -stimulated [^{3}H]thymidine incorporation. This is consistent with the inhibition of DNA synthesis in fibroblasts by PD098059 that has been reported previously (Dudley *et al.*, 1995) and corroborates the findings of other investigators using oligonucleotide antisense and $p44^{mapk}$ kinase-deficient mutants to show that activation of $p42^{mapk}$ and $p44^{mapk}$ by thrombin and growth factors is an absolute requirement for triggering G_o-arrested fibroblasts to enter the cell cycle (Pagès *et al.*, 1993).

Consistent with a role for a MAP kinase-dependent pathway in the transmission of mitogenic signals, the effect of depletion of cellular PKC levels by phorbol ester pretreatment on DNA synthesis caused by thrombin and PDGF paralleled the effect on MAP kinase activation. Thus, PMA-sensitive PKC isoforms are unlikely to be involved, or play a minor role in the mitogenic response to thrombin and PDGF, respectively. This inference is also implied by the relative weak efficacy of PMA to stimulate [^{3}H]thymidine incorporation compared to thrombin or PDGF (Fig. 3.6 & Table 3.2). The magnitude of the MAP kinase signal, however, does not appear to always directly correlate with DNA synthesis as is exemplified by thrombin. In BPA fibroblasts, thrombin is as efficient as PDGF as a mitogen (Fig. 3.6 & Table 3.2) yet at maximally-effective concentrations and exposure times, stimulates MAP kinase activity to a lesser extent (Table 3.1). Conceivably, full activation of MAP kinase may not be necessary to cause an optimal mitogenic response. Alternatively, as discussed above, the strength of the MAP kinase signal at later time points, where the level of MAP kinase activation by thrombin and PDGF appear to be comparable (Figs. 3.1.1 & 3.2.1), may be a more important determinant in reflecting the efficacy of a particular stimulus as a mitogen. However, the sensitivity of thrombin-stimulated DNA synthesis but not MAP kinase activation to PTX lends support to the possibility that additional signalling pathways other than the MAP kinase cascade are involved in transducing growth signals in BPA fibroblasts. While the involvement of a G_i/G_o protein has been implicated previously in the mitogenic effect of thrombin in fibroblasts by other investigators using PTX (Chambard *et al.*, 1987, Gupta *et al.*, 1992) or microinjection of functionally inhibitory antibodies to $G\alpha_{i2}$ and $G\alpha_o$ (Lamorte *et al.*, 1993, Baffy *et al.*, 1994), clearly, in BPA fibroblasts this pathway operates independently of MAP kinase.

Other authors have proposed that the full mitogenic action of thrombin in vascular smooth muscle and endothelial cells may be mediated by an indirect mechanism involving the

release of peptide growth factors such as PDGF and basic fibroblast growth factor (bFGF) that act in an autocrine manner (Stouffer *et al.*, 1993, Weiss & Maduri, 1993, Herbert *et al.*, 1994). However, in the present study, the time course for thrombin-stimulated [^{3}H]thymidine incorporation using pulse labelling revealed that peak incorporation of [^{3}H]thymidine for both thrombin and PDGF occurred at the same point (24 hours) and was not delayed (not shown). This suggests that thrombin itself, and not a factor that is synthesised and secreted in response to thrombin, serves as the primary stimulus for mitogenesis.

3.9.4 Role of a thrombin receptor in thrombin-signalling in BPA fibroblasts

Most, if not all of the cellular effects of thrombin are believed to mediated by a G-protein-coupled thrombin receptor which has been cloned in several mammalian species and cell types (Rasmussen *et al.*, 1991, Vu *et al.*, 1991, Zhong *et al.*, 1992). The thrombin receptor is thought to interact with at least two families of G proteins, the pertussis toxin (PTX)-sensitive G_i and the non-PTX sensitive G_q subtype coupled to PI-PLC. However, as described above, in this system, neither activation of the phospholipase C-Ca^{2+}-PKC pathway nor a route involving G_i/G_o appear to be involved in transducing signals required for activation of MAP kinase in response to thrombin. The unique features of thrombin-induced activation of MAP kinase in BPAFs in terms of kinetics, concentration required and signalling pathways evoked presents the question: how does the thrombin receptor couple to downstream effectors in BPA fibroblasts? While α_s is another class of G protein subunit known to be recruited by G-protein-coupled receptors, there is no evidence in the literature to indicate that the thrombin receptor couples to this species. Furthermore, activation of G_s is linked to the stimulation of adenylyl cylase causing an increase in cyclic AMP levels. Although in some cell types, cAMP accummulation causes an activation of MAP kinase (Faure *et al.*, 1994, Frödin *et al.*, 1994), elevation of cAMP in fibroblasts appears to confer either a negative (Burgering *et al.*, 1993b, Wu *et al.*, 1993a) or negligible effect (Kahan *et al.*, 1992, McKenzie & Pouysségur, 1996) on MAP kinase activation, and inhibits mitogenesis (Magnaldo *et al.*, 1989, McKenzie & Pouysségur, 1996). Recently, it has been shown that the thrombin receptor can couple to PTX-insensitive G-proteins G_{12} and G_{13} belonging to a distinct class separate from α_s, α_i and α_q in platelet membranes (Offermans *et al.*, 1994). While little is known about the cellular effectors regulated by this class of G-protein, MAP kinase is unlikely to be a candidate since mutationally activated G_{12} and G_{13} stimulate DNA synthesis but did not

significantly increase MAP kinase activity in Rat-1 fibroblasts (Voyno-Yasenetskaya *et al.*, 1994).

The specificity of thrombin-induced responses in BPA fibroblasts was demonstrated using hirudin, the most potent natural inhibitor of thrombin derived from the European medicinal leech *Hirudo medicinalis*. The unique specificity and high affinity of hirudin towards thrombin results from numerous interactions both within the catalytic centre of thrombin and at an anion-binding exosite found on the surface distinct from the active site (Grütter *et al.*, 1990, Rydel *et al.*, 1991). The carboxyl tail of hirudin which binds to the latter region shows sequence similarity to a region on the extracellular domain of the cloned thrombin receptor immediately carboxyl to the thrombin cleavage site which structure-function studies reveal is important for thrombin binding (Liu *et al.*, 1991). Thus, hirudin not only inhibits the catalytic activity of thrombin but also the binding of thrombin to the receptor. The finding that hirudin completely inhibited both thrombin-stimulated MAP kinase activation and [^{3}H]thymidine incorporation at a concentration reported to abolish the amidolytic activity of thrombin (Herbert *et al.*, 1993) confirmed that these effects were not a consequence of non-specific actions of thrombin and is consistent with the possible involvement of the proteolytically activated thrombin receptor.

The role of the thrombin receptor in mediating the responses to thrombin in BPA fibroblasts was investigated using the synthetic 14-mer peptide SFLLRNPNDKYEPF or thrombin receptor-activating peptide (TRAP-14). This sequence corresponds to amino acids within the N-terminal extracellular domain of the cloned human thrombin receptor which, when revealed following proteolytic cleavage by thrombin, functions as a "tethered ligand" to effect receptor activation (Vu *et al.*, 1991) by interacting with other receptor domains (Gerstzen *et al.*, 1994). TRAP-14 has been shown to mimic a number of the cellular responses of thrombin (Vu *et al.*, 1991, Hung *et al.*, 1992a, 1992b) implicating the involvement of the cloned thrombin receptor. However, in BPA fibroblasts, unlike thrombin, TRAP-14 was unable to stimulate the activation of MAP kinase or DNA synthesis. One reason for this could be that the receptor-activating sequence for an equivalent bovine thrombin receptor, which has yet to be cloned, could be different to the human TRAP-14 used here. However, TRAP-14 has previously been reported to evoke signalling events in bovine pulmonary artery endothelial cells (Lum *et al.*, 1993). Furthermore, variations in receptor-activating sequences may not be critical either for the activation of thrombin receptors from different species, or multiple subtypes of the thrombin receptor in different cell types, as human TRAP-14 caused strong

activation of MAP kinase in rat aortic smooth muscle cells. The predicted receptor-activating sequence for the thrombin receptor which has been cloned in this tissue (Zhong *et al.*, 1992) differs from the human sequence by one residue within the first six amino acids believed to be important for receptor activation with the replacement of the first leucine at position 3 (SFLLRN***) with phenylalanine (SFFLRN***) while the remaining eight amino acids in the corresponding region in the rat show no sequence homology (Zhong *et al.*, 1992). The weight of evidence in the literature suggests that this substitution does not appear to impart species specificity (van Obberghen-Schilling *et al.*, 1993). Taken together, this evidence suggests that the actions of thrombin in BPA fibroblasts do not appear to be mediated by a mechanism resembling the tethered-ligand model described for proteolytic activation of the cloned human and rodent thrombin receptors. Rather, in this cell type, the effect of thrombin may be due to its enzymatic activity on other substrates or by signal generated by interaction with the cloned receptor further to those produced by proteolysis.

A number of other studies have also dissociated cellular responses to receptor activation by thrombin and TRAPs. Lum and co-workers (1993) have demonstrated that TRAP -dependent and -independent pathways are involved in thrombin-induced enhancement of vascular endothelium permeability inferring that dual signals can be initiated by thrombin at the cell surface possibly by thrombin receptor -dependent and -independent mechanisms. Similarly, in hamster lung fibroblasts, where a biphasic activation of MAP kinase in response to thrombin has been described (Vouret-Craviari *et al.*, 1993b), TRAP stimulates only the first rapid phase of activity at around 5 minutes and not the additional persistent activation of MAP kinase seen with thrombin which is required for growth. This finding led to the proposal that activation of the thrombin receptor is not sufficient to account for the full mitogenic effect of thrombin (Vouret-Craviari *et al.*, 1993b). In view of the findings with TRAP-14, here, it is conceivable that delayed sustained kinetics of thrombin-stimulated MAP kinase activity and DNA synthesis are not a consequence of activation of the currently-defined thrombin receptor but rather a signal generated by a novel mechanism. The possibility that thrombin may exert additional actions by interacting with other uncharacterized thrombin-responsive cell-surface proteins may also be indicated by the high concentrations required. These responses may involve a distinct binding site on the cell membrane for a region encompassing residues 365-378 within loop-B on the surface of the human thrombin B chain and distant from the active centre. A peptide sequence corresponding to this region has been shown to be responsible for a non-catalytic-dependent component of thrombin-stimulated growth in a macrophage-like

tumour cell line (Bar-Shavit *et al.*, 1986) and human umbilical vein endothelial cells (Herbert *et al.*, 1993) over the concentration range for thrombin-stimulated DNA synthesis in BPA fibroblasts. Even though hirudin abolished thrombin-stimulated MAP kinase activation and DNA synthesis in BPA fibroblasts, the contribution of a non-catalytic action of thrombin in mediating these responses via a second receptor site cannot be excluded since hirudin has also been found to block the mitogenic effect of esterolytically-inactive thrombin in macrophages (Bar-Shavit *et al.*, 1986).

3.9.5 CONCLUDING REMARKS

Taken together, these results indicate that in BPA fibroblasts, thrombin and PDGF activate components of the well-characterized MAP kinase cascade resulting in the sustained activation of MAP kinase which appears to be obligatory for these agonists to stimulate DNA synthesis in this cell type. For thrombin, signalling by the MAP kinase pathway displays unusual characteristics defined by the slow kinetics of MAP kinase activation which does not appear to employ either the PKC or PTX-sensitive G protein -dependent signalling pathways that have been described for other ligands that act on G-protein coupled receptors, and may represent the involvement of a novel cell surface receptor or distinct G proteins. The magnitudes of the respective MAP kinase signal and the mitogenic response to thrombin relative to PDGF, and the finding that thrombin stimulated DNA synthesis could be dissociated from MAP kinase activity by PTX suggests that although activation of MAP kinase may be required for cell replication, one or more other signalling systems transmit mitogenic signals in BPA fibroblasts.

CHAPTER 4

CHARACTERIZATION OF AGONIST-STIMULATED ACTIVATION OF THE P70 RIBOSOMAL S6 PROTEIN KINASE PATHWAY IN BOVINE PULMONARY ARTERY FIBROBLASTS

As well as the Raf/MEK/MAP kinase pathway, signal transduction by other protein kinase phosphorylation cascades is believed to be important in cell growth and division. One such pathway that has emerged recently involves the 70 and 85 kDa serine/threonine-specific ribosomal protein S6 kinases (reviewed in Section 1.7). Both isoforms of the same kinase represent alternative splice products derived from a single gene (Grove *et al.* 1991) and hereafter referred to collectively as $p70^{s6k}$. Mounting evidence suggests that $p70^{s6k}$ plays multiple roles in mitogenic signalling (reviewed by Ferrari & Thomas, 1994, Chou & Blenis, 1995) which lie on a distinct pathway from the p42 and p44 MAP kinases (Ballou *et al.*, 1991). In a similar way to MAP kinase, $p70^{s6k}$ is rapidly activated in response to most mitogenic stimuli, including growth factors, cytokines, phorbol esters and oncogene products. Activation of $p70^{s6k}$ is believed to be important for cell cycle progression as serum-stimulated fibroblasts are arrested in G_1 phase when microinjected with antibodies raised against $p70^{s6k}$ (Lane *et al.*, 1993, Reinhard *et al.*, 1994). Furthermore, the G_1 to S phase transition is also delayed or prevented in certain cell types, including T lymphocytes and fibroblasts, by treatment with the immunosuppressant rapamycin, found to potently and selectively block activation of $p70^{s6k}$(Calvo *et al.*, 1992, Chung *et al.*, 1992, Price *et al.*, 1992, Migita *et al.*, 1996). $P70^{s6k}$ is believed to be the major physiological kinase phosphorylating the S6 polypeptide of the 40S ribosomal subunit upon mitogenic stimulation in mammalian cells (Chung *et al.*, 1992, Flotov & Thomas, 1992). S6 phosphorylation is thought to specifically increase the rate of translation of a family of mRNAs containing a polypyrimidine tract near the 5' untranslated terminus (Jefferies *et al.*, 1994) that encode for certain proteins required for efficient G_1 progression, such as ribosomal proteins and elongation factors (Terada *et al.*, 1994). In addition to effects on protein synthesis, $p70^{s6k}$ may also be important in regulating gene transcription since the transcription factor cAMP-responsive element modulator (CREMτ) has been shown to be a physiological substrate for $p70^{s6k}$ and activation of CREMτ by serum is blocked by rapamycin (de Groot *et al.*, 1994). The use of rapamycin as a pharmacological probe has implicated the involvement of the $p70^{s6k}$ pathway in a multitude other cellular processes involved in growth, including the regulation of other proteins involved in protein synthesis such as the translation repressor eIF-4E-BPI (PHAS-1) (Graves *et al.*, 1995, Lin *et al.*, 1995), and translation elongation factor eEF-2 (Redpath *et al.*, 1996) as well as components of the cell cycle machinary (Morice *et al.*, 1993, Nourse *et al.*, 1994). For

many of these effects, however, a definitive role for $p70^{s6k}$ has not been established and may reflect a bifurcation of rapamycin-sensitive signalling pathways.

The mechanism(s) by which $p70^{s6k}$ becomes activated in response to stimuli is not entirely clear but appears to involve phosphorylation at multiple serine/threonine residues by more than one protein kinase (Han *et al.*, 1995, Weng *et al*, 1995b). The identity of the protein kinase directly upstream of $p70^{s6k}$ is still unknown, although roles for upstream signalling molecules more distal in the pathway leading to $p70^{s6k}$ activation have emerged. While early studies characterizing $p70^{s6k}$ regulation demonstrated roles for both PKC - dependent and independent pathways (Susa *et al.*, 1992, Anderson, 1993, Chung *et al.*, 1994) most attention of late has focused on phosphatidylinositol (PI) 3-kinase. Receptor mutants preventing PI 3-kinase binding and activation, and the structurally unrelated PI 3-kinase inhibitors wortmannin and LY294002, have been shown to inhibit $p70^{s6k}$ activation in response to growth factors and cytokines (Cheatham *et al.*, 1994, Chung *et al.* 1994, Monfar *et al.*, 1995). Not only do these, and many other investigations that followed, implicate an obligatory role for PI 3-kinase in the regulation of $p70^{s6k}$ by a range of extracellular stimuli, but also provide a molecular target for PI 3-kinase, a signalling molecule recognized for a long time as a playing a critical role in mitogenesis (Kaplan *et al.*, 1987, Coughlin *et al.*, 1989). It is well established that PI 3-kinase signalling by RTKs is attributable to a 110 kDa catalytic subunit (p110) which becomes activated by binding to phosphorylated YXXM motifs in the cytoplasmic domains of the activated receptor or their substrates via a 85 kDa (p85) SH2/SH3 domain-containing regulatory subunit (Morgan *et al.*, 1990, Hiles *et al.*, 1992, Carpenter *et al.*, 1993). Characterization of PI 3-kinase activities in response to G-protein-coupled receptor agonists such as thrombin, however, is currently unclear and has been proposed to involve both the heterodimeric p85/PI 3-kinase which may be dependent on the small G-protein Rho for activation (Zhang *et al.*, 1993a) and a distinct form of PI 3-kinase activated directly by the $\beta\gamma$ subunits of dissociated G-proteins (Stephens *et al.*, 1994, Thomason *et al.*, 1994, Stoyanov *et al.*, 1995)

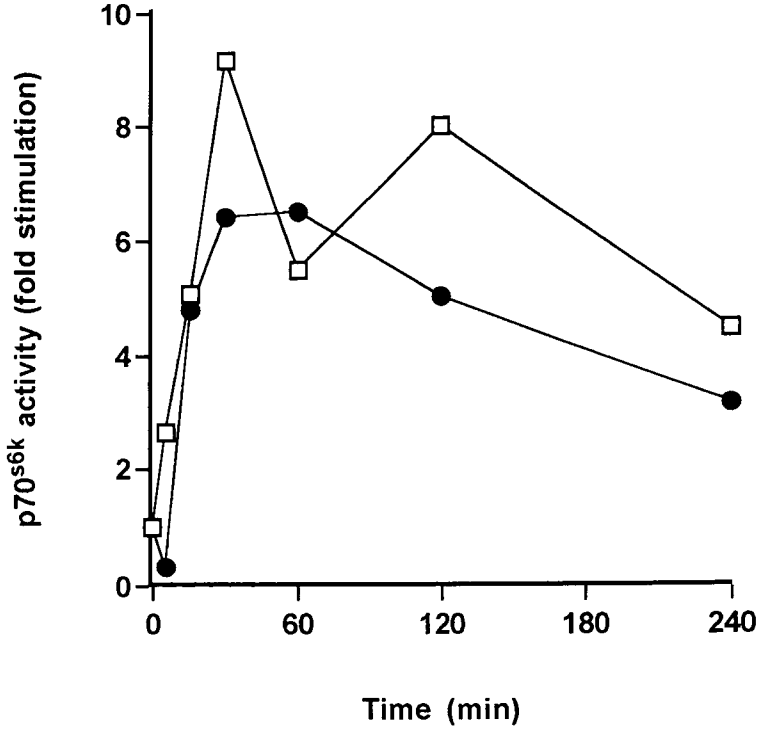
Although activation of the $p70^{s6k}$ pathway in response to growth factors that activate receptor tyrosine kinases (RTKs) has been well documented, there is less evidence to implicate $p70^{s6k}$ in signalling by G-protein-coupled receptor agonists. Moreover, no studies to date have addressed the involvement of this signalling pathway in the mitogenic effect of thrombin. The aim of this study is to characterize the activation of $p70^{s6k}$ by thrombin and PDGF in BPA fibroblasts and determine the processes regulating $p70^{s6k}$ activity, in particular, whether a PI

3-kinase is involved. In addition, the role of the $p70^{s6k}$ pathway in thrombin and PDGF -stimulated DNA synthesis in BPA fibroblasts will be assessed.

4.2 Thrombin and PDGF-stimulated $p70^{s6k}$ activation in BPA fibroblasts

The activity of $p70^{s6k}$ kinase was assayed by the ability of immunoprecipitated $p70^{s6k}$ from thrombin and PDGF -stimulated BPA fibroblasts to phosphorylate *in vitro* a peptide representing a portion of the 40S ribosomal subunit S6 protein (RRRLSSLRA), believed to contain the major *in vivo* sites of phosphorylation by $p70^{s6k}$ (Flotov & Thomas, 1992) as described in Section 2.4.4. Immunoprecipitation and immunoblotting with a polyclonal antibody raised against the C-terminus (amino acids 485-502) of $p70^{s6k}$ detected two proteins, one migrating at approximately 70 kDa and another, of lower abundance and of approximately 85 kDa which is likely to be the minor splice variant isoform of the same kinase that also contains the same epitope recognized by the $p70^{s6k}$ antibody (Fig. 4.2). Addition of thrombin to quiescent bovine PA fibroblasts stimulated $p70^{s6k}$ kinase phosphotransferase activity in a time and concentration -dependent manner resulting in approximately 6 fold increase in activity at maximal stimulation. The time course for thrombin-stimulated $p70^{s6k}$ activation was monophasic (Fig. 4.1A). Enzyme activity increased rapidly after 5 min, reached a maximum between 30-60 min, and slowly declined thereafter. Activity was still elevated above basal levels after 4 h exposure to thrombin. Stimulation of $p70^{s6k}$ activity required a thrombin concentration exceeding 3 nM and increased steadily with increasing concentrations up to 300 nM (Fig. 4.1B). At higher thrombin concentrations, cell monolayers became detached from the plate within 30 minutes. In comparison, PDGF exhibited time-dependent biphasic kinetics of $p70^{s6k}$ activation (Fig. 4.1A) as has been observed by other authors in Swiss 3T3 cells (Susa *et al.*, 1992). The first phase of activity was maximal at approximately 30 minutes, began to fall soon after before peaking again at 2 h. This late phase of activity was sustained beyond 4 h. Kinase activity in response to PDGF was also concentration-dependent reaching a maximum at 30 ng/ml (results not shown). Activation of $p70^{s6k}$ *in vivo* is believed to occur following phosphorylation by an upstream kinase (Han *et al.*, 1995, Weng *et al*, 1995b). As it has been previously shown that hyperphosphorylated forms of $p70^{s6k}$ exhibit reduced mobility on SDS-PAGE (Chung *et al.* 1992, Wilson *et al.*, 1996), agonist-stimulated activation of $p70^{s6k}$ was also assessed from the electrophoretic mobility of the protein on $p70^{s6k}$ immunoblots (Fig. 4.2). Both thrombin and

A.


p70s6k activity (fold stimulation)
10
8
6
4
2
0
0
60
120
180
240
Time (min)

B.

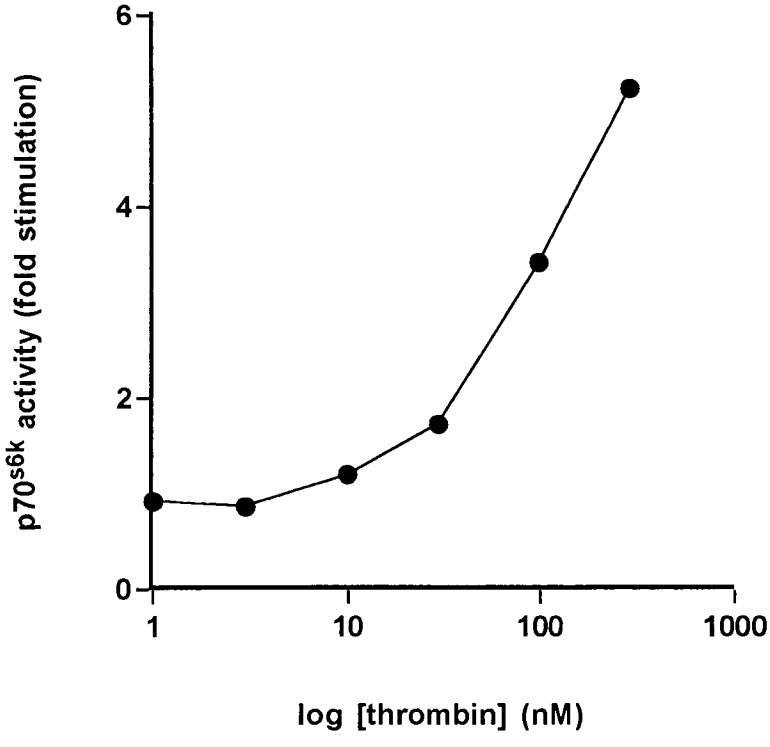

p70s6k activity (fold stimulation)
6
4
2
0
1
10
100
1000
log [thrombin] (nM)

Figure 4.1 Thrombin and PDGF -stimulated $p70^{s6k}$ activity in BPA fibroblasts. Cells were stimulated with **A)** thrombin (300 nM) (●) or PDGF (30 ng/ml) (□) for the times indicated or **B)** thrombin for 30 minutes at the concentrations indicated and immunoprecipitates of $p70^{s6k}$ from cell lysates were assayed for $p70^{s6k}$ activity as outlined in Section 2.4.4. Kinase activity is expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into ribosomal S6 peptide from representative experiments carried out on three separate occasions with similar results. (control: 81.1 fmol Pi min^{-1} mg^{-1} protein (300 nM thrombin); 144.1 pmol Pi min^{-1} mg^{-1} protein (30 ng/ml PDGF)).

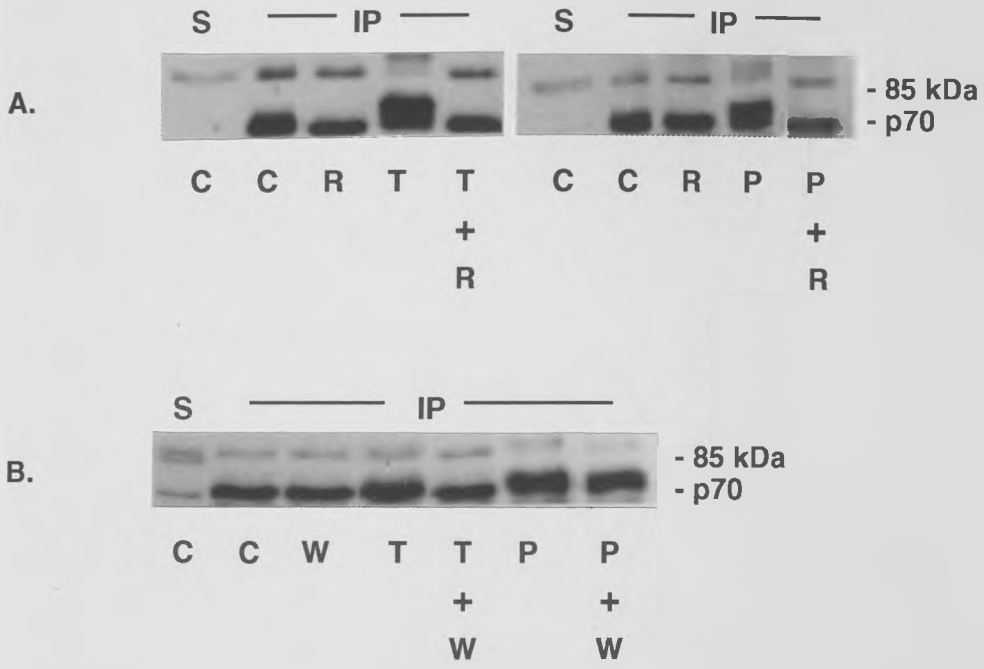


Figure 4.2 Effect of rapamycin and wortmannin on agonist-stimulated $p70^{s6k}$ electrophoretic mobility in BPA fibroblasts. Cells were pretreated with **A)** 50 nM rapamycin (R) or **B)** 100 nM wortmannin (W) for 30 minutes prior to exposure to thrombin (300 nM for 30 minutes) (T), PDGF (30 ng/ml for 30 minutes) (P) or vehicle (C). Immunoprecipitates of $p70^{s6k}$ from cell lysates (IP) or supernatents (S) were immunoblotted for $p70^{s6k}$ as outlined in Section 2.3. Bands corresponding to $p70^{s6k}$ and the mobility of the 85 kDa molecular weight marker are indicated on the right of immunoblots, each of which is a representative example from three independent experiments.

PDGF treatment resulted in the characteristic retardation in the migration of $p70^{s6k}$ on polyacrylamide gel giving rise to at least four intermediate bands suggesting that these agonists induce multisite phosphorylation of $p70^{s6k}$. Mobility shifts were also observed associated with the minor 85 kDa band.

Phosphotransferase activity in $p70^{s6k}$ immunoprecipitates was also evaluated using KKRNRTLTK, a synthetic peptide substrate which shows a high degree of selectivity for $p70^{s6k}$, having a 50-fold higher V_{max}/K_m than $p90^{rsk}$ (Leighton *et al.*, 1995). When used as a substrate in this assay, KKRNRTLTK was found to be phosphorylated to an equal extent as RRRLSSLRA under identical assay conditions (not shown), although this peptide does not represent a physiological substrate for $p70^{s6k}$.

4.3 The effects of rapamycin, wortmannin and PTX on agonist-stimulated $p70^{s6k}$ activity in BPA fibroblasts

The specificity of the kinase activity in $p70^{s6k}$ immunoprecipitates was checked using the macrolide antibiotic rapamycin, a highly selective inhibitor of the $p70^{s6k}$ pathway. Pretreatment of BPA fibroblasts for 30 min with a specific inhibitor of $p70^{s6k}$ activation, rapamycin, at a concentration (50 nM) that abolition serum-stimulated $p70^{s6k}$ activity in NIH 3T3 fibroblasts (Weng *et al.*, 1995b), completely blocked $p70^{s6k}$ kinase activity in response to thrombin and PDGF and decreased basal $p70^{s6k}$ activity by approximately one half (Fig. 4.3). A similar effect was also observed with $p70^{s6k}$ activity by PDGF. The role of PI 3-kinase in thrombin-evoked $p70^{s6k}$ activation was investigated using wortmannin, a fungal metabolite shown to be a potent and specific inhibitor of PI 3-kinase (Yano *et al.*, 1993). Treatment with wortmannin lowered basal $p70^{s6k}$ activity in unstimulated cells and inhibited thrombin-stimulated $p70^{s6k}$ activity in a dose-dependent manner (Fig. 4.3 & 4.4); half maximal inhibition was apparent at 41.5±6.5 nM and comparable to the IC_{50} for wortmannin inactivation of serum and EGF -stimulated $p70^{s6k}$ described by other authors (Han *et al.*, 1995, Petristch *et al.*, 1995). At 100 nM, wortmannin completely abolished the thrombin response. In contrast to thrombin, $p70^{s6k}$ activity induced by PDGF was unaffected by wortmannin at concentrations up to 100 nM (Fig. 4.3 & 4.4). Inhibition of thrombin-stimulated $p70^{s6k}$ phosphotransferase activity by rapamycin and wortmannin, and of PDGF-stimulated kinase activity by rapamycin, was reflected by the reversal of thrombin and PDGF -induced $p70^{s6k}$ mobility shifts on SDS-PAGE (Fig. 4.2). Neither rapamycin (50 nM) nor

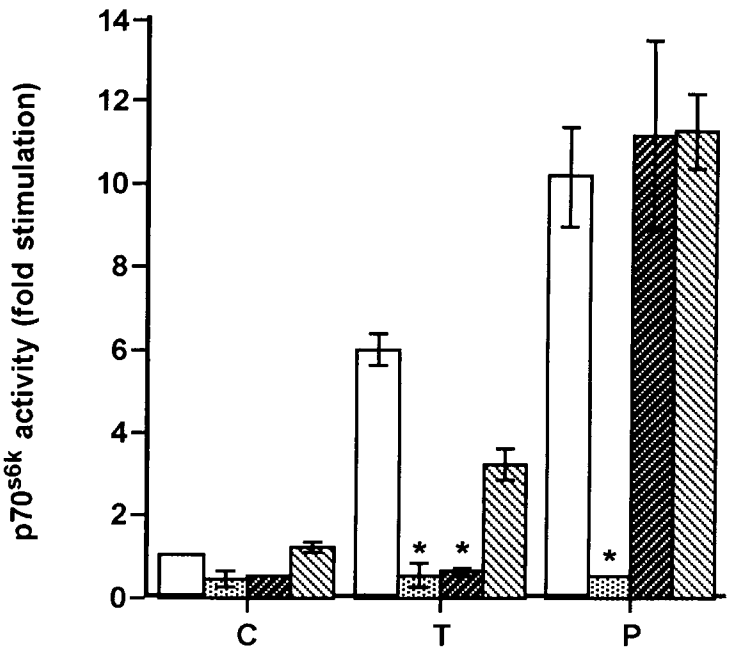


Figure 4.3 Effect of rapamycin, wortmannin and pertussis toxin on agonist-stimulated $p70^{s6k}$ activity in BPA fibroblasts. Cells were pretreated with vehicle (□), 50 nM rapamycin (⊡) or 100 nM wortmannin (▨) for 30 minutes, or 100 ng/ml pertussis toxin (▧) for 18 hours, prior to exposure to thrombin (300 nM for 30 minutes) (T), PDGF (30 ng/ml for 30 minutes) (P) or vehicle (C). Immunoprecipitates of $p70^{s6k}$ from cell lysates were assayed for $p70^{s6k}$ activity as describd in Section 2.4.4. Activity is expressed as mean±s.e.m. fold stimulation relative to control of [^{32}P]phosphate incorporated into ribosomal S6 peptide from three separate experiments. * denotes significant effect of inhibitor treatment at $p < 0.05$ level using Student's paired t test.

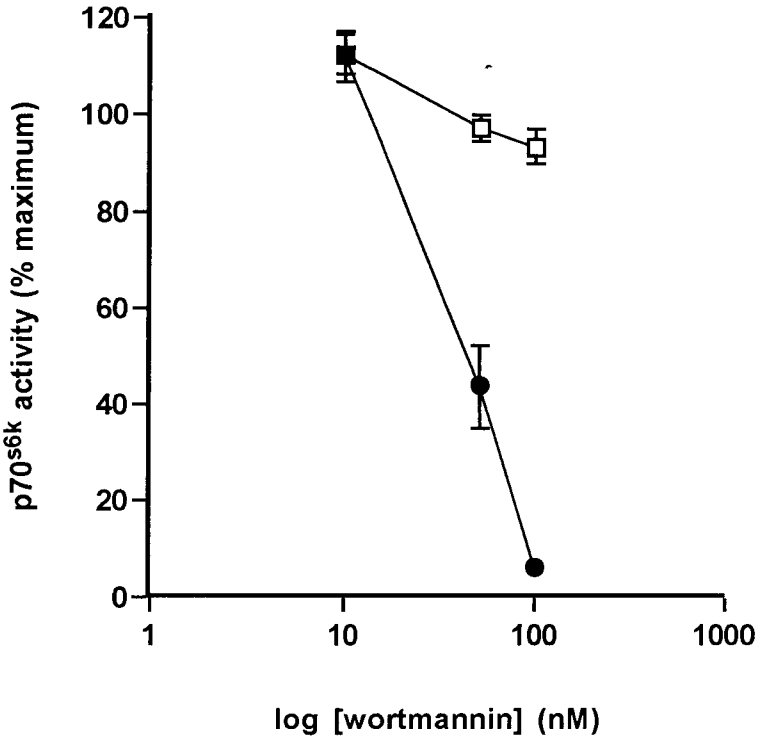


Figure 4. 4 Wortmannin inhibition of $p70^{s6k}$ activation by thrombin in BPA fibroblasts. Cells were pretreated for 30 minutes with wortmannin at the concentrations indicated prior to incubation with thrombin (300 nM) (●) or PDGF (30 ng/ml) (□) for 30 minutes. Immunoprecipitates of $p70^{s6k}$ from cell lysates were assayed for activity as described in Section 2.4.4. Results are expressed as mean±s.e.m. percentage of maximal thrombin or PDGF -stimulated $p70^{s6k}$ activity in the absence of wortmannin from two separate experiments. (maximum activity: 224.4±16.6 fmol Pi min^{-1} mg $protein^{-1}$ (300 nM thrombin); 645.9±66.0 fmol Pi min^{-1} mg $protein^{-1}$ (30 ng/ml PDGF).

wortmannin (100 nM) affected the time-dependent activation of p42 and p44 MAP kinase in response to either thrombin or PDGF, as assessed by mobility on SDS-PAGE (Fig. 4.9), further supporting the notion that $p70^{s6k}$ and MAP kinase lie on divergent pathways (Ballou *et al.*, 1991, Chung *et al.*, 1992, Ming *et al.*, 1994).

As $p70^{s6k}$ activation in response to other G protein-coupled receptor agonists has been shown to be pertussis toxin (PTX) sensitive (Wilson *et al.*, 1996) and thrombin-induced PI 3-kinase activity in platelets is partially inhibited by PTX (King *et al.*, 1991), the role of PTX-sensitive G-proteins in $p70^{s6k}$ activation by thrombin in BPA fibroblasts was assessed. Pretreatment of PA fibroblasts for 18 h with 100 ng/ml pertussis toxin (PTX) caused 48.3±5.4 % reduction in $p70^{s6k}$ activity stimulated by thrombin. Conversely, kinase activity following PDGF exposure was unaffected by prior PTX treatment (Fig. 4.3).

4.4 Agonist-stimulated phosphatidylinositol kinase activity in BPA fibroblasts

Since thrombin-stimulated $p70^{s6k}$ activation was wortmannin-sensitive, the ability of thrombin to activate PI 3-kinase in BPA fibroblasts was addressed. In preliminary studies, PI kinase activity was assayed in immunoprecipitates of the p85α subunit by measuring the incorporation of [^{32}P] into phosphatidylinositol (PI) *in vitro*. In this system, however, phosphorylation of PI was found to be markedly elevated in the p85 extract from unstimulated cells such that an increase in lipid kinase activity in response to agonist stimulation was unable to be detected. Following cell stimulation with growth factors that activate receptor tyrosine kinases, such as PDGF, PI 3-kinase activity is associated with the fraction of proteins phosphorylated on tyrosine residues (Coughlin *et al.*, 1989, Ruderman *et al.*, 1990). This is thought to reflect the interaction between the SH2 domains of p85 with specific phosphotyrosyl docking motifs such as those found on activated receptor- and non-receptor tyrosine kinases (Fry, 1994). Increased PI 3-kinase activity has also been observed in antiphosphotyrosine immunoprecipitates following activation of G protein -mediated systems (Stephens *et al.*, 1993). To evaluate whether p85/PI 3-kinase activity was associated with the phosphotyrosyl protein fraction in agonist-stimulated BPA fibroblasts, anti-phosphotyrosine immunoprecipitates were prepared from lysates of cells stimulated with either thrombin or PDGF and assayed for recovery of p85 and PI kinase activity *in vitro*. Western blotting analysis with anti-p85α antibody failed to reveal the presence of p85α in immunocomplexes of tyrosine phosphorylated proteins from BPA fibroblasts treated with thrombin (300 nM) at

time points up to 30 minutes (Figs. 4.5.1). Furthermore, there was no evidence of PI kinase activity in these immunoprecipitates (Fig. 4.5.3). In contrast, a six fold increase in anti-phosphotyrosine-immunoprecipitable PI kinase activity was extracted from lysates of cells exposed to 30 ng/ml PDGF for 5 minutes (Figs. 4.5.2 & 4.5.3). An examination of the time course revealed that phosphorylation of PI was stimulated as a early as 30 seconds after PDGF addition, was maximal at 5 minutes (6 fold stimulation) and declined thereafter yet was still elevated above unstimulated level at 30 minutes. Recovery of p85α in phosphotyrosine immunoprecipitates from cells treated with PDGF was also time dependent and maximal at 2 minutes inferring that tyrosine phosphorylation of p85 precedes enzyme activity (Fig. 4.5.1). Pretreatment of cells with wortmannin (50 nM) for 30 minutes totally blocked PDGF-stimulated PIP formation in BPA fibroblasts (Fig. 4.5.3). The [^{32}P]phospholabelled PI product on separation by TLC, co-migrated with the product of kinase activity in immunoprecipitates of the p85α regulatory subunit of PI 3-kinase inferring that the identity of this species is likely to be phosphatidylinositol 3-phosphate. Although the effect of wortmannin on the tyrosine phosphorylation status of p85α was not addressed, other studies in this laboratory have shown that in bovine tracheal smooth muscle cells, agonist stimulated PI kinase activity but not tyrosine phosphorylation of the regulatory subunit is sensitive to inhibition by wortmannin (P. Scott, personal communication) consistent with the notion that wortmannin inhibits PI 3-kinase by binding covalently to the p110 catalytic subunit (Yano *et al.*, 1993, Woscholski *et al.*, 1994).

A novel mechanism for 3-kinase activation has been described in thrombin-stimulated platelets and PDGF-stimulated fibroblasts involving an interaction between the p85 subunit and the 125 kDa cytoskeletal protein focal adhesion kinase ($p125^{FAK}$), a tyrosine kinase that localizes with integrins at focal contacts (Chen & Guan, 1994b, Giunebault *et al.*, 1995). For thrombin in platelets, this association has been shown to be independent of p85 tyrosine phosphorylation and involves the binding of the *src* homology (SH) 3 domain of p85α to the proline-rich regions of $p125^{FAK}$ (Giunebault *et al.*, 1995). To explore whether thrombin or PDGF could stimulate PI 3-kinase activity via an interaction with $p125^{FAK}$ in BPA fibroblasts, p85α recovery and PI kinase activity was measured in anti- $p125^{FAK}$ immunoprecipitates from Triton-X-100 -soluble fractions of cells exposed to thrombin or PDGF. SDS-PAGE and Western blotting analysis with anti- $p125^{FAK}$ antibody confirmed the recovery in anti-$p125^{FAK}$ immunoprecipitates and the depletion from supernatents, of a single immunoreactive band migrating at an apparent molecular weight of approximately 125 kDa (Fig. 4.6A). Equivalent

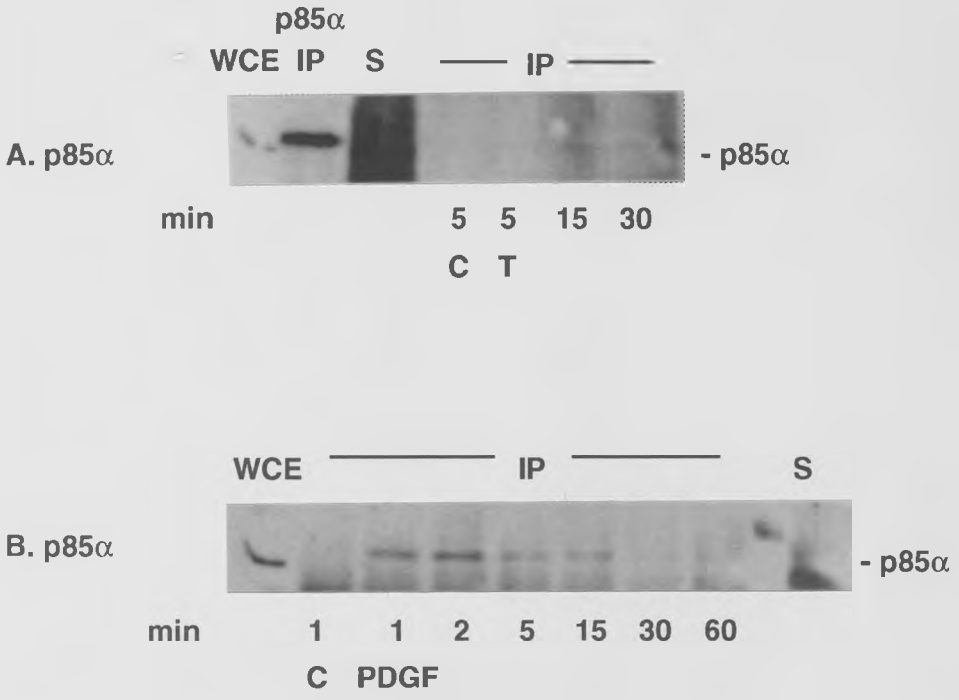


Figure 4.5.1 Recovery of p85α PI 3-kinase in anti-phosphotyrosine immunoprecipitates from agonist-stimulated BPA fibroblasts. Cells were treated with A) thrombin (300 nM) (T), B) PDGF (30 ng/ml) (P) or vehicle (C) for the times indicated and phosphotyrosyl proteins in cell lysates immunoprecipitated with anti-phosphotyrosine antibody. Immunoprecipitates (IP), supernatents (S) and whole cell extracts (WCE) were resolved by SDS-PAGE and immunoblotted for the p85α PI 3-kinase regulatory subunit as outlined in Section 2.3. An immunoprecipitate of p85α was run in the second lane in panel A (p85α IP). Bands corresponding to p85α are shown on the right of immunoblots, each of which is representative of one experiment performed twice.

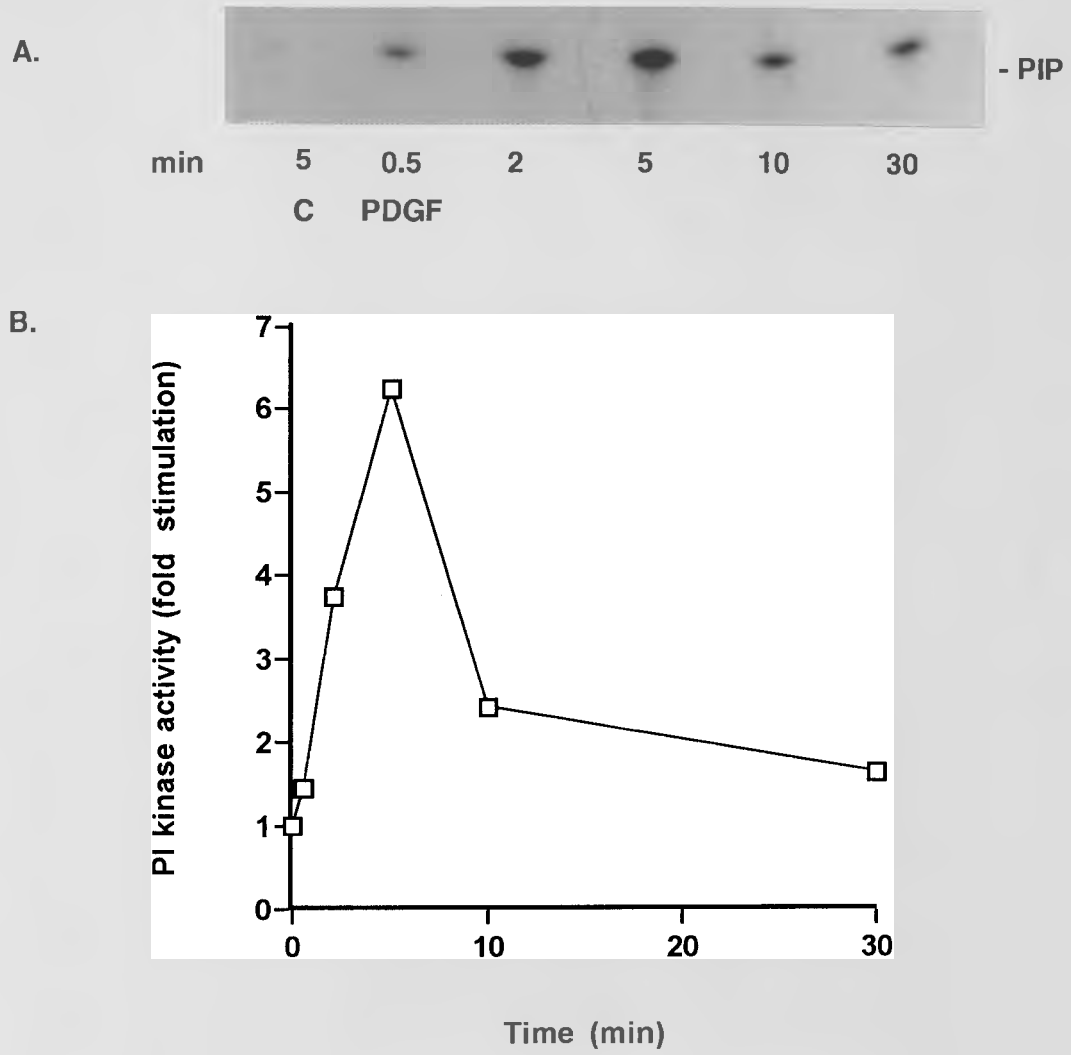


Figure 4.5.2 Time course for PDGF-stimulated PI kinase activity in phosphotyrosine immunoprecipitates in BPA fibroblasts. Cells were treated with PDGF (30 ng/ml) (P) or vehicle (C) for the times indicated. The fraction of tyrosine phosphorylated proteins from total cell extracts was immunoprecipitated with anti-phosphotyrosine antibody and assayed for PI kinase activity as described in Section 2.4.7. Phosphatidylinositol [^{32}P]phosphate (PIP) was resolved by TLC and detected by autoradiography (**A**). Radioactive spots were scraped from TLC plates, quantified by liquid scintillation counting and PI kinase activity expressed as fold stimulation relative to control (**B**).

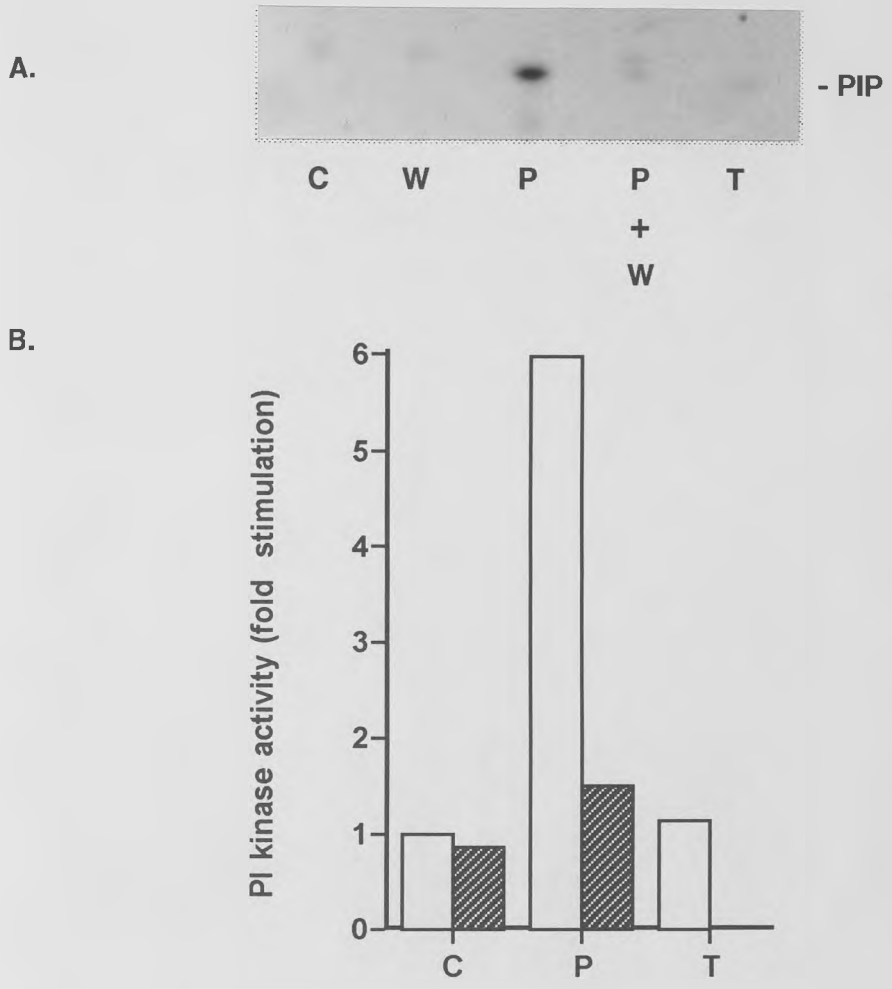
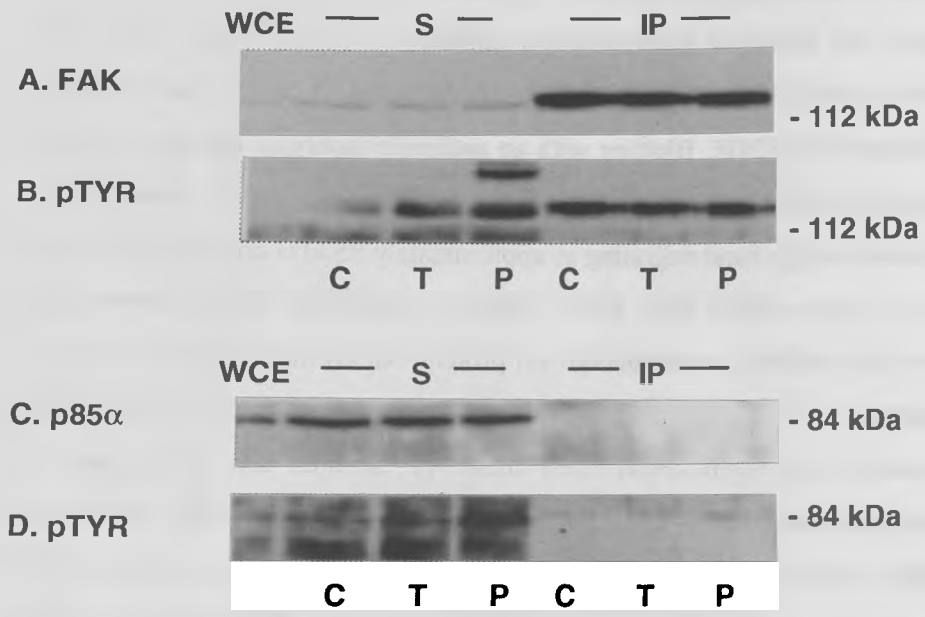


Figure 4.5.3 Effect of wortmannin on PDGF-stimulated PI kinase activity in BPA fibroblasts. Cells were pretreated with vehicle (open bars) or wortmannin (100 nM) (W) for 30 minutes (shaded bars) prior to exposure to PDGF (30 ng/ml, 5 minutes) (P). Immunoprecipitates of phosphotyrosine proteins from cell lysates were assayed for PI kinase activity as described in Section 2.4.7. Phosphatidylinositol [^{32}P]phosphate (PIP) was resolved by TLC and detected by autoradiography (**A**). PI kinase activity in cells exposed to thrombin (300 nM, 30 minutes) (T) was assessed in the same way and is shown on the right of the panel. Radioactive spots were scraped from TLC plates, quantified by liquid scintillation counting and PI kinase activity expressed as fold stimulation relative to control (**B**).

amounts of $p125^{FAK}$ protein was immunoprecipitated from fibroblasts treated with vehicle, thrombin or PDGF for 15 minutes. As $p125^{FAK}$ is a major tyrosine phosphorylated protein in response to integrin activation (Kornberg *et al.*, 1992, Chen & Guan, 1994b) and agonists stimulation (Zachary *et al.*, 1992, Rankin & Rozengurt, 1994) the extent of tyrosine phosphorylation of $p125^{FAK}$ was assessed by reprobing anti- $p125^{FAK}$ immunoblots with antiphosphotyrosine antibody. A high level of basal tyrosine phosphorylation was associated with a phosphotyrosine-containing protein in $p125^{FAK}$ immunoprecipitates from unstimulated cells which displayed the predicted electrophoretic mobility of $p125^{FAK}$(Fig. 4.6B). This protein did not show a detectable increase in tyrosine phosphorylation in cells that had been exposed to either thrombin or PDGF. Blotting with an anti-p85α antibody did not reveal the presence of immunodetectable p85 in anti-$p125^{FAK}$ immunoprecipitates from stimulated or unstimulated cells while a single band migrating at approximately 85 kDa could be detected in the supernatents from these samples (Fig. 4.6C). Again, on reprobing the p85 immunoblot with antiphosphotyrosine antibody, a phosphotyrosyl protein that co-migrating with p85 was evident in supernatents but not $p125^{FAK}$ -immunoprecipitates (Fig. 4.6D). While PDGF stimulated the recovery of p85 in phosphotyrosine immunoprecipitates, detecting an increase in p85 tyrosine phosphorylation by PDGF in $p125^{FAK}$ -depleted lysates where the protein levels are much higher, was beyond the sensitivity of immunodetection. When anti- $p125^{FAK}$ immunoprecipitates were assayed for PI kinase activity, there was no significant increase in PI kinase phosphorylation from extracts of BPA fibroblasts treated with either thrombin (300 nM) or PDGF (30 ng/ml) for 5 or 15 minutes (Fig. 4.6E)

4.5 The effect of PKC downregulation on agonist-stimulated $p70^{s6k}$ activation in BPA fibroblasts

Early studies investigating signal transduction by $p70^{s6k}$ identified a role for PKC in the activation of $p70^{s6k}$ (Susa *et al.*, 1992, Anderson, 1993). The possible involvement of PKC in the activation of $p70^{s6k}$ by thrombin and PDGF was explored using the PKC downregulation protocol described in Section 3.6. When BPA fibroblasts were pretreated with 1 μM PMA for 48 hours, a regimen found to downregulate immunodetectable PKC α and ε isoforms (see Section 3.6), activation of $p70^{s6k}$ in response to PMA was abolished, confirming the desensitization of a PMA-sensitive pathway regulating $p70^{s6k}$ activity. Under conditions where PKC isoforms were depleted by PMA pretreatment, $p70^{s6k}$ activities after 30 minute


WCE
S
IP
A. FAK
- 112 kDa
B. pTYR
- 112 kDa
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WCE
S
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C. p85α
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D. pTYR
- 84 kDa
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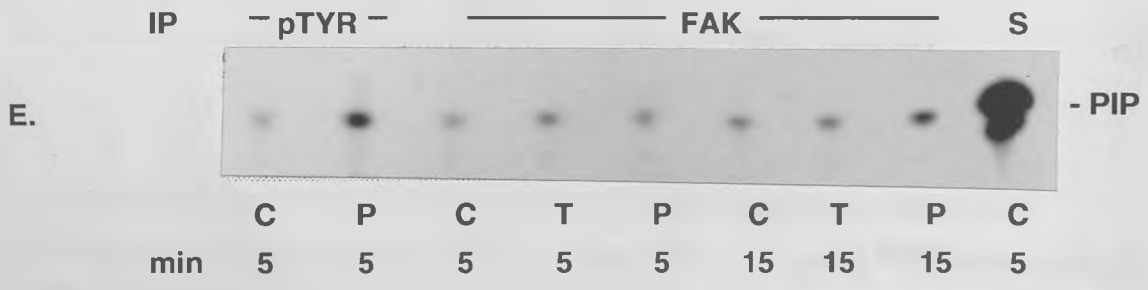
IP
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S
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- PIP
C P C T P C T P C
min 5 5 5 5 5 15 15 15 5

Figure 4.6 Neither p85α nor PI kinase activity is associated with anti p125^{FAK} immunoprecipitates from agonist-stimulated BPA fibroblasts. Cells were treated with thrombin (T) (300 nM), PDGF (P) (30 ng/ml) or vehicle (C) for 15 minutes (**A-D**) or 5 and 15 minutes (**E**). Immunoprecipitates of p125^{FAK} prepared from cell lysates (IP), supernatents (S) or whole cell extracts (WCE) were immunoblotted for p125^{FAK} (**A**), p85α, (**C**) and blots reprobed for phosphotyrosine (**B** & **D**) as described in Sections 2.3. The mobilities of molecular weight markers in kDa are indicated on the right of immunoblots. P125^{FAK} immunoprecipitates were assayed with phosphotyrosine (pTYR) immunoprecipitates for PI kinase activity as described in Section 2.4.7. Phosphatidylinositol [^{32}P]phosphate (PIP) was resolved by TLC and detected by autoradiography (**E**). Both the immunoblots and autoradiograph are representative of two separate experiments.

exposure to both thrombin and PDGF were attenuated by 74.8±4.4 % and 82.3±7.9 % respectively (Table 4.1). Similar affects of PMA pretreatment were observed during the later phase of PDGF-stimulated $p70^{s6k}$ activation occurring after 1 hour exposure to PDGF (not shown).

4.6 Effect of rapamycin and wortmannin on agonist-stimulated DNA synthesis in BPA fibroblasts

As $p70^{s6k}$ has been reputed to be involved in cell cycle re-entry in fibroblasts (Lane *et al.*, 1993, Reinhard *et al.*, 1994) the role of a $p70^{s6k}$-dependent pathway in thrombin and PDGF -stimulated DNA synthesis in BPA fibroblasts was evaluated. To corroborate the findings of growth studies conducted in Section 3.5.1 of Chapter 3, thrombin (300 nM) and PDGF (30 ng/ml) were found to be equally effective at stimulating DNA synthesis in quiescent PA fibroblasts causing approximately 65 fold increase in [^{3}H]thymidine incorporation during the final 4 hours of a 24 hour exposure to these agonists. At a concentration that abolished agonist-stimulated $p70^{s6k}$ activity (50 nM), rapamycin inhibited the mitogenic effect of thrombin by 33.6±2.0 % while the comparable increase in [^{3}H]thymidine incorporation in response to PDGF was attenuated by 69.6±2.0 % (Fig. 4.7). Despite the marked differences in inhibiting $p70^{s6k}$ activation by thrombin and PDGF, wortmannin caused a concentration-dependent inhibition of both thrombin and PDGF-stimulated [^{3}H]thymidine incorporation with IC_{50} values of approximately 16 nM and 28 nM respectively (Fig. 4.8.). Complete inhibition of both of these responses was evident at 100 nM wortmannin.

4.7 DISCUSSION

This section has investigated signal transduction via the $p70^{s6k}$ pathway in BPA fibroblasts and the role of this pathway in the strong mitogenic effect of thrombin and PDGF in this cell type reported in Section 3.5.1. Activation of $p70^{s6k}$ was found to be prevalent in BPA fibroblasts exposed to both thrombin and PDGF, and abolished by the macrolide rapamycin. That rapamycin is specific for dissecting the roles of the $p70^{s6k}$ pathway in cellular responses without affecting components of other signalling pathways was exemplified by the demonstration that both thrombin and PDGF-stimulated activation of p42 or p44 MAP

Treatment	$p70^{s6k}$ activity mean±s.e.m. fold stimulation	
	- PMA	+ PMA
C	1.0	0.9±0.0
PMA	5.9±0.6	0.5±0.2*
T	8.0±2.8	1.9±0.4*
P	15.1±1.1	2.8±1.4*

Table 4.1 Effect of PMA pretreatment on agonist-stimulated $p70^{s6k}$ activity in BPA fibroblasts. Cells were pretreated with or without 1 μM PMA for 48 hours prior to exposure to thrombin (300 nM for 30 minutes) (T), PDGF (30 ng/ml for 30 minutes) (P), PMA (100 nM for 30 minutes) or vehicle (C). Immunoprecipitates of $p70^{s6k}$ from cell lysates were assayed for $p70^{s6k}$ activity as described in the Section 2.4.4. Activity is expressed as mean±s.e.m. fold stimulation relative to control of radioactive phosphate incorporated into ribosomal S6 peptide from three separate experiments. * denotes significant effect of PMA pretreatment at $p < 0.05$ level using Student's paired t test. (control: 20.7±6.74 fmol Pi min^{-1} mg^{-1} protein).

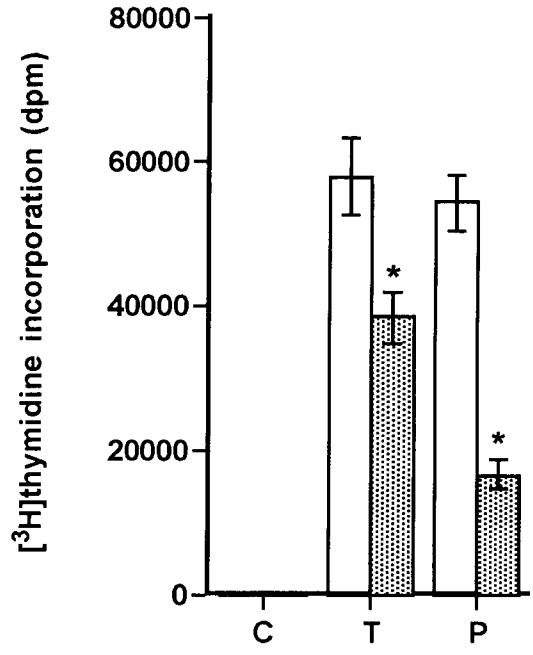


Figure 4.7 Effect of rapamycin on agonist-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Cells were untreated (open bars) or pretreated with 50 nM rapamycin for 30 minutes (shaded bars) prior to incubation with thrombin (300 nM) (T), PDGF (30 ng/ml) (P) or vehicle (C) for 24 hours. Incorporation of [^{3}H]thymidine during the final 4 hours of agonist treatment was measured as described in Section 2.5. Values shown are dpm±s.e.m. from three separate experiments performed in triplicate. * denotes significant effect of rapamycin treatment at $p < 0.05$ level using Student's paired t test.

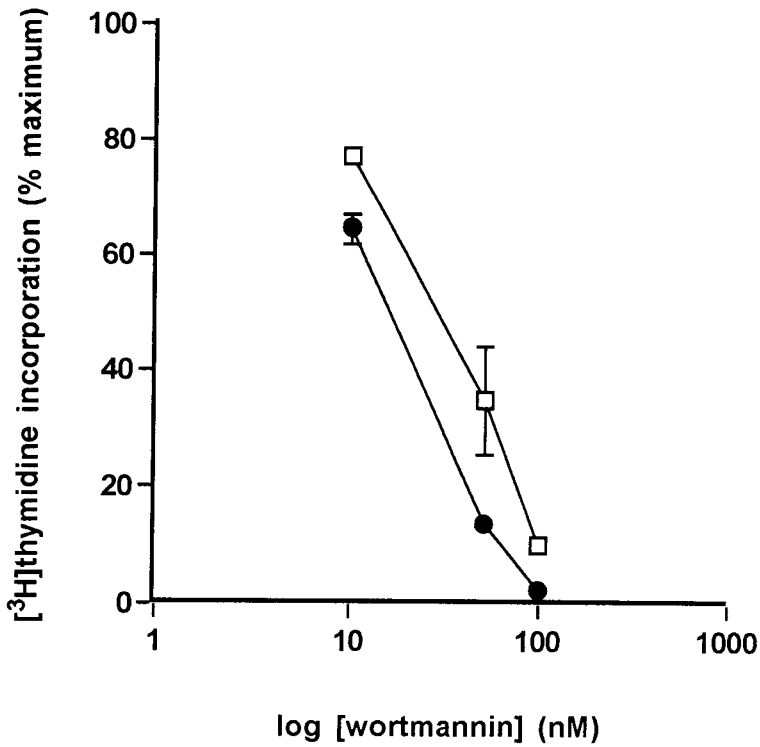


Figure 4.8 Effect of wortmannin on agonist-stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Cells were pretreated with vehicle or wortmannin at the indicated concentrations for 30 minutes prior to exposure to thrombin (300 nM) (●) or PDGF (30 ng/ml) (□) for 24 hours. Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNA replicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. percentage of maximal thrombin or PDGF -stimulated [^{3}H]thymidine incorporation in the absence of wortmannin from 3 independent experiments each performed in triplicate. (maximum [^{3}H]thymidine incorporation: 57529±3060 dpm (300 nM thrombin); 55176±1397 dpm (30 ng/ml PDGF).

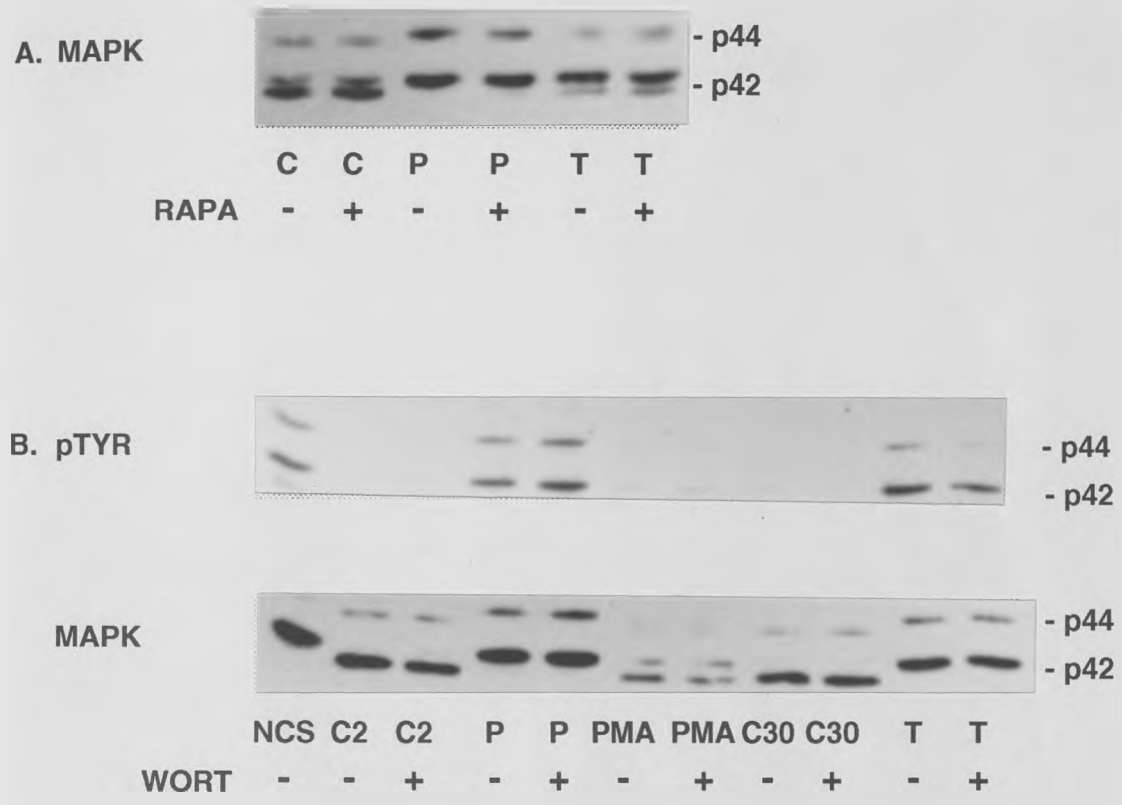


Figure 4.9 Effect of rapamycin and wortmannin on agonist-stimulated MAP kinase activation in BPA fibroblasts. Cells were pretreated with vehicle (-) or **A)** rapamycin (50 nM) (RAPA) or **B)** wortmannin (100 nM) (WORT) for 30 minutes (+) prior to exposure to PDGF (30 ng/ml) (P) or NCS (10%) for 10 minutes, thrombin (300 nM) (T) for 30 minutes or PMA (100 nM) for 5 minutes. Whole cell lysates were immunoblotted for MAP kinase (**A**) or phosphotyrosine (upper panel in **B**) and then reprobed for MAP kinase (lower panel in **B**) as outlined in the Section 2.3. Each blot is a representative example from at least three independent experiments.

kinases was not affected by exposure of BPA fibroblasts to rapamycin. Although this does not exclude the possibility that MAP kinase is upstream of $p70^{s6k}$, and phosphorylation sites within the C-terminus of $p70^{s6k}$ lie within consensus sequences recognized by MAP kinase itself (Ferrari *et al.*, 1992), there is no clear evidence that MAP kinase activates $p70^{s6k}$ *in vivo* (Mukhopadhyay *et al.*, 1992). Besides, as discussed in detail below, the differential effects on MAP kinase and $p70^{s6k}$ activation of both wortmannin and PKC downregulation, further support the notion that these two signalling molecules lie on a distinct axis (Ballou *et al.*, 1991, Chung *et al.*, 1992, Ming *et al.*, 1994).

4.7.1 Role of PI 3-kinase in thrombin and PDGF -stimulated $p70^{s6k}$ activation

Compared to the signalling processes controlling activation of the MAP kinase cascade, the mechanisms regulating $p70^{s6k}$ on stimulation of tyrosine kinase-encoded receptors and G-protein-coupled receptors are relatively ill-defined to date. Recently, much attention has centred on PI 3-kinase. An obligatory role for this signalling molecule in the regulation of $p70^{s6k}$ by growth factors that activate receptor tyrosine kinases (RTK) was originally inferred by the demonstration that PDGF and insulin-stimulated $p70^{s6k}$ activity is inhibited by the structurally unrelated PI 3-kinase inhibitors wortmannin and LY294002 (Cheatham *et al.*, 1994, Chung *et al.*, 1994). Furthermore, under conditions where PDGF receptor-associated PI 3-kinase activity was abolished by point mutation of the PDGF receptor kinase insert domain at the autophosphorylation site Tyr740/751 responsible for PI 3-kinase binding, PDGF-dependent $p70^{s6k}$ activation has been shown to be noticeably reduced (Chung *et al.*, 1994). The range of stimuli evoking PI 3-kinase-dependent $p70^{s6k}$ activation, as judged by the sensitivity to inhibition by wortmannin, now extends to agonists acting on G-protein-coupled receptors (Wilson *et al.*, 1996) and cytokine receptors (Karnitz *et al.*, 1995, Monfar *et al.*, 1995, Crawley *et al.*, 1996, Kilgour *et al.*, 1996). Recently, additional evidence to strongly support a role for PI 3-kinase in $p70^{s6k}$ activation was provided by Weng *et al* (1995a) showing that constitutively active PI 3-kinase stimulates phosphorylation indirectly on Thr^{252} a key residue within the catalytic domain controlling activity. In the present study, wortmannin was used to investigate the involvement of PI 3-kinase in the activation of $p70^{s6k}$ by thrombin and PDGF. The ability of wortmannin to completely block thrombin-stimulated $p70^{s6k}$ activity in BPA fibroblasts suggests that for this agonist, regulation of $p70^{s6k}$ activity requires PI 3-kinase. This finding is in agreement with those by Lenormand *et al.*, (1996) showing that

thrombin-mediated stimulation of $p70^{s6k}$ is abolished by wortmannin in CCL39 hamster lung fibroblasts.

Thrombin and other G protein-coupled receptor agonists have been shown to increase PI 3-kinase activity in antiphosphotyrosine and anti-src type immunoprecipitates (Gutkind *et al.*, 1990, Yamamoto & Lapetina, 1990) indicating that protein tyrosine kinases may be involved in the regulation of PI 3-kinase by G protein-coupled receptors. Activation of PI 3-kinase by these agents may reflect tyrosine phosphorylation of the p85α subunit of PI 3-kinase (Gout *et al.*, 1993) brought about as a consequence of the interaction via the SH2 domains of p85 with tyrosyl-phosphorylated Y-X-X-M binding sequences of activated receptor or non-receptor tyrosine kinases as has been described for many growth factors (Backer *et al.*, 1992, Carpenter *et al.*, 1993). However, in this study, the ability of thrombin to stimulate an increase in PI kinase activity or the recovery of p85α in anti-phosphotyrosine immunoprecipitates from PA fibroblasts was not readily detectable, corroborating the findings of other investigators who have been unable to demonstrate tyrosine phosphorylation of p85 in thrombin-stimulated vascular smooth muscle cells (Molloy *et al.*, 1996) and platelets (Giunebalt *et al.*, 1995, Zhang *et al.*, 1996).

It has been reported that PI 3-kinase associated with a pool of p85α that is not tyrosine phosphorylated and found in the cytoskeletal fraction of platelets exposed to thrombin may become activated by a novel mechanism (Giunebalt *et al.*, 1995). An increase in PI 3-kinase activity was demonstrated following binding of the SH3 domains of non-tyrosine phosphorylated p85α with a proline rich sequence of focal adhesion kinase ($p125^{FAK}$). $p125^{FAK}$ is a novel cytosolic protein tyrosine kinase that is localized at focal-adhesions forming at the termini of actin stress fibres (Schaller *et al.*, 1992). Like a number of other cytoskeletal proteins, the cellular signalling events mediated by $p125^{FAK}$ are not fully understood, although $p125^{FAK}$ appears to play a key role in the reorganization of the actin cytoskeleton and may contribute to cellular transformation by v-src in fibroblasts (Guan & Shalloway, 1992), indicating a possible involvement in mitogenesis. Furthermore, $p125^{FAK}$ is likely to be a point of convergence for signalling pathways activated by the integrin family of cell surface receptors and agonist receptors (Zachary & Rozengurt, 1992) since $p125^{FAK}$ becomes tyrosine phosphorylated as a consequence of cell-matrix and cell-cell interactions (Kornberg *et al.*, 1992, Chen & Guan, 1994b) or by G-protein coupled receptor neuropeptides (Zachary *et al.*, 1992, Sinnett-Smith *et al.*, 1993) and PDGF (Rankin & Rozengurt, 1994, Chen & Guan 1994a). Many of the biochemical events associated with the cytoskeleton of thrombin

stimulated platelets such as PI 3-kinase activity (Sultan *et al.*, 1991, Giunebalt *et al.*, 1995) and $p125^{FAK}$ tyrosine phosphorylation (Lipfert *et al.*, 1992) are dependent on the engagement of the platelet-specific α_{IIb}/b_3 integrin and therefore the platelet may not be a universal model for FAK-dependent PI 3-kinase activation by thrombin and other agonists in different cell types. However, p85/PI 3-kinase has also been shown to be activated by association with $p125^{FAK}$ in PDGF-stimulated fibroblasts, although in this case, by a mechanism independent of $p125^{FAK}$ tyrosine phosphorylation or the integrity of the cytoskeleton (Chen & Guan, 1994a). It is unlikely, though, that PI 3-kinase activation resulting from this type of interaction occurs in BPA fibroblasts, or for thrombin, provides the signal for $p70^{s6k}$ activation, since neither thrombin or PDGF were able to stimulate detectable p85 recovery or PI kinase activity in anti- $p125^{FAK}$ immunoprecipitates. While it is possible that the physical interaction between PI 3-kinase and $p125^{FAK}$ may be a transient event, Chen & Guan (1994a) demonstrated that PDGF-stimulated PI 3-kinase activity in anti- $p125^{FAK}$ immunoprecipitates was evident after 30 seconds but sustained past 1 hour, a time course which encompasses the exposure times for thrombin and PDGF used here (5 and 15 minutes).

Despite the unsuccessful efforts to measure extractable PI 3-kinase activity from thrombin-treated cells, PI kinase activity was evident in antiphosphotyrosine immunoprecipitates from PDGF treated cells and likely to be attributed to the activity of the p85-PI 3-kinase heterodimer as the p85α regulatory subunit of PI 3-kinase was co-immunoprecipitated with this activity, the PIP product when separated by TLC comigrated with the product of PI kinase activity in p85α immunoprecipitates, and the formation of phosphatidylinositol phosphate was completely blocked by 100 nM wortmannin. At this concentration, wortmannin does not significantly inhibit mammalian PI 4-kinase (Okada *et al.*, 1994, Woscholski *et al* 1994), indicating that the phosphorylation was most likely to be occuring predominantly on the 3-OH and not the 4-OH position.

Taken together, these findings suggest that a PI 3-kinase distinct from the p85/PI 3-kinase activated by PDGF could be involved in thrombin-stimulated $p70^{s6k}$ activation. Novel PI 3-kinase activities have been described in platelets and myeloid cells that are stimulated directly by G-protein βγ subunits (Stephens *et al.*, 1994 , Thomason *et al.*, 1994) and sensitive to inhibition by wortmannin at concentrations in the range used here (Stephens *et al.*, 1995). A PI 3-kinase isoform sharing some of these properties has recently been cloned, and designated $p110_\gamma$ (Stoyanov *et al.*, 1995). Furthermore, activation of this isoform occurs independently of the p85 regulatory subunit. Another PI 3-kinase, p170, has been cloned from

mouse 3T3LI adipocytes and exhibits unique substrate specificity and lower sensitivity to wortmannin (Virbasius *et al.* 1996). It is not possible to speculate whether this or a G-protein-regulated PI 3-kinase isoform is responsible for mediating the wortmannin-sensitive $p70^{s6k}$ activation by thrombin in PA fibroblasts, but in support of the latter possibility, it was also found that thrombin-stimulated $p70^{s6k}$ activation was partially inhibited by PTX suggesting an involvement of a heterotrimeric G-protein of the G_i or G_o family. ADP ribosylation of the α subunit of these G-protein species by PTX would prevent binding of GTP to allow dissociation of the heterotrimeric complex to generate free $\beta\gamma$ subunits required for PI 3-kinase activation. Members of this family have also been implicated in thrombin-stimulated PI 3-kinase activity in platelets (King *et al.*, 1991) and other signalling responses evoked by thrombin including $p21^{ras}$ and MAP kinase activation in other fibroblast types (Kahan *et al.*, 1992, van Corven *et al.*, 1993) as outlined in Chapter 3. An important experiment that would need to be conducted to strengthen the hypothesis that a novel PI 3-kinase was involved in thrombin-stimulated $p70^{s6k}$ activation in BPA fibroblasts would be to show that exposure of cells to thrombin stimulates PI 3-kinase activity as assessed by the accumulation of cellular PI-3,4,5-P_3 measured by *in vivo* labelling and lipid analysis. This response would be expected to have the same sensitivity to inhibition to wortmannin as thrombin-stimulated $p70^{s6k}$ activation.

Surprisingly, PDGF-stimulated $p70^{s6k}$ activity in PA fibroblasts was unaffected by wortmannin at a concentration that abolished PI kinase activity in immunoprecipitates containing tyrosine phosphorylated p85. This finding suggests that in this cell type, the p85-dependent PI 3-kinase regulated by RTKs is not involved in $p70^{s6k}$ activation. Other groups using a mutant of the p85 regulatory subunit lacking the binding site for p110, or a mutant of the PDGF receptor that prevents p85 binding, have also challenged the notion that the heterodimeric p85/p110 PI 3-kinase is upstream of $p70^{s6k}$ (Ming *et al.* 1994, Hara *et al.*, 1995). In light of this evidence and the findings with PDGF in the present study, it is conceivable that while in many RTK signalling systems activation of PI 3-kinase may be the predominant pathway that activates $p70^{s6k}$, this may not be a universal phenomenon.

The use of wortmannin in dissecting a role for a PI 3-kinase in thrombin-stimulated $p70^{s6k}$ activation must be interpreted with caution since other investigations have cast doubt on the specificity of this inhibitor. Similar to thrombin in this investigation, previous studies have also described wortmannin-sensitive $p70^{s6k}$ activation without strong PI 3-kinase activity associated with p85/p110 (Kido *et al.*, 1995). This finding and another report showing that

wortmannin, but not a dominant negative p85 mutant, inhibits insulin-stimulated $p70^{s6k}$ activity, while both regimens markedly reduce insulin-induced PI 3-kinase (Hara *et al.*, 1995), suggest that wortmannin may affect other targets apart from PI 3-kinase. These may include known or as-yet unidentified lipid or protein kinases related to PI 3-kinase (Wymann, *et al.*, 1996) that may be distinguished in being required for regulating $p70^{s6k}$ activation by thrombin. A recent observation that the catalytic subunit of DNA-dependent kinase, a molecule with a PI 3-kinase-like domain, is also sensitive to wortmannin supports such a hypothesis (Hartley *et al.*, 1995). Although inhibition of myosin light chain kinase (MLCK) has been shown at five-fold higher concentrations of wortmannin than those used in this study (Yano *et al.*, 1993), a recent publication reports that wortmannin is a more potent inhibitor of phospholipase A_2 (PLA_2) than PI 3-kinase (Cross *et al.*, 1995). In spite of this, the concentrations of wortmannin used here are in the range required to inhibit $G_{\beta\gamma}$-stimulated PI 3-kinase activity in myeloid-derived cells (Stephens *et al.*, 1994). While there is no evidence to date to indicate that MLCK or PLA_2 are involved in $p70^{s6k}$ regulation, the possibility that either of these, or a wortmannin-sensitive kinase other than PI 3-kinase is an upstream regulator of $p70^{s6k}$ in response to thrombin cannot be ruled out. In spite of this, the use of wortmannin can clearly discriminate between two distinctly different mechanisms by which thrombin and PDGF regulate $p70^{s6k}$.

4.7.2 Role of PKC in thrombin and PDGF -stimulated $p70^{s6k}$ activation

From studies with PDGF it was apparent that a wortmannin-insensitive pathway could also regulate $p70^{s6k}$ activation in PA fibroblasts. Before PI 3-kinase-dependent $p70^{s6k}$ signalling came to light, early studies characterizing how mitogens signal to $p70^{s6k}$ demonstrated that the late phase of epidermal growth factor (EGF)-stimulated $p70^{s6k}$ activity could be abated by a specific PKC inhibitor in Swiss 3T3 fibroblasts (Susa *et al.*, 1992) indicating a role for PKC in receptor-mediated $p70^{s6k}$ activation. More recently, this possibility has been supported by the finding that mutant PDGF receptors which fail to associate with PLC_γ do not fully activate $p70^{s6k}$ (Chung *et al.*, 1994). However, since these reports, the possible involvement of PKC in $p70^{s6k}$ activation has not been pursued. As outlined in Chapter 3, both the thrombin and PDGF receptors have been shown to couple to different isoforms of phospholipase C (PLC) (Brass *et al.*, 1986, Kondo *et al.*, 1993) and through the production of DAG activates a number of PKC isoforms. The demonstration that

$p70^{s6k}$ activity could be stimulated by PMA, implied that $p70^{s6k}$ can be regulated by PKC in BPA fibroblasts. As thrombin and PDGF stimulated kinase activities were markedly reduced when PMA-dependent $p70^{s6k}$ activation was desensitized by prior exposure to phorbol ester, these agonists may share a common pathway employing conventional PKC isoforms, possibly α and ε, to activate $p70^{s6k}$. The effect of PMA pretreatment cannot be attributed to a general deleterious effect on the cell because this regime did not affect MAP kinase stimulation and DNA synthesis in response to thrombin (Section 3.6). In contrast to the results of the present study, other investigations using PKC downregulation protocols have found PDGF-evoked $p70^{s6k}$ activity to be unaffected or only marginally abated (Susa *et al.* 1992, Chung *et al.*, 1994). Furthermore, in any one cell type, the involvement of PKC may depend on the stimulus since $p70^{s6k}$ activation in 3T3-F442A preadipocytes by growth hormone but not EGF is sensitive to inhibition by the PKC inhibitor chelyrethrine (Anderson *et al.*, 1993). An explanation for the discrepancies in the literature and in the present study may be that differential pathways regulated by both G-protein-coupled receptors and RTKs can activate $p70^{s6k}$ in different systems and that the contribution of PKC to this signalling pathway may depend on whether the necessary effectors are expressed and become activated.

It is unclear how PKC mediates $p70^{s6k}$ activation in response to thrombin and PDGF although it is unlikely that PKC phosphorylates $p70^{s6k}$ directly. For PDGF, the PKC-dependent route to $p70^{s6k}$ activation defines a p85/PI 3-kinase independent/wortmannin insensitive pathway. Very recent evidence indicates that a minor wortmannin-insensitive pathway leading to activation of $p70^{s6k}$ may involve Raf-1 since an estradiol-regulated oncogenic Raf-1 chimer is capable of activating $p70^{s6k}$ (Lenormand *et al.*, 1996) inferring that MAP kinase and $p70^{s6k}$ signalling pathways can branch out at the level of Raf-1. This publication was surprising in light of earlier reports indicating that dominant negative Raf-1 did not affect $p70^{s6k}$ activation by EGF and PMA (Ming *et al.*, 1994). While PKC can phosphorylate and activate Raf-1 directly (Kolch *et al.*, 1993), and it was demonstrated in Section 3.4 that Raf-1 becomes strongly activated in response to thrombin and PDGF in BPA fibroblasts, it is unlikely that this is the route by which the PKC-dependent component of thrombin and PDGF -stimulated $p70^{s6k}$ activation occurs as MAP kinase activation by these agents appears to be entirely or largely independent of PKC (see Section 3.9.2).

For thrombin, the regulation of $p70^{s6k}$ is more complex incorporating both PKC-dependent and wortmannin-sensitive pathways. In the case of thrombin, from the amount of definitive data in the present study, it is not clear whether PKC acts upstream of, downstream

of, or in parallel to the wortmannin-sensitive target to induce $p70^{s6k}$ activation. However, evidence in the literature showing that activation of $p70^{s6k}$ by phorbol esters is insensitive to wortmannin but can be blocked by rapamycin (Chung *et al.*, 1994, Han *et al.*, 1995), suggests that the wortmannin-sensitive input to $p70^{s6k}$ is not downstream of PKC. Recent studies have indicated that the products of PI 3-kinase, the 3-phosphorylated phosphoinositides, may serve as second messengers in signal transduction. Phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) have been shown to activate the non-conventional PKC (nPKC) isoforms ε (Toker *et al.*, 1994, Moriya *et al.*, 1996) δ and η (Toker *et al.*, 1994) and the atypical PKC isoform ζ (Nakanishi *et al.*, 1993) *in vitro*. A role for lipid products of PI 3-kinase activating PKC *in vivo* has been implied by the finding that phosphorylation of the PKC substrate pleckstrin correlates with sustained PI-3,4-P_2 production in thrombin-stimulated platelets and is inhibited by wortmannin (Toker *et al.*, 1995). Furthermore PI-3,4-P_2 and PI-3,4,5-P_3 stimulates pleckstrin phosphorylation when applied to permeablized platelets. As PKC δ, ε and η are also activated by DAG, and PKC ε was shown to be susceptible to downregulation in BPA fibroblasts following PMA pretreatment (Section 3.6), it is possible that these PKCs may serve as potential mediators of a PI 3-kinase-dependent $p70^{s6k}$ pathway in response to thrombin, requiring the generation of 3-phosphorylated phosphoinositides for their activation. The specific type and cellular location of the thrombin-activated PI 3-kinase may also determine the site of generation and species of membrane-anchored phosphoinositide second messengers formed. Clearly, for PDGF, however, activation of $p70^{s6k}$ does not require PI 3-kinase, and either these or other PMA-sensitive PKC isoforms involved in $p70^{s6k}$ activation may be activated by another mechanism, such as DAG derived from PLCγ or PLD activities. The complexity of signalling events downstream of cell surface receptors limits the ability to analyze the pathways leading to the activation of individual PKC members. However, Moriya *et al.*, (1996) have shown that PDGF can activate PKCε through redundant and independent signalling pathways involving PLCγ or PI 3-kinase suggesting that for this receptor, the coupling systems may allow activation of PKC even in the face of PI 3-kinase inhibition by wortmannin.

Another possible downstream effector of PI 3-kinase involved in $p70^{s6k}$ activation by thrombin may be the serine/threonine-specific protein kinase B (Akt), the cellular homologue of the transforming kinase v-Akt. Growth factor-induced activation of this kinase has been shown to require PI 3-kinase (Burgering & Coffer, 1995, Franke *et al.*, 1995) and transfection of a constitutively active Gag-PKB fusion protein increase $p70^{s6k}$ phosphorylation and

activity, indicating that PKB may be involved in activating $p70^{s6k}$ *in vivo* (Burgering & Coffer, 1995). The mechanism of PKB activation has not been resolved, but PI 3 kinase may generate PI-3-P which by interacting with the pleckstrin homology domain of PKB activates PKB directly (Franke *et al.*, 1995), or provide PI-3,4,5-P_3 which serves as a docking site for recruitment of Akt-1 from the cytosol to the plasma membrane (James *et al.*, 1996) where it is activated by phosphorylation (Burgering & Coffer, 1995) by an as yet unidentified kinase. PI 3-kinase dependent PKB activation, however, has only been demonstrated for insulin and growth factors such as PDGF, which in BPA fibroblasts, clearly activates $p70^{s6k}$ by a wortmannin-insensitive manner, and not for phorbol esters or the G_i -protein coupled receptor agonist LPA (Burgering & Coffer, 1995), suggesting that this pathway may not operate for thrombin.

4.7.3 Role of FKBP12 rapamycin associated protein (FRAP) in thrombin and PDGF -stimulated $p70^{s6k}$ activation

Models for the differential pathways regulating $p70^{s6k}$ by thrombin and PDGF would have to accommodate a role for a rapamycin-sensitive component which in BPA fibroblasts appears to be necessary for both of these agonists to activate $p70^{s6k}$. Rapamycin does not inhibit $p70^{s6k}$ directly but by forming a complex with one of a family of intracellular proteins termed FK506-binding protein (FKBP) 12 (Fruman *et al.*, 1995) which in turn, targets FRAP (FKBP-rapamycin -associated protein) a mammalian homologue of the yeast gene products called Target of Rapamycin (TOR) 1 and 2 (Brown *et al.*, 1994). While FRAP shows structural homology to the kinase domain of PI 3-kinase, no direct demonstration of PI 3-kinase activity has been reported. Recent studies, however, have indicated that FRAP exhibits intrinsic serine/threonine protein kinase activity, which, in combination with an N-terminal region, is required for rapamycin-sensitive activation of $p70^{s6k}$ by FRAP *in vivo* (Brown *et al.*, 1995a). However, many questions remain unanswered at present regarding the FRAP pathway. How the kinase activity of FRAP regulates $p70^{s6k}$ is unclear at present although this is likely to involve the activation of an intermediary kinase, as to date, FRAP has not been shown to phosphorylate $p70^{s6k}$ directly. The way that the rapamycin sensitive step is linked to cell surface receptors is also ill-defined. With regard to the data presented here, while an obligatory role for the rapamycin target is implicated, it is not clear whether the PKC-dependent and wortmannin-sensitive components of agonist-stimulated $p70^{s6k}$ activation act

above, or in parallel to, the step blocked by rapamycin. Of the available evidence in the literature, rapamycin prevents $p70^{s6k}$ activation by all reported stimuli, including phorbol esters (Chung *et al.*, 1994, Monfar *et al.*, 1995) indicating that FRAP is likely to function proximal to $p70^{s6k}$ and downstream of PKC. In contrast, the wortmannin-mediated inhibition of $p70^{s6k}$ is believed to be independent of FRAP. Autophosphorylation of FRAP is not blocked by wortmannin (Brown *et al.*, 1995a) ruling out the possibility that FRAP is a common target for wortmannin and rapamycin in mediating inhibition of $p70^{s6k}$ activation. The likelihood that wortmannin and rapamycin act to block different inputs is supported by recent studies identifying the domains of the $p70^{s6k}$ molecule required for its mitogen-stimulated activation and rapamycin and wortmannin inhibition. From these investigations it was concluded that multiple phosphorylation events from at least two different sources appear to be required for full activation of $p70^{s6k}$ (Han *et al.*, 1995, Weng *et al.*, 1995b), implying that more than one kinase cascade is likely to converge on $p70^{s6k}$ to activate the kinase directly. Reflected on the present studies, this evidence would support a model where, rather than lie upstream of PKC as outlined above, the wortmannin-sensitive pathway leading to thrombin-stimulated $p70^{s6k}$ activation in BPA fibroblasts defines a pathway parallel to PKC, although the intermediate steps of this axis are unclear. Identification of the $p70^{s6k}$ activating-kinase(s) will help to elucidate the different routes to $p70^{s6k}$ activation.

4.7.4 The role of $p70^{s6k}$ in agonist-stimulated DNA synthesis

The functional relevance of activation of $p70^{s6k}$ in BPA fibroblast to the mitogenic response of thrombin and PDGF was assessed using rapamycin. The inhibition of thrombin and PDGF-stimulated [^{3}H]thymidine incorporation by rapamycin demonstrated that the $p70^{s6k}$ pathway is involved in relaying mitogenic signals by both of these agents and agrees with many previous studies implicating $p70^{s6k}$ and growth factor-induced proliferation of a number of cell types (Chung *et al.*, 1992, Price *et al.*, 1992) including fibroblasts (Migita *et al.*, 1996). The different extents to which rapamycin inhibited DNA synthesis for the two agonists suggests that a $p70^{s6k}$-dependent component may be of greater importance to the mitogenic action of PDGF than thrombin and was reflected in the magnitude of $p70^{s6k}$ activation by the two agonists. Clearly, as DNA synthesis on exposure to agonists was stimulated substantially even in the presence of rapamycin, other $p70^{s6k}$-independent signalling pathways, including the

MAP kinase cascade described in Chapter 3 are also likely to be important in BPA fibroblast mitogenesis.

Using the other inhibitors and PKC depletion strategy outlined, it was difficult to make definitive conclusions about the role of the different inputs to $p70^{s6k}$ in thrombin and PDGF-stimulated DNA synthesis. The disproportionate contributions that $p70^{s6k}$ makes to the net growth responses to thrombin and PDGF may explain partly why PKC downregulation attenuated PDGF but not thrombin -stimulated DNA synthesis (discussed in Chapter 3). In addition, inhibition of $p70^{s6k}$ by PKC downregulation may not be reflected in the extent of the mitogenic response as PKC may impose negative control at later stages of the cell cycle. PKC activation at a critical point prior to S phase entry has been demonstrated to inhibit G1/S phase transition and vascular smooth muscle cell proliferation following growth factor stimulation (Huang & Ives, 1987, Sasaguri *et al.*, 1993). Thus, removal by PKC downregulation of the effect of $p70^{s6k}$ in initiating cell cycle re-entry may be compensated for by the loss of PKC-dependent inhibition of cell cycle progression at later time points. The observation that $p70^{s6k}$ activation by thrombin was partially inhibited by PTX indicates that the $p70^{s6k}$ pathway can account in part for the PTX-sensitive component of thrombin-stimulated DNA synthesis described in Section 3.7. In this case, however, the extent to which thrombin-stimulated [^{3}H]thymidine incorporation was inhibited by PTX (40%) could not be accounted for entirely by the input from $p70^{s6k}$ that was sensitive to PTX (50% of the rapamycin-sensitive component), suggesting that for the proportion of thrombin-evoked responses that are PTX-sensitive, signals diverge soon after $G_{i/o}$ proteins to activate both $p70^{s6k}$ -dependent and independent pathways. Similarly, the existence of more than one separate PI 3-kinase-regulated growth pathway that bifurcates upstream of $p70^{s6k}$ provides a plausible reason why the wortmannin-sensitive component of thrombin-stimulated growth (Fig. 4.8) is considerably greater than can be explained by inhibition of $p70^{s6k}$ activity alone. For PDGF, this wortmannin component appears to be entirely independent of $p70^{s6k}$ implying that other molecular downstream targets of PI 3-kinase activity are involved in mitogenic signalling. Other investigators have also been able to dissociate wortmannin-sensitive responses from $p70^{s6k}$ activity. For example, insulin-stimulated glucose transport, glycogen synthase activation and inhibition of glycogen synthase kinase (GSK)-3 is antagonized by wortmannin but is unaffected by rapamycin (Cross *et al.*, 1994, Welsh *et al.*, 1994, Yamamoto-Honda *et al.*, 1995). Unlike these findings, however, the effects of wortmannin on DNA synthesis in BPA fibroblasts could not be attributable to inhibition of MAP kinase.

4.7.5 CONCLUDING REMARKS

To conclude, this study supports a role for $p70^{s6k}$ in the mitogenic effect of thrombin and PDGF in BPA fibroblasts although thrombin relies considerably less on this component. In this cell type activation of $p70^{s6k}$ by thrombin, in common with PDGF, appears to depend largely on an input from PKC. As thrombin and PDGF -stimulated $p70^{s6k}$ activities show differential sensitivity to inhibition by wortmannin and PTX, however, distinct regulatory mechanisms also appear to be involved. Overall, these results are suggestive of an involvement of a G protein of the G_i family and a PI 3-kinase different from p85/p110 in thrombin-stimulated $p70^{s6k}$ activation.

CHAPTER 5

ROLE OF THE STRESS-ACTIVATED PROTEIN KINASES IN THROMBIN-STIMULATED DNA SYNTHESIS IN BOVINE PULMONARY ARTERY FIBROBLASTS

5.1 INTRODUCTION

It is now established that novel mammalian protein kinases distantly related to p42/p44 MAP kinase, including the c-Jun N-terminal kinase (JNK) and p38 subfamilies of stress-activated protein (SAP) kinases (Dérijard *et al.*, 1994, Kyriakis *et al.*, 1994, Rouse *et al.*, 1994), are elements of parallel signalling cascades (see Section 1.8). While the extensive characterization of p42 and p44 MAP kinases has established that these proteins are central components of signal transduction cascades activated by growth factors and G-protein-coupled receptor agonists involved in a number of cellular functions including proliferation, less is understood about the regulation and functional significance of signalling pathways involving the homologous SAP kinases. Initial studies indicated that both JNK (SAP kinase-1) and p38 (SAP kinase-2) could be distinguished from the p42 and p44 MAP kinases not only by distinct dual phosphorylation motifs and substrate specificities, but also by the different range of extracellular stimuli reported to cause their activation. These include the pro-inflammatory mediators tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and LPS and several forms of environmental stress such as UV irradiation, high osmolarity, heat shock (reviewed by Cano & Mahadevan, 1995), and ischemia/reperfusion (Pombo *et al.*, 1994). It is now evident that these pathways involving SAP kinases become activated in response to an increasingly large number of extracellular cues. While growth factors acting on tyrosine kinase receptors are generally poor stimuli for JNK and p38 activation (Minden *et al.*, 1994, Raingeaud *et al.*, 1995), a number of studies of late, have demonstrated that G-protein coupled receptor agonists are able to activate these cellular targets. For example, JNK has been shown to be activated by carbachol in NIH 3T3 cells transfected with the acetylcholine muscarinic receptor (Coso *et al.*, 1995a), angiotensin II in rat liver epithelial cells (Zohn *et al.*, 1995) and endothelin and thrombin in airway smooth muscle cells (Shapiro *et al.*, 1996). Thrombin has also been shown to activate p38 in platelets (Kramer *et al.* 1995, Saklatvala *et al.*, 1996) indicating that SAP kinases may be integral components of signal transduction pathways utilized by the thrombin receptor in other cell types.

The biological significance of activation SAP kinases is starting to become apparent. One of the cellular roles of JNK is to phosphorylate the transcription factors c-Jun (Dérijard *et al.*, 1994) and activating transcription factor-2 (ATF2) (Gupta *et al.*, 1995, van Dam *et al.*, 1995), resulting in their increased transcriptional activity. The major function of c-Jun appears to be to autoregulate expression of c-Jun itself (Karin, 1995). That c-Jun becomes

phosphorylated in response to mitogens and is involved in the G_0/G_1 transition and progression through late G_1/S (Ryseck *et al.*, 1988) infers that JNK may be involved in mitogenic signalling. Indeed, activation of JNK by stimulation of G-protein coupled receptors by carbachol, thrombin and endothelin has been shown to correlate with DNA synthesis and expression of genes important for cell growth (Coso *et al.* 1995a, Shapiro *et al.*, 1996). Despite this, a growing body of evidence indicates that JNK activation by diverse stimuli in different cells plays an obligatory role in temporal growth arrest, and in some types of susceptible cells, the induction of programmed cell death or apoptosis (Verheij *et al.*, 1996, Xia *et al.*, 1995). Thus, the nature of the response to JNK activation may depend on the cell type involved and the particular stimulus. In mammalian cells, p38/reactivating kinase (RK) phosphorylates and activates MAP kinase-activated protein (MAPKAP) kinase-2 *in vitro* and *in vivo* (Rouse *et al.,* 1994), one of whose intracellular targets is the 27 kDa heat shock protein (Hsp27) (Stokoe *et al.*, 1992). Although research is currently being conducted to establish definitive functions for these downstream effectors, the p38 pathway has been linked to LPS-and TNF-induced cytokine expression in monocytes (Lee *et al.*, 1994, Beyaert *et al.*, 1996) and aggregation of platelets in response to thrombin, collagen and a thromboxane analogue (Saklatvala *et al.*, 1996). As with JNK, however, due to conflicting data, the precise role of p38 in regulating cell growth is unclear. Although p38 has been implicated in the apoptosis of neurons upon growth factor removal (Xia *et al.*, 1995), another study has concluded that p38 protects against TNFα -induced apoptosis in neutrophils (Paul *et al.*, submitted). In support of a role for the p38 pathway in signalling by mediators which promote growth, Hsp27 is a major substrate of phosphorylation following stimulation of cells with mitogens, including thrombin and PDGF (Saklatvala *et al.*, 1991, Zhou *et al.*, 1993) as well as cytokines and heat shock. Furthermore, functioning of the yeast homologue of p38, HOG-1, is required to allow yeast to proliferate in conditions of high osmolarity (Brewster *et al.*, 1993), although a direct role for p38 in normal mammalian cell growth has not been established.

Information on signalling via SAP kinases by G-protein-coupled receptor agonists is relatively sparse, and evidence in the literature of roles for signal transduction pathways involving these classes of MAPK homologue in the control of the cell cycle is apparently conflicting. The aim of this study, therefore, is to assess whether JNK and/or p38 become activated in response to thrombin in BPA fibroblasts and whether this activation can account for the mitogenic effect of thrombin under physiological conditions.

5.2 Agonist-stimulated JNK activation in BPA fibroblasts

Thrombin, PDGF and sorbitol were screened for their ability to activate JNK in BPA fibroblasts. The phosphorylation of c-Jun by activated JNK requires a binding interaction between JNK and the δ-domain of the NH_2-terminal region of c-Jun separate from the sites of phosphorylation (Dai *et al.*, 1995, Dérijard *et al.*, 1994, Hibi *et al.*, 1993). The high affinity of JNK for its substrate was exploited to purify JNK from the lysates of agonist-treated cells by binding to a recombinant GST-tagged truncated N-terminus of c-Jun (c-Jun_{5-89}), containing the binding domain and Ser^{63} and Ser^{73} phophorylation sites, immobilized on glutathione (GSH)-sepharose beads (Dai *et al.*, 1995). Initial experiments were conducted to check the efficiency of affinity purification of JNK by Western blotting analysis. Proteins associated with GST-c-Jun_{5-89} from extracts of cells treated with vehicle, thrombin, PDGF or sorbitol were immunoblotted for JNK with polyclonal anti-JNK1/2 antiserum. Two bands migrating at approximately 46 and 54 kDa, corresponding to the molecular weights of variant JNK isoforms differing in the length of their carboxy termini and derived from alternative processing of transcripts from separate genes (Gupta *et al.*, 1996), were detected in both affinity precipitates and supernatents (Fig. 5.1A). The incomplete recovery of the 46 and 54 kDa proteins was not due to a limitation of substrate binding since increasing the concentration of GST-c-Jun_{5-89}/GSH-sepharose beads did result in additional capture of these proteins (not shown) suggesting that under the conditions used, only a fraction of the total cellular content of JNK binds to GST-c-Jun_{5-89}, as has been reported by other investigators (Dai *et al.*, 1995). *In vitro* JNK activity was assayed in affinity precipitates by assessing the incorporation of [^{32}P]phosphate into GST-c-Jun_{5-89} fusion protein. No detectable increase in c-Jun_{5-89}-associated kinase activity was observed in extracts from BPA fibroblasts treated with either thrombin or PDGF for exposure times up to 30 minutes compared to vehicle-treated cells (Fig. 5.1B & C). In contrast, over a 20 fold stimulation in the phosphorylation of a protein, which when resolved by SDS-PAGE, migrated at the appropriate molecular weight for GST-c-Jun_{5-89} (37 kDa), was consistently seen when cells were exposed to sorbitol (0.5 M) for 30 minutes (Fig. 5.1B & C). The level of the 46 and 54 kDa proteins extracted in affinity precipitates was similar in cells treated with vehicle, thrombin, PDGF and sorbitol indicating that the effect of sorbitol was not due to an increase in the binding affinity of JNK for its substrate.

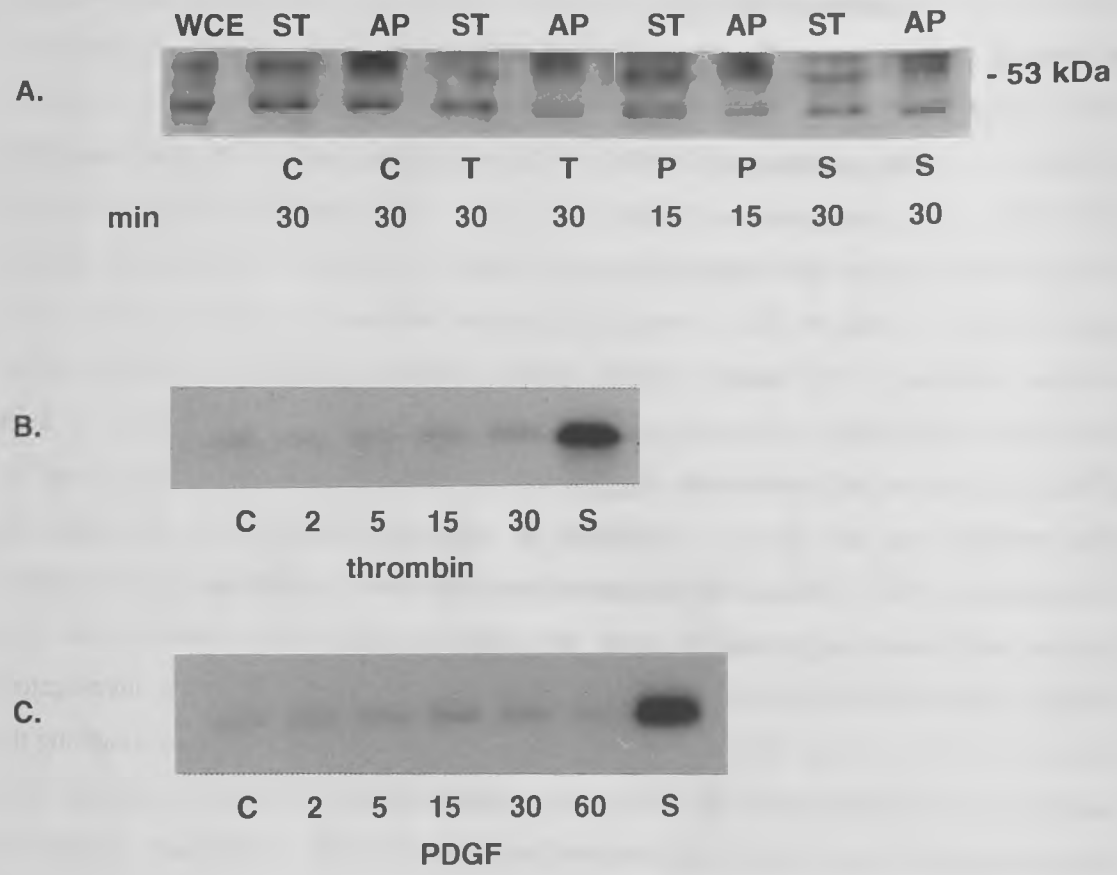

WCE ST AP ST AP ST AP ST AP
A.
- 53 kDa
C C T T P P S S
min 30 30 30 30 15 15 30 30
B.
C 2 5 15 30 S
thrombin
C.
C 2 5 15 30 60 S
PDGF

Figure 5.1 Recovery of JNK and effect of thrombin and PDGF on JNK activation in affinity precipitates from agonist-stimulated BPA fibroblasts. Cells were exposed to thrombin (300 nM) (T), PDGF (30 ng/ml) (P), sorbitol (0.5 M) (S) or vehicle (C) for the times indicated in minutes. Lysates were incubated with GST-c-Jun_{5-89} immobilized on glutathione-sepharose. Affinity precipitates (AP), supernatents (ST) and whole cell extracts (WCE) were resolved by SDS-PAGE and immunoblotted for JNK (**A**) as outlined in Section 2.3. The mobility of the 53 kDa molecular weight marker is indicated on the right of the immunoblot. In other experiments, affinity precipitates were prepared from lysates of cells treated with **B**) thrombin (300 nM) or **C**) PDGF (30 ng/ml) for the times indicated in minutes, or sorbitol (0.5 M) (S) or vehicle (C) for 30 minutes, and assayed for *in vitro* JNK activity as outlined in Section 2.4.5. Incorporation of [^{32}P]phosphate into GST-c-Jun_{5-89}, resolved by SDS-PAGE, was detected by autoradiography. Each of the autoradiographs shown is representative of two separate experiments.

5.3.1 Agonist-stimulated MAPKAP kinase-2-associated kinase activity in BPA fibroblasts

By analogy to JNK, p38 has also been shown to interact strongly with its substrate MAPKAP kinase-2 (Bogoyevitch *et al.*, 1996). The effect of agonists on p38 activation in BPA fibroblasts was measured by a solid phase kinase assay in an identical manner to JNK, except that in this case, p38 was affinity purified from cell lysates by binding to GST-tagged full length MAPKAPK-2 immobilized on GSH-sepharose beads. To check the recovery of p38 in affinity precipitates, protein bound to GST-MAPKAPK-2/GSH -sepharose complexes was eluted with Laemmli sample buffer, resolved by SDS-PAGE and immunoblotted with an anti-p38 -specific antibody. A single band migrating at an apparent molecular weight of 38-40 kDa was detected in the fraction precipitated with the GST-MAPKAP kinase-2/GSH-sepharose beads but not in supernatents (Fig. 5.2.1A). In contrast, when Western blots were probed with anti-p42/p44 MAP kinase antibody, two immunoreactive bands, one migrating at approximately 42 kDa and another, of lower abundance and of approximately 44 kDa, were detected in the supernatents but not in the bead fractions (Fig. 5.2.1B). Affinity precipitates were assayed for associated phosphotransferase activity against the bound GST-MAPKAP kinase-2. Exposure of BPA fibroblasts to thrombin or PDGF resulted in the time-dependent increase in [^{32}P]phosphorylation of a protein in affinity precipitates which when resolved by SDS-PAGE, migrated at an apparent molecular weight corresponding to GST-MAPKAP kinase-2 (approximately 79 kDa). Thrombin-stimulated GST-MAPKAP kinase-2-associated kinase activity extracted in affinity precipitates increased steadily after 5 minutes and continued to rise up to 60 minutes (15 fold stimulation) (Fig. 5.2.2A & C), after which, the time course was not recorded. For PDGF, kinase activity rose rapidly after 2 minutes to reach a maximum at 15 minutes (approximately 12 fold increase over activity in vehicle-treated cells) and returned to basal levels by 60 minutes (Fig. 5.2.2B & C). For both agonists studied, sorbitol was used as a positive control and routinely caused between 15 and 25 fold increase in GST-MAPKAP kinase-2-associated kinase activity. SDS-PAGE immunoblotting verified that equivalent amounts of p38 were recovered in affinity precipitates from the lysates of cells treated with either vehicle, thrombin, PDGF or sorbitol at the exposure times found to cause maximal GST-MAPKAP kinase-2-associated kinase activity (Fig. 5.2.1A) confirming that binding of p38 to its substrate was not affected by agonist stimulation. Attempts to further characterize p38 activation in BPA fibroblasts in response to thrombin and PDGF were

Figure 5.2.1 Recovery of p38 in affinity precipitates from agonist-stimulated BPA fibroblasts. Cells were exposed to thrombin (300 nM) (T), PDGF (30 ng/ml) (P), sorbitol (0.5 M) (S) or vehicle (C) for the times indicated. Lysates were incubated with GST-MAPKAP kinase-2 immobilized on glutathione-sepharose. Affinity precipitates (AP), supernatents (ST) and whole cell extracts (WCE) were resolved by SDS-PAGE and immunoblotted for p38 (A) or p42/p44 MAP kinases (B) as outlined in Section 2.3. Bands corresponding to p42 and p44 and the p38 MAP kinase homologue are shown on the right of immunoblots.

A.

- GST-MAPKAPK-2

C2 2 5 15 30 60 C60 S

thrombin

B.

- GST-MAPKAPK-2

C2 2 5 15 30 60 C60 S

PDGF

C.

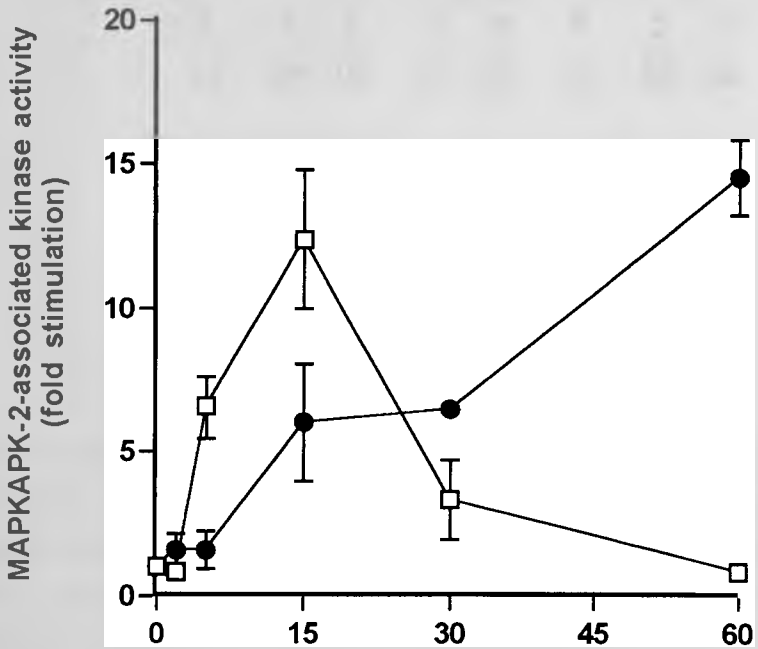


Figure 5.2.2 Agonist-stimulated MAPKAP kinase-2-associated kinase activity in BPA fibroblasts. Cells were treated with **A)** thrombin (300 nM) or **B)** PDGF (30 ng/ml) for 2, 5, 15, 30 and 60 minutes, 0.5 M sorbitol (S) for 30 minutes or vehicle (C) for 2 and 60 minutes. Affinity precipitates were prepared from cell lysates with GST-MAPKAP kinase-2 immobilized on glutathione-sepharose and assayed for associated kinase activity *in vitro* as outlined in Section 2.4.6. Incorporation of [^{32}P]phosphate into GST-MAPKAP kinase-2, resolved by SDS-PAGE, was detected by autoradiography (**A** & **B**). The extent of GST-MAPKAP kinase-2 phosphorylation in cells treated with thrombin (●) or PDGF (□) was quantified by densitometric analysis (**C**) and is expressed as mean±s.e.m. fold stimulation relative to control of two separate experiments for each time course.

hampered by the unsuitability of commercially available antisera for immunoprecipitation of p38.

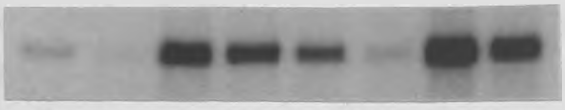
5.3.2 Effect of SB203580 on thrombin and PDGF -stimulated MAPKAP kinase-2-associated kinase activity

The specificity of the kinase activity associated with GST-MAPKAP kinase-2 affinity precipitates from agonist-stimulated fibroblasts was checked using SB203580 (4-(4-flurophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)-imadazole. This compound has emerged as a highly specific inhibitor of p38 both *in vitro* and *in vivo*, having no effect on the activities of 12 other protein kinases *in vitro* including the p42/p44 and JNK MAP kinase homologues (Cuenda *et al.*, 1995, Lee *et al.*, 1994, Lee & Young, 1996). Pretreatment of BPA fibroblasts for 30 minutes with SB203580, at a concentration (20 μM) reported to cause complete inhibition of *in vitro* p38 activity from PC-12 and HeLa cells exposed to chemical, osmotic and thermal stress (Cuenda *et al.*, 1995), was found to completely inhibit the phosphorylation of GST-MAPKAP kinase-2 by affinity purified p38 from lysates of cells treated with PDGF for 15 minutes (Fig. 5.2.3). In contrast, kinase activity extracted with GST-MAPKAP kinase-2/GSH-sepharose beads from cells stimulated with thrombin or sorbitol was inhibited by approximately 40 % and 35 %, respectively, by prior treatment with 20 μM SB203580 (Fig. 5.2.3), although in these experiments, the relative increase in kinase activity evoked by these stimuli (approximately 18 fold for thrombin and 24 fold for sorbitol) was greater than for PDGF (8 fold).

5.4 Effect of SB203580 on thrombin and PDGF -stimulated DNA synthesis

The highly specific inhibition of p38 by SB203580 has been exploited to probe for physiological roles of p38. To evaluate whether p38 activation by thrombin and PDGF plays a significant role in the mitogenic reponse to these agents, the effect of SB203580 on thrombin and PDGF-stimulated DNA synthesis was tested. Treatment of quiescent BPA fibroblasts with varying concentrations of the p38 inhibitor for 30 minutes prior to incubation with thrombin (300 nM) or PDGF (30 ng/ml) for 24 hours, caused a concentration-dependent inhibition of [^{3}H]thymidine incorporation (Fig. 5.3). Mitogenic responses to both agonists were abolished at 20 μM SB203580 with an IC_{50} value of 5.6±1.0 μM for thrombin. As

A.



- GST-MAPKAPK-2

	C	C	T	T	P	P	S	S
	30	30	60	60	15	15	30	30
SB203580	-	+	-	+	-	+	-	+

B.

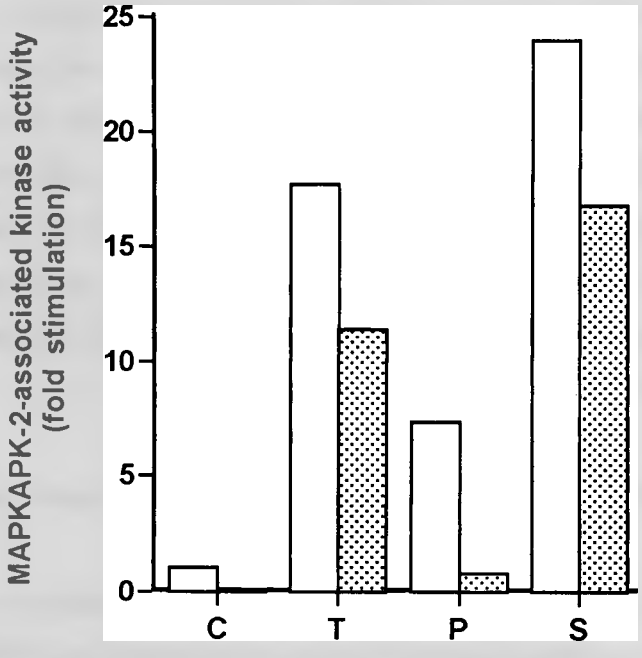


Figure 5.2.3 Effect of SB203580 on agonist-stimulated MAPKAP kinase-2-associated kinase activity in BPA fibroblasts. Serum-deprived cells were pretreated with vehicle (- or open bars) or SB203580 (20 μM) (+ or hatched bars) for 30 minutes prior to exposure to thrombin (300 nM for 60 minutes) (T), PDGF (30 ng/ml for 15 minutes) (P), sorbitol (0.5 M for 30 minutes) (S) or vehicle (C) for 30 minutes. Affinity precipitates were prepared from cell lysates with GST-MAPKAP kinase-2 immobilized on glutathione-sepharose and assayed for associated kinase activity *in vitro* as outlined in Section 2.4.6. Incorporation of [^{32}P]phosphate into GST-MAPKAP kinase-2, resolved by SDS-PAGE, was detected by autoradiography (**A**). The extent of GST-MAPKAP kinase-2 phosphorylation was quantified by densitometric analysis (**B**) and is expressed as fold stimulation relative to control for one assay representative of four separate experiments.

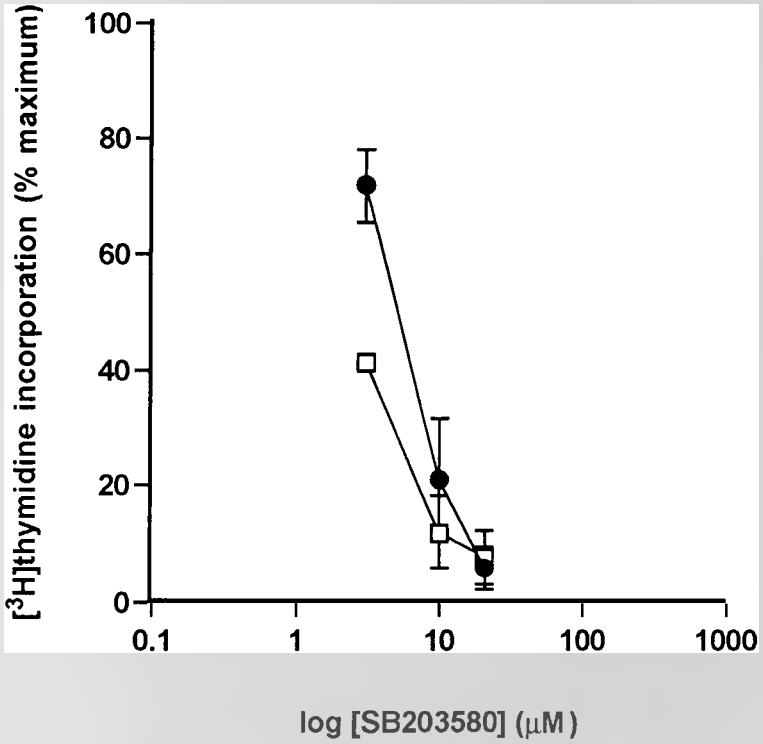


Figure 5.3 Effect of SB203580 on thrombin and PDGF -stimulated [^{3}H]thymidine incorporation in BPA fibroblasts. Cells were pretreated with vehicle or SB203580 at the indicated concentrations for 30 minutes prior to exposure to thrombin (300 nM) (●) or PDGF (30 ng/ml) (□) for 24 hours. Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNA replicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. percentage of maximal thrombin or PDGF -stimulated [^{3}H]thymidine incorporation in the absence of SB203580 from two independent experiments each performed in triplicate.

greater than 50 % inhibition of PDGF-stimulated [^{3}H]thymidine incorporation was observed at the lowest concentration of SB203580 tested (3 μM), the IC_{50} for this response could not be determined accurately. However, this value can be estimated to be less than 3 μM and in the range reported by others to cause half-maximal inhibition of TNF-induced IL-6 expression in L929 and HeLa cells *in vivo* (Beyaert *et al.*, 1996).

5.5 DISCUSSION

The purpose of this study was to ascertain whether the mitogenic stimuli characterized for BPA fibroblasts were able to activate the stress-activated protein kinase pathways and whether signalling via these routes accounts for the ability of these agents to stimulate DNA synthesis in this cell type.

5.5.1 Agonist-stimulated activation of JNK

The JNK subfamily of SAP kinases were originally identified as kinases phophorylating the N-terminus of the transcription factor c-Jun in response to UV irradiation (Dérijard *et al.*, 1994), inhibitors of protein synthesis, heat shock and TNF (Kyriakis *et al.*, 1994). Soon after these initial discoveries, hyperosmolarity (Galcheva-Gargova *et al.*, 1994), ischemia/reperfusion (Pombo *et al.*, 1994) and IL-1 (Gupta *et al.*, 1996) were soon added to the list of stimuli able to evoke strong activation of JNKs, defining these kinases as transducers of intracellular signals evoked by cellular stresses and pro-inflammatory cytokines. While JNK has also been shown to become activated in response to growth factors acting on tyrosine kinase receptors, which are strong activators of the p42/p44 MAP kinase pathway (Dérijard *et al.*, 1994, Kyriakis *et al.*, 1994), generally, this class of agonist evokes a much lower level of JNK activity compared to the stimuli listed above. Evidence in the literature of late, however, has indicated that as well as stress-related stimuli and inflammatory mediators, JNK is activated effectively by G-protein-coupled receptor agonists. For example, stimulation by carbachol of muscarinic receptors expressed in NIH 3T3 fibroblasts or Cos-7 cells has been shown to cause an increase in JNK activity (Coso *et al.*, 1995a). Furthermore, JNK becomes activated in response to angiotensin II in rat liver epithelial cells (Zohn *et al.*, 1995) and endothelin and thrombin in rat airway smooth muscle cells (Shapiro *et al.*, 1996). A role for G proteins in the latter response was inferred by the

finding that JNK activation could also be evoked by aluminium fluoride (AlF_4^-)(Shapiro *et al.*, 1996), an agent which is thought to activate G proteins directly by interacting with GDP in the nucleotide binding site of the α subunit and mimic the γ phosphate of GTP (Bigay *et al.*, 1985). In addition, heterotrimeric G-protein $\beta\gamma$ subunits have been shown to activate JNK by a Ras and Rac 1-dependent pathway (Coso *et al.*, 1996). It was therefore of interest to investigate the possibility of whether activation of the JNK pathway represented a novel signalling mechanism for thrombin in BPA fibroblasts. As JNK binds with high affinity to c-Jun (Dai *et al.*, 1995, Dérijard *et al.*, 1994, Hibi *et al.*, 1993) agonists were screened for their ability to activate JNK in BPA fibroblasts by assaying for c-Jun -associated kinase activity. The present study demonstrated that exposure of cells to thrombin for durations up to 30 minutes did not stimulate any detectable increase in JNK activity as assessed by the inability of the protease to increase the phosphorylation of GST-c-Jun_{5-89} in affinity precipitates. JNK activity was also found to be unaffected by PDGF. A similar observation has been made by other investigators in NIH 3T3 fibroblasts (Coso *et al.*, 1995a) and is in line with other reports showing that growth factors acting on tyrosine kinase receptors are either ineffective agents for inducing JNK activation or weakly stimulate JNK activity in only a few cell types (Dérijard *et al.*, 1994, Kyriakis *et al.*, 1994). As exposure of cultured cells to hyperosmolar media has been shown to markedly stimulate JNK activity (Galcheva-Gargova *et al.*, 1994), an increase in extracellular osmolarity induced by a high concentration of sorbitol was used as a positive control stimulus for the JNK assay. In contrast to the mitogens tested, and in agreement with the literature reports, sorbitol was found to cause a very strong increase in the phosphorylation of GST-c-Jun_{5-89} confirming that the assay could effectively detect changes in GST-c-Jun_{5-89} -associated kinase activity. The demonstration by immunoblotting analysis with anti-JNK1/2 -specific antiserum of the presence of proteins corresponding to the JNK isoforms of approximately 46 and 54 kDa in affinity precipitates from sorbitol-exposed cells, strongly suggest that the sorbitol-evoked increase in c-Jun_{5-89} -phosphorylating activity is attributed to JNK1/2. As the amount of both the 46 and 54 kDa isoforms of JNK extracted by affinity purification was not affected by exposure of cells to either of the stimuli tested, the failure of thrombin and PDGF to stimulate JNK activity cannot be due to poor expression or ineffective recovery of JNK in BPA fibroblasts. However, other investigators have established that the JNKs of approximately 46 and 54 kDa represent a mixture of at least ten variant isoforms derived from alternative splicing of three gene products that exhibit markedly different binding affinities for c-Jun (Gupta *et al.*, 1996). This may explain why only a

fraction of the total cellular content of JNK could be extracted from BPA fibroblasts by affinity purification when the amount of GST-c-Jun_{5-89} was not limiting. Thus, the possibility that specific JNK isoforms may become activated in response to thrombin or PDGF, but are not detected under the assay conditions used here because they bind very weakly to GST-c-Jun_{5-89}, cannot be ruled out.

5.5.2 Agonist-stimulated activation of p38 MAP kinase

p38 belongs to a new subfamily of mammalian SAP kinases that was originally identified by virtue of its high sequence homology to the gene product of HOG1 (Han *et al.*, 1994), a kinase required for adaptation to osmotic stress in *Saccharomyces cerevisiae* (Brewster *et al.*, 1993). In addition to osmotic stress, p38, like JNK, has been shown to activated by other environmental stresses including UV light (Raingeaud *et al.*, 1995) and heat shock (Rouse *et al.*, 1994) as well as inflammatory mediators such as endotoxin (Han *et al.*, 1994) and the cytokines TNFα and IL-1 (Raingeaud *et al.*, 1995). Recently, it has been reported that thrombin causes a marked activation of p38 in platelets (Kramer *et al.*, 1995b, Saklatvala *et al.*, 1996), indicating that the range of stimuli able to activate p38, like JNK in specific cell types, may extend to certain G-protein coupled receptor agonists. The potential for thrombin and other mitogenic stimuli to activate the p38 pathway is also suggested by reports that thrombin, serum and FGF phosphorylate the 27 kDa heat shock protein Hsp27 *in vivo* by stimulating a Hsp27 protein kinase activity in CCL39 fibroblasts (Zhou *et al.*, 1993). While the identity of this kinase was not identified, other reports have indicated that MAPKAP kinase-2, a substrate of p38 (Rouse *et al.*, 1994) is a likely candidate since this kinase can phosphorylate Hsp27 *in vitro* (Stokoe *et al.*, 1992). The finding that Hsp27 also becomes phosphorylated in response to heat shock, TNFα and IL-1 (Freshney *et al.*, 1994, Kaur *et al.*, 1989), stimuli which have been documented to activate MAPKAP kinase-2 (Rouse *et al.*, 1994, Engel *et al.*, 1995) and that MAPKAP kinase-2 phosphorylates Hsp27 on the same serine residues as these stimuli (Stokoe *et al.*, 1992), indicates that the p38 substrate may be the enzyme responsible for phosphorylating Hsp27 *in vivo*.

Given the evidence that a signalling pathway involving p38 may couple to the thrombin receptor, the present study investigated the ability of thrombin to activate p38 in BPA fibroblasts. The data provides strong evidence that in this cell type, thrombin stimulates the activation of p38 in a time-dependent manner on the basis that: 1) A marked increase in

GST-MAPKAP kinase-2 phosphorylation was observed in affinity precipitates from cells exposed to thrombin 2) Affinity precipitates contained p38 as detected by immunoblotting with anti-p38 -specific antiserum. While p38 and JNK are often activated in parallel, clearly, thrombin can activate p38 independently of JNK in BPA fibroblasts, as has been observed by others in platelets (Kramer *et al.*, 1995b). Surprisingly, PDGF also stimulated MAPKAP kinase-2 -associated kinase activity, although the magnitude of this response was not as strong as that for thrombin. While PDGF has previously been reported to phosphorylate Hsp27 (Saklatvala *et al.*, 1991), no studies up until now have addressed whether this growth factor activates p38, although other receptor tyrosine kinase ligands have generally been shown to cause either a modest or negligible increase in p38 activity (Rouse *et al.*, 1994, Raingeaud *et al.*, 1995). Because of this unexpected discovery, the specificity of the MAPKAP kinase-2 phosphotransferase activity in affinity precipitates was assessed using SB203580. This agent is a member of a class of pyridinyl imidazole compounds which were originally developed as inhibitors of cytokine production by inflammatory stimuli (Lee *et al.*, 1994). Subsequently, this action has been attributed to the ability of SB203580 to specifically inhibit p38. The complete inhibition by SB203580 of MAPKAP kinase-2 -associated kinase activity extracted from cells treated with PDGF is consistent with the kinase activity being attributable to p38. However, for thrombin and sorbitol, SB203580 only partially inhibited the purified kinase activity. This may reflect the relatively greater magnitude of activation of p38 by these stimuli relative to PDGF. The limitation in the amount of SB203580 available for these experiments precluded testing the inhibitor at higher concentrations. Besides, from the available evidence in the literature, inhibition of SB203580-sensitive responses is usually complete at 10 μM, and therefore, the concentration used in this study (20 μM) should be sufficient to cause maximal inhibition. It is unlikely that the disparate sensitivities of agonist-stimulated MAPKAP kinase-2 -associated kinase activity to SB203580 can be explained by distinct processes by which the different stimuli may activate p38 since the inhibitor is thought to act by binding directly to, and specifically blocking the kinase activity of p38, preventing phosphorylation of its substrates (Lee *et al.*, 1996), rather than interfering with the activation of p38 by upstream kinases (Cuenda *et al.*, 1995, Lee & Young, 1996). It is conceivable that the bovine homologue of p38, when activated strongly by thrombin or sorbitol may not be inhibited so effectively by SB203580, although the observation that both thrombin and PDGF -stimulated DNA synthesis in BPA fibroblasts (see below) was completely blocked by this compound does not support this possibility. Furthermore, the sensitivity of this response to low micromolar

concentrations of SB203580 indicates that incomplete inhibition of kinase activity by thrombin or sorbitol cannot be due to the poor solubility of SB203580 or an insufficient time of exposure to the inhibitor. It is possible that the SB203580-insensitive phosphotransferase activity measured in affinity precipitates may be due to another kinase which can bind to the immobilized MAPKAP kinase-2. While MAPKAP kinase-2 has been shown to be a substrate of p42 and p44 MAP kinases *in vitro* (Stokoe *et al.*, 1992), only p38 appears to activate this kinase *in vivo* (Cuenda *et al.*, 1995). Besides, even though the kinetics of p38 activation by thrombin and PDGF resemble temporal activation of p42/p44 MAP kinases by the same agonists (Sections 3.2), phosphorylation of GST-MAPKAP kinase-2 cannot be attributed to the classical MAP kinase homologues since p42/p44 MAP kinases were not detected in affinity precipitates by immunoblotting, indicating that these isoforms are unable to bind to immobilized MAPKAP kinase-2. While no other kinases have been described that use MAPKAP kinase-2 as a substrate *in vivo*, the possibility that the phosphotransferase activity measured in the solid phase assay was due to a kinase other than p38 which can bind with high affinity to, and phosphorylate MAPKAP kinase-2 cannot be excluded. Recently, novel protein kinases designated SAP kinase-3 and p38β have been cloned from the rat and human, showing high sequence homology to p38 and sharing the TGY sequence in the activation domain (Jiang *et al.*, 1996, Mertens *et al.*, 1996). Furthermore, p38β was shown to phosphorylate MAPKAP kinase-2 *in vitro* (Jiang *et al.*, 1996). Whether either of these kinases, or a bovine equivalent, exhibit different sensitivity than p38 to inhibition by SB203580 and account for the GST-MAPKAP kinase-2 -associated kinase activity in response to thrombin and sorbitol in the present study, requires further investigation. Thus, although taken together, these results strongly indicates that thrombin and PDGF stimulate p38 activity in BPA fibroblasts, the data must be interpreted with caution since the measured kinase activity could not be shown by pharmacological intervention to be unequivocally attributable entirely to p38.

5.5.3 Putative regulation of p38 in BPA fibroblasts

Unlike the extensive characterization of the upstream regulatory mechanisms involved in the activation of p42 and p44 MAP kinases (outlined in Chapters and 2) the sequential cascade leading to the activation of p38 has not been fully elucidated. In an analogous manner to activation of the p42/p44 MAP kinase by MEK-1/2 (see Section 1.8.2) activation of p38

kinase requires dual phosphorylation, but in this case on Thr^{180} and Tyr^{182} within a TGY motif in the activation domain (Raingeaud *et al.*, 1995). A MAP kinase kinase homologue capable of directly activating both p38 and JNK *in vitro* has been identified and designated MKK4 (also referred to as SEK-1/JNKK/SAPKK1)(Sanchez *et al.*, 1994, Dérijard *et al.*, 1995, Lin *et al.*, 1995, Meier *et al.*, 1996). While MKK4 may represent a common signalling element mediating sorbitol-evoked JNK and p38 activation in BPA fibroblasts, clearly, this dual-specificity kinase is unlikely to be the upstream regulator of p38 activity evoked by thrombin or PDGF since JNK would be expected to be activated in parallel by these agonists. Rather, the preferential activation of p38 and not JNK indicates that a MAP kinase kinase specific to the p38 pathway must be involved. One such kinase, designated MKK3 (or SAPPK2) has been defined as a specific activator of p38 *in vitro*, being incapable of phosphorylating JNK or p42/p44 MAP kinases (Dérijard *et al.*, 1995, Meier *et al.*, 1996). In additional experiments, attempts were made to measure MKK3 activity in BPA fibroblasts exposed to thrombin and PDGF to assess the role of this kinase as an activator of p38. While conditions were optimized to successfully immunoprecipitate MKK3 with an anti-MKK3 -specific antibody, indicating that BPA fibroblasts express this protein, no significant increase in MKK3 phosphotransferase activity *in vitro* was detectable by an immunocomplex kinase assay using recombinant p38 as a substrate (not shown). Recently, a novel mammalian MAP kinase kinase, designated MKK6 (or SAPKK3), has been cloned and characterized (Cuenda *et al.*, 1996, Han *et al.*, 1996, Raingeaud *et al.*, 1996, Stein *et al.*, 1996). Like MKK3, MKK6 was found to exhibit substrate specificity for the p38 subgroup of MAP kinases and tissue-specific distribution, found predominantly in skeletal muscle and leucocytes (Han *et al.*, 1996, Raingeaud *et al.*, 1996, Stein *et al.*, 1996). Furthermore, transfection of constitutively active MKK6 but not MKK3 was found to activate endogenous p38 indicating that the former is more likely to be the physiological activator of p38 (Raingeaud *et al.*, 1996). MKK6 would be an attractive candidate to activate p38 independently of JNK in response to thrombin and PDGF in BPA fibroblasts, although the upstream activator(s) of either this dual-specificity kinase or MKK3 are undefined at present. While MEKK1 is believed to play a role primarily as the physiological activator of MKK4 (Minden *et al.*, 1994, Yan *et al.* 1994), there is no evidence that this kinase is an upstream regulatory in the pathway leading to specific activation of p38 as neither MKK3 nor MKK6 is a physiological substrates of MEKK1 (Lin *et al.*, 1995, Stein *et al.*, 1996). Similarly, while the Rho family GTPases Rac1 and Cdc42, have been implicated upstream of the p38 (Bagrodia *et al.*, 1995, Zhang *et al.*, 1995) and

JNK pathways (Bagrodia *et al.*, 1995, Coso *et al.*, 1995b, Minden *et al.*, 1995) by regulating MEKK1 and MKK4 (Minden *et al.* 1995, Han *et al.*, 1996) possibly via activating p21-activated kinases (PAK) (Manser *et al.*, 1994, Bagrodia *et al.*, 1995), Rac1 and Cdc42 do not control activation of MKK6 (Han *et al.*, 1996). Thus there appears to be two distinct routes leading to p38 activation, one involving MKK6/3 and another potentially involving Rac1/Cdc42, PAK, MEKK1 and MKK4. Because in BPA fibroblasts, thrombin and PDGF stimulate p38 independently of JNK, which is also regulated by the latter pathway, it is likely that the MKK6/3-dependent pathway is activated.

5.5.4 Role of SAP kinases in agonist-stimulated DNA synthesis in BPA fibroblasts

Use of cells transfected with constitutively-activated or dominant-interfering mutant forms of various components of the SAP kinase signalling cascades and pharmacological inhibitors has only recently shed light on the biological significance of these pathways. The involvement of either JNK or p38 family members in cell cycle regulation has not been addressed extensively since up until now, most growth factors have been either ineffective or weak activators of these kinases. Furthermore, activation of the JNK and p38 pathways in certain cell types such as rat PC-12 pheochromocytoma cells, has been linked with the induction of programmed cell death, or apoptosis (Xia *et al.*, 1995). Recently, it has been reported that bioactive peptides that act on specific G-protein-coupled receptors activate JNK in cell types in which they behave as mitogens (Zohn *et al.*, 1995, Shapiro *et al.*, 1996). Although the functional significance of this signalling event in the growth-promoting effects of these agonists was not addressed, in one study stimulation of JNK activity in airway smooth muscle cells by endothelin-1 and thrombin correlated with DNA synthesis (Shapiro *et al.*, 1996). In the present investigation, however, the JNK pathway cannot be involved in the mitogenic response of thrombin or PDGF in BPA fibroblasts since neither of these stimuli activated JNK in this cell type. While these agonists appear to strongly stimulate p38 in BPA fibroblasts, at present, there is no evidence to directly link this MAP kinase homologue to mammalian cell mitogenesis, although the counterpart of p38 in *Saccharomyces cerevisiae*, HOG-1, plays a permissive role in yeast growth under hyperosmolar conditions (Brewster *et al.*, 1993). Although the MKK3-dependent p38 pathway, like JNK, has been implicated in the apoptotic response of PC-12 cells on NGF withdrawal, this effect requires the concurrent inhibition of the p42/p44 MAP kinase pathway (Xia *et al.*, 1995). In direct contrast to PC-12

cells, however, work from this laboratory has indicated that p38 protects against TNFα - induced apoptosis in neutrophils (Paul *et al.*, submitted). Thus, depending on the cell type and the contributions of other MAP kinase family members activated by a particular stimulus, p38 can inflict apparently opposing effects on the fate of the cell. Just as the p42/p44 MAP kinase pathway appears to be critical for cell survival in certain cell types such as neurons (Xia *et al.*, 1995) and required for mitogenesis in others, including BPA fibroblasts (Section 3.5.2), it is tempting to speculate that the protective function of the p38 pathway in neutrophils may be manifested as a growth response in a mitogen-responsive cell type. Indeed, despite being unable to complete inhibit MAPKAP kinase-2 -associated kinase activity, SB203580 abolished both thrombin and PDGF -stimulated DNA synthesis with IC_{50} values which resembled the inhibition of other SB203580-sensitive responses *in vivo* (Beyaert *et al.*, 1996). These results strongly suggest that the mitogenic effects of thrombin and PDGF in BPA fibroblasts requires p38, identifying for the first time a physiological role for p38 in normal mammalian cell growth.

It is unclear how p38 may mediate the mitogenic response to thrombin and PDGF. MAPKAP kinase-2 is one substrate for p38 *in vitro* (Stokoe et al., 1992) and while SB203580 has been shown to inhibit the activation of MAPKAP kinase-2 and the phosphorylation of Hsp27 in response to cellular stress and inflammatory mediators *in vivo* (Cuenda *et al.*, 1995), the role of p38 or its downstream effectors in cell cycle control is not obvious. In transient hostile environmental conditions, Hsp27 phosphorylation may serve a protective function to aid cell survival, possibly by promoting actin polymerization (Lavoie *et al.*, 1995). The significance of Hsp27 under normal conditions in response to physiological agonists such as thrombin and PDGF, however, is not known precisely, although the correlation between expression of Hsp27 and the growth properties and prognostic markers of certain tumours (Ciocca *et al.*, 1983) may provide a possible link with normal cell growth. Recently, another substrate of p38 *in vivo*, a novel serine-threonine protein kinase designated MAPKAP kinase-3, has been identified (McLaughlin *et al.*, 1996), although the cellular targets of this signalling molecule have not been discovered yet.

The mechanism by which the p38 pathway transmits growth-promoting signals evoked by thrombin and PDGF is likely to involve the control of gene expression. Like MAPKAP kinase-2, the transcription factor ATF-2 has been identified as a substrate of p38 *in vitro*. Recently, a study has reported that gene transcription mediated by both ATF2 and $p62^{TCF}$/Elk-1 is enhanced following transfection with constitutively-active MKK6, indicating

that these transcription factors are likely to be nuclear targets of p38 *in vivo* (Raingeaud *et al.*, 1996). $p62^{TCF}$/Elk-1 is an ETS domain transcription factor that binds together with serum response factor to the serum response element (SRE) leading to the induction of expression of specific genes, including c-*fos*, and is also a target for the p42/p44 MAP kinases (Gille *et al.*, 1992, Marais *et al.*, 1993). ATF-2 binds to cAMP-responsive element (CRE) -like elements (T(G/T)ACGTCA) in the promoters of many genes, leading to their induction. In homodimer or heterodimer complexes bound to partner subunits such as c-Jun or NF-κB, ATF2 can mediate transcription of other genes including c-*jun* (van Dam *et al.*, 1995). Detailed knowledge is lacking, however, about how expression of these, or other target genes regulated by the p38-activated transcription factors coordinate the complex genetic program controlling the cell cycle.

5.5.5 CONCLUDING REMARKS

The present study provides data to indicate that in BPA fibroblasts, thrombin and PDGF are effective activators of p38 MAP kinase, an element of a signalling cascade that up until now has been reported to operate primarily in response to stress-related events and pro-inflammatory mediators. Activation of p38 by these agonists, unlike many of the archetypal stimuli, occurred independently of JNK activation, suggesting that thrombin and PDGF are likely to employ a discrete signalling pathway upstream of p38. Furthermore, p38 may play a role in thrombin and PDGF-stimulated DNA synthesis in BPA fibroblasts indicating that re-entry into the cell cycle may be one of the functional consequences of activation of the p38 pathway in certain cell types.

CHAPTER 6

INVESTIGATION INTO THE ROLE OF PROTEINASE-ACTIVATED RECEPTOR (PAR) -2 IN TRYPSIN-MEDIATED ACTIVATION OF MAP KINASE IN BPA FIBROBLASTS

6.1 INTRODUCTION

It has been well recognized from studies conducted primarily by Pouysségur's group in CCL39 fibroblasts, that thrombin-derived peptides can mimic thrombin in evoking a number of early phospholipid-generated second messenger signalling events (Vouret-Craviari *et al.*, 1992, McKenzie *et al.*, 1992) and the rapid and transient activation of MAP kinase (Meloche *et al.*, 1992). Unlike thrombin, however, peptides are unable to cause the late sustained phase of MAP kinase activation that is believed to be important for mitogenesis (Meloche *et al.*, 1992), indicating that this effect may be mediated by an additional surface receptor beside the cloned tethered-ligand thrombin receptor. Data presented in Chapter 3 demonstrated that in BPA fibroblasts, thrombin only evokes delayed kinetics for MAP kinase activation which is not imitated by TRAP-14, inferring that this cell type may serve as a good model to study signalling events coupled to the putative alternative thrombin-responsive receptor. However, activation of the signal transduction pathways described in these earlier studies required concentrations of thrombin which can be considered relatively high compared to those shown to elicit biochemical responses in other cell types. This raises the question of whether the cellular actions of thrombin observed in BPA fibroblasts under these conditions arise as a consequence of the protease acting on a surrogate receptor rather than a second thrombin receptor.

In the course of characterizing cellular signals induced by thrombin in BPA fibroblasts, a second protease-sensitive receptor, distinct from the thrombin receptor and designated proteinase-activated receptor-2 (PAR-2) was cloned from a mouse genomic library (Nystedt *et al.*, 1994). A highly homologous human counterpart of the mouse PAR-2 receptor receptor has now also been identified (Nystedt *et al.*, 1995a, Böhm *et al.*, 1996). The discovery of PAR-2 has led to the proposal that the thrombin receptor may serve as a prototype for a large family of receptors activated by other proteases (for reviews see Coughlin, 1994, Hollenburg, 1996). Like the thrombin receptor, PAR-2 belongs to the superfamily of receptors possessing seven transmembrane spanning domains and coupled to heterotrimeric G proteins and is believed to be activated by the same unique mechanism requiring proteolytic cleavage of an extracellular N-terminal exodomain to reveal a new N-terminus containing a receptor-activating sequence which serves as a "tethered ligand", interacting with other as yet undefined sites of the receptor. Although the endogenous activator of PAR-2 *in vivo* has not been established, this receptor is activated by trypsin at nanomolar

concentrations in a relatively selective manner. In addition, the synthetic agonist peptides SLIGRL and SLIGKV, representing the first 6 amino acids adjacent to the putative trypsin cleavage site of the murine and human PAR-2, respectively, have been shown to mimic the actions of trypsin (Nystedt *et al.*, 1994, 1995b). The sites of expression and biological actions of PAR-2 are relatively poorly characterized compared to the thrombin receptor. However, PAR-2 mRNA has been detected in the kidney, intestine, stomach and eye (Nystedt *et al.*, 1994a, Böhm *et al.*, 1996), although this apparent tissue specificity may partially reflect the highly vascularized nature of these organs as PAR-2 has also been shown to be expressed in rat aortic tissue (Al-Ani *et al.*, 1995) and endothelial cells (Mirza *et al.*, 1996) and is speculated to play a role in regulating blood vessel tone (Al-Ani *et al.*, 1995, Hwa *et al.*, 1996, Saifeddine *et al.*, 1996). PAR-2 may not be confined to the circulatory system, as transcripts for the receptor have been detected in human keratinocytes, rat intestinal epithelial cells and pancreatic acini where roles in wound healing (Santulli *et al.*, 1995), small intestine function and pancreatic exocrine secretion (Böhm *et al.*, 1996) have been implicated. While data is accumulating regarding the functional roles of this novel receptor, the intracellular consequences of PAR-2 activation are not well characterized and at present limited to studies demonstrating intracellular calcium mobilization and inositol phosphate formation in a number of cell types (Nystedt *et al.*, 1995b, Santulli *et al.*, 1995, Böhm *et al.*, 1996, Mari *et al.*, 1996).

In order to evaluate whether the unusual effects seen with high concentrations of thrombin in BPA fibroblasts could be attributed to cross-reactivity with PAR-2, the following series of experiments set out to determine whether PAR-2 is expressed by BPA fibroblasts, and whether these receptors are functional in these cells by characterizing the activation of the MAP kinase signalling pathway and mitogenic response evoked by trypsin and the PAR-2-activating peptide.

6.2 Bovine pulmonary arterial fibroblasts respond to trypsin by a PAR-2-independent mechanism

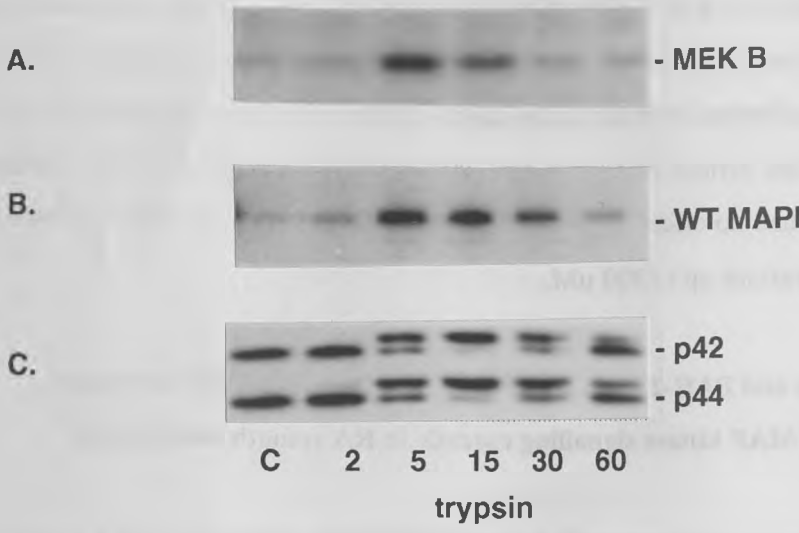
Exposure of BPA fibroblasts to trypsin caused a strong activation of p42 and p44 MAP kinases in a time and concentration -dependent manner as assessed by *in vitro* kinase activity and by retardation of MAP kinase electrophoretic mobility indicative of enzyme phosphorylation. In response to a saturating concentration of trypsin (30 nM), kinase activity

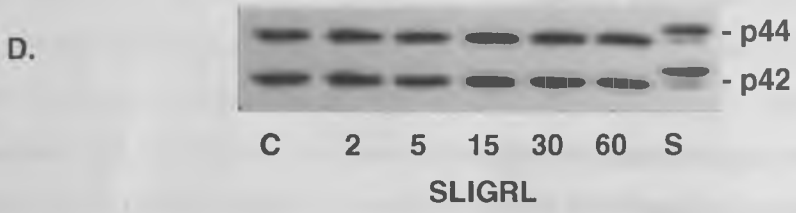
rose rapidly after 2 minutes, peaked at 15 minutes (4.5 fold stimulation) and was sustained up to 1 hour (Fig. 6.1C & E). This effect was accompanied by transient activation of c-Raf-1 (Fig. 6.1A) and MEK-1/2 (Fig. 6.1B) which were both maximal at 5 minutes and returned to unstimulated levels by 30 and 60 minutes, respectively. To investigate whether these effects were mediated by activation of PAR-2, the ability of the PAR-2-activating peptide sequence SLIGRL to mimic the actions of trypsin was investigated. Exposure of BPA fibroblasts to SLIGRL, however, had no effect on MAP kinase (Fig. 6.1D & E) or MEK-1/2 (not shown) activities at concentrations up to 300 μM.

6.3.1 Trypsin and PAR-2-derived-activating peptide SLIGRL activation of the MAP kinase signalling cascade in RA smooth muscle cells

Other cell types were screened for their sensitivity to trypsin to find a suitable model for investigating PAR-2-dependent signalling events and to check that SLIGRL was effective. Trypsin was found to also activate p42/p44 MAP kinase in rat aortic (RA) smooth muscle cells. In this cell type, stimulation of kinase activity was evident as early as 2 minutes, maximal at 5 minutes (approximately 4 fold) and returned to basal levels by 30 minutes (Fig. 6.2.1B & C). Trypsin-stimulated MAP kinase activity was abrogated when the protease was treated with 1 mg/ml soybean trypsin inhibitor (SBTI) for 5 minutes before application to cells (Table 6.1). Inhibition of MAP kinase activation was also reflected in the prevention by SBTI of trypsin-induced MAP kinase mobility shifts on SDS-PAGE (not shown). Trypsin also stimulated time-dependent activation of the MAP kinase activator MEK-1/2 (Fig. 6.2.1A & C). Activation of MEK-1/2 by the protease preceded MAP kinase activation and was more transient, returning to basal levels between 10-15 minutes. MEK-1/2 activation, in turn, was preceded by the activation of the canonical MEK-1/2 activator c-Raf-1 (not shown).

In contrast to BPA fibroblasts, exogenous application of SLIGRL also stimulated p42 and p44 MAP kinase (Fig. 6.2.2B & C), MEK-1/2 (Fig. 6.2.2A & C) and c-Raf-1 (not shown) activities in RA smooth muscle cells with time courses which closely resembled activation by trypsin. To check that activation of MAP kinases was not a non-specific effect of the peptide, the analogue LSIGRL, in which the first two residues were exchanged, was tested as a control peptide. At a concentration and exposure time (300 μM at 5 minutes) found to evoke a maximum response to SLIGRL, the effect LSIGRL on MAP kinase activity was negligible (Table 6.1).


A.
- MEK B
B.
- WT MAPK
C.
- p42
- p44
C 2 5 15 30 60
trypsin


D.
- p44
- p42
C 2 5 15 30 60 S
SLIGRL

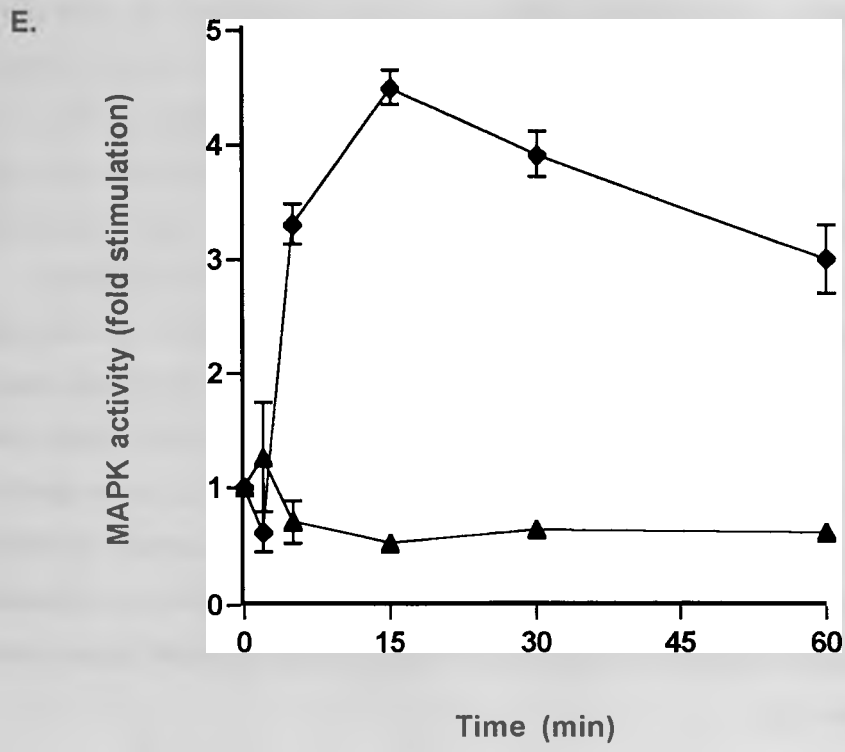
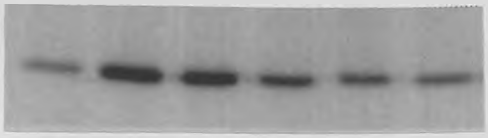

E.
MAPK activity (fold stimulation)
0
1
2
3
4
5
0
15
30
45
60
Time (min)

Figure 6.1 Effect of trypsin on MAP kinase, MEK-1/2 and c-Raf-1 activities in BPA fibroblasts. Cells were stimulated with 30 nM trypsin (◆) or 300 μM SLIGRL (▲) for the times indicated indicated, FCS (10 %) for 10 minutes (S) or with vehicle for 15 minutes (C). Lysates were analysed for c-Raf-1 (**A**), MEK-1/2 (**B**) and MAP kinase (**E**) kinase activities by the incorporation of [^{32}P]phosphate into kinase-inactive MEK-1 (MEK-B), wild type MAP kinase (WT MAPK) and EGFR$^{661-680}$ as substrates, respectively, or resolved by SDS-PAGE and immunoblotted for MAP kinase (**C** & **D**) as outlined in Sections 2.4.1, 2.4.2 & 2.4.3. The autoradiographs and immunoblot shown are each representative of three separate experiments. Kinase activity is expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into peptide substrate for each time-course representative of three separate experiments (control: 44.2±15.8 pmol Pi min^{-1} mg^{-1} protein).

A.



C 2 5 10 15 30

B.

C 2 5 15 30 60

C.

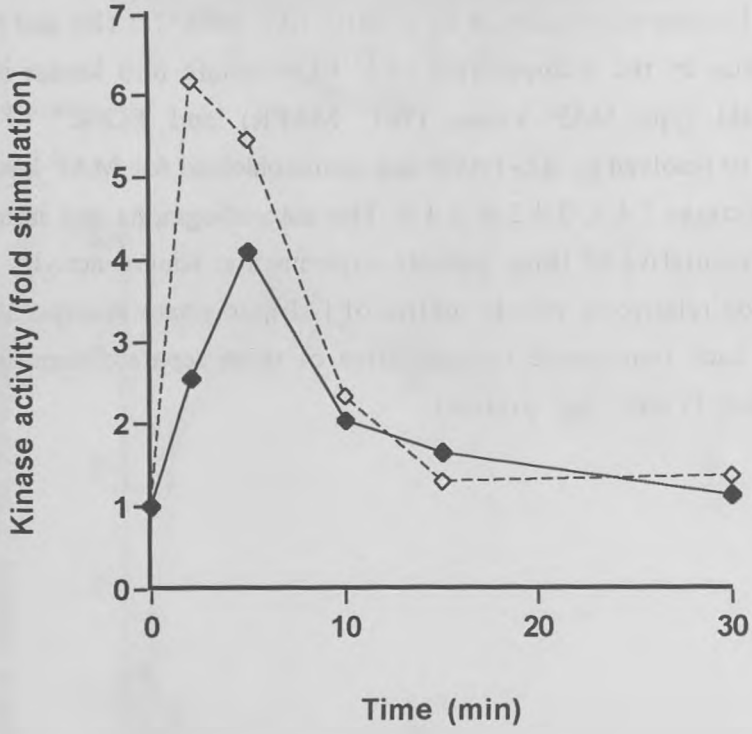
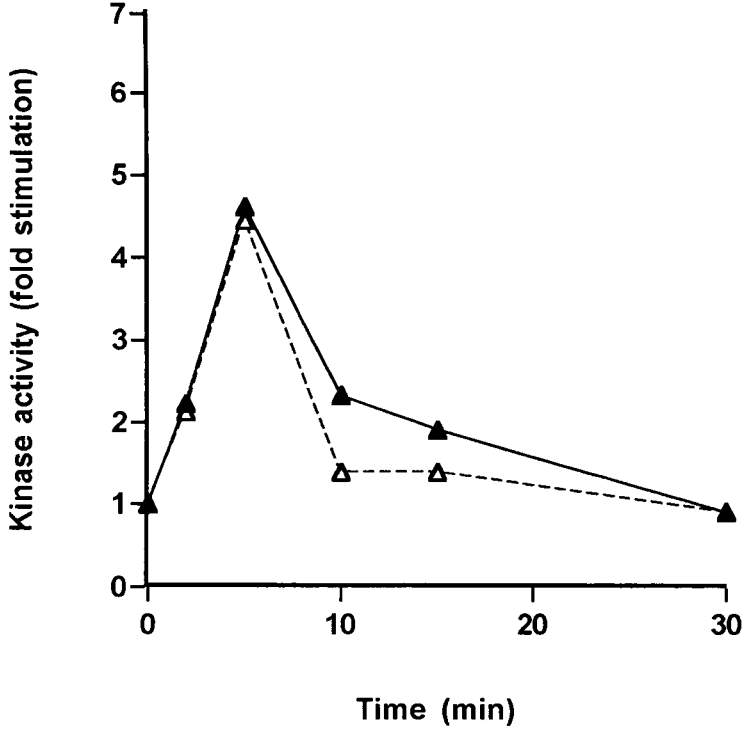


Figure 6.2.1 MAP kinase and MEK-1/2 activation by trypsin in RA smooth muscle cells. Cells were exposed to trypsin (30 nM) for the indicated times in minutes or with vehicle for 5 minutes (C). Lysates were assayed for *in vitro* MAP kinase activity assessed by incorporation of [^{32}P]phosphate into EGFR$^{661-680}$ or resolved by SDS-PAGE and immunoblotted for MAP kinase. Immunoprecipitates of MEK-1/2 were prepared from cell lysates and assayed for *in vitro* MEK activity measured by a coupled kinase assay using recombinant wild type MAP kinase (WT MAPK) and EGFR$^{661-680}$ as sequential substrates as outlined in Section 2.4.2. **A)** autoradiograph showing [^{32}P]phosphorylation of WT MAPK by MEK-1/2; **B)** immunoblot showing electrophoretic shifts associated with the 42 and 44 kDa MAP kinase isoforms; **C)** MAP kinase (◆) and MEK-1/2 activities (◇) expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one time-course representative of three separate experiments (control: 36.2 pmol Pi min^{-1} mg^{-1} protein (MAPK); 0.48 pmol Pi min^{-1} mg^{-1} protein (MEK-1/2).

C.


Kinase activity (fold stimulation)
0
1
2
3
4
5
6
7
0
10
20
30
Time (min)

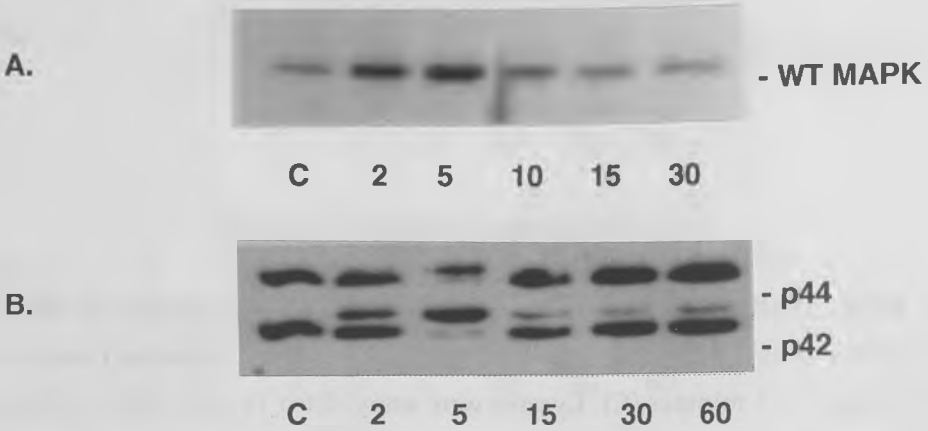

A.
- WT MAPK
C 2 5 10 15 30
B.
- p44
- p42
C 2 5 15 30 60

Figure 6.2.2 MAP kinase and MEK-1/2 activation by PAR-2-activating peptide in RA smooth muscle cells. Cells were exposed to SLIGRL (300 μM) for the indicated times in minutes or with vehicle for 5 minutes (C). Lysates were assayed for *in vitro* MAP kinase activity assessed by incorporation of [^{32}P]phosphate into EGFR$^{661-680}$ or resolved by SDS-PAGE and immunoblotted for MAP kinase. Immunoprecipitates of MEK-1/2 were prepared from cell lysates and *in vitro* MEK activity measured by a coupled kinase assay using recombinant wild type MAP kinase (WT MAPK) and EGFR$^{661-680}$ as sequential substrates as outlined in Section 2.4.2. **A)** autoradiograph showing [^{32}P]phosphorylation of WT MAPK by MEK-1/2; **B)** immunoblot showing electrophoretic shifts associated with the 42 and 44 kDa MAP kinase isoforms; **C)** MAP kinase (▲) and MEK-1/2 activities (Δ) expressed as fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from one time-course representative of three separate experiments (control: 36.2 pmol Pi min^{-1} mg^{-1} protein (MAPK); 0.48 pmol Pi min^{-1} mg^{-1} protein (MEK-1/2).

Treatment	MAPK activity mean±s.e.m. fold stimulation
C	1.0
SBTI	1.02±0.01
T	7.00±0.57
T +SBTI	0.94±0.02
SLIGRL	5.17±0.58
LSIGRL	1.13±0.01

Table 6.1 Agonist-stimulated MAP kinase activity in RA smooth muscle cells. Cells were treated with 30 nM trypsin (T), 300 μM SLIGRL, 300 μM LSIGRL or vehicle (C) for 5 minutes. In some instances, trypsin or vehicle was incubated for 5 minutes at room temperature with 1 mg/ml soybean trypsin inhibitor (SBTI) prior to application to cells. Cell lysates were assayed for *in vitro* MAP kinase activity as outlined in Section 2.4.1. Activity is expressed as mean±s.e.m. fold stimulation relative to vehicle control of [^{32}P]phosphate incorporated into EGFR$^{661-680}$ from three separate experiments. (C: 36.9±7.4 pmol Pi min^{-1} mg^{-1} protein).

Both trypsin and SLIGRL responses were also dependent on concentration. While maximal MAP kinase activities evoked by each agonist were comparable (Fig. 6.2.1C & 6.2.2C), the concentration of peptide required for this was considerably greater than that for the protease (Fig. 6.2.3), as has been reported previously in other cell systems (Nystedt *et al.*, 1994, Santulli *et al.*, 1995). Peak MAP kinase activation in response to trypsin occurred at 30 nM while the concentration causing an half-maximal response was 12.1±3.4 nM and comparable to the EC_{50} for trypsin-stimulated Ca^{2+} mobilization in lung adenocarcinoma cells (Böhm *et al.*, 1996) and inositol phosphate formation in keratinocytes (Santulli *et al.*, 1995). Peptide-evoked MAP kinase activation, in contrast, was maximal at 300 μM with an EC_{50} of approximately 62.5±4.5 μM μM.

6.3.2 Effect of PMA pretreatment and forskolin on agonist-stimulated activation of the MAP kinase signalling pathway in RA smooth muscle cells

To further investigate whether activation of MAP kinase, MEK and Raf-1 by trypsin and SLIGRL in RA smooth muscle cells resulted from signalling via a *bona fide* cascade conserved for other receptor-mediated systems, and not a consequence of a non-specific action, the effect on trypsin and SLIGRL-stimulated kinase activities of PKC downregulation by PMA pretreatment and an elevation in intracellular cAMP by forskolin was examined. Both of these interventions have been reported to inhibit activation of MAP kinase in response to various agonists in a number of cell types at the level of c-Raf-1 (Burgering *et al.*, 1993b, Cook & McCormick, 1993, Wu *et al.*, 1993a). *In vitro* phosphorylation of recombinant MEK by c-Raf-1 and of MAP kinase by MEK-1/2 immunopurified from RA smooth muscle cells following exposure to trypsin or PAR-2-derived peptide, was markedly reduced when cells were pretreated for 24 h with 100 nM PMA (Fig. 6.3A), which has previously been shown in this laboratory to downregulate protein kinase C (PKC) α and ε isoforms in RA smooth muscle cells (Malarkey *et al.*, 1996). This correlated with approximately 95% inhibition by PMA pretreatment of MEK activity evoked by either agonist. A similar effect was observed after prior treatment of RA smooth muscle cells with 10 μM forskolin (Fig. 6.3B).

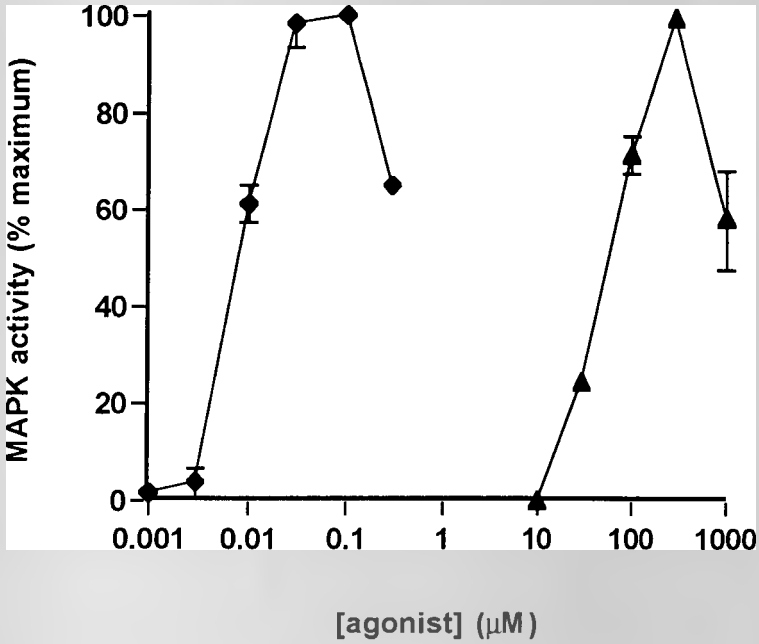


Figure 6.2.3 Trypsin and PAR-2-activating peptide activation of MAP kinase in RA smooth muscle cells. Cells were stimulated with trypsin (◆) or SLIGRL (▲) for 5 minutes at the concentrations indicated and MAP kinase activity in lysates determined as outlined in Section 2.4.1. Results are expressed as mean±S.E.M percentage of maximum activity evoked by each agonist from three separate experiments. (maximum activity: 216.4±7.6 pmol Pi min^{-1} mg^{-1} protein (100 nM trypsin); 199.8±1.9 pmol Pi min^{-1} mg^{-1} protein (300 μM SLIGRL).

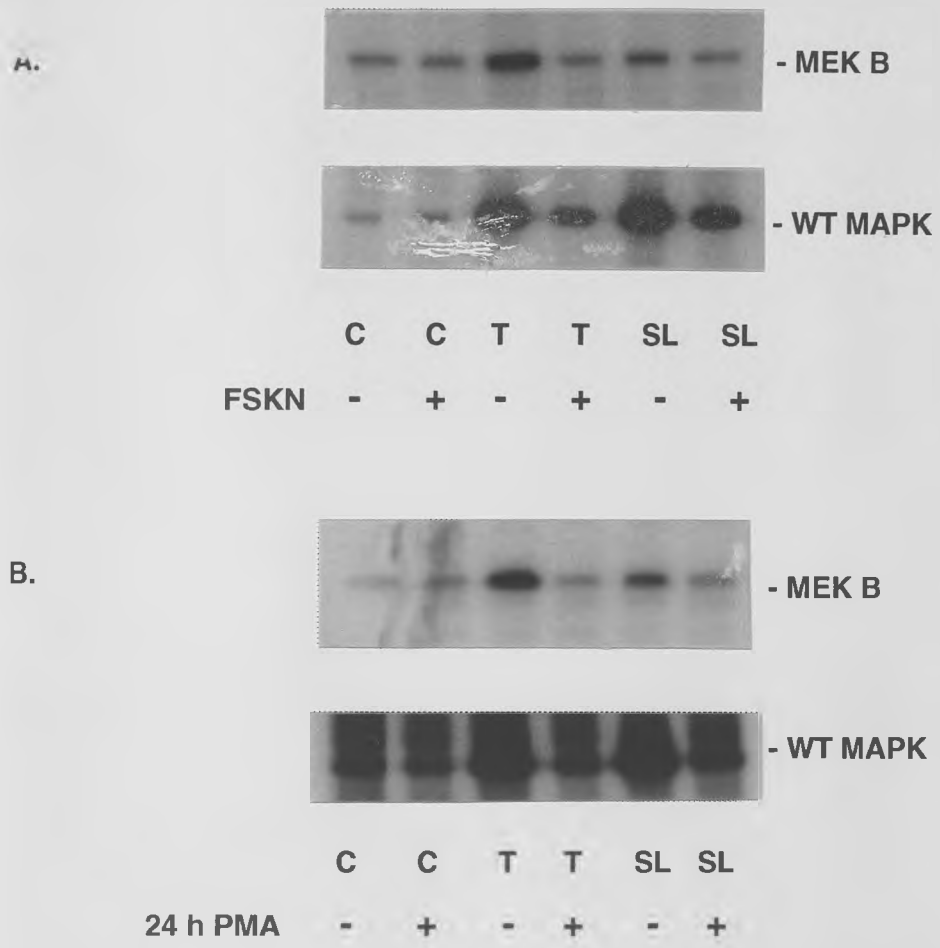


Figure 6.3 Effect of PMA pretreatment and forskolin on agonist-stimulated c-Raf-1 and MEK activity in RA smooth muscle cells. Cells were treated with vehicle (-) or either 10 μM forskolin (FSKN) for 30 minutes (**A**) or 100 nM PMA for 24 hours (**B**) (+) prior to exposure to 30 nM trypsin (T), 300 μM SLIGRL (SL) or vehicle (C) for 5 minutes. Lysates were analysed for c-Raf-1 (upper panel) and MEK-1/2 (lower panel) kinase activities by the incorporation of [^{32}P]phosphate into kinase-inactive MEK-1 (MEK-B) and wild type MAP kinase (WT MAPK) substrates, respectively, as outlined in Sections 2.4.2 & 2.4.3. Each of the autoradiographs depicted is representative of three separate experiments.

6.3.3 Effect of SLIGRL pretreatment on agonist-stimulated activation of MAP kinase in RA smooth muscle cells

Desensitization is the regulatory process by which receptors exhibit decreased responsiveness to further stimulation following prior exposure to an agonist. Since previous evidence has shown that the prototype PAR, the thrombin receptor, undergoes homologous desensitization when preactivated with either thrombin or its peptide ligand (Hoxie *et al.*, 1993, Hein *et al.*, 1994), a PAR-2 desensitization protocol was designed to investigate whether this receptor undergoes similar desensitization and if trypsin signalling events were mediated by activation of PAR-2. For the thrombin receptor, brief exposure to thrombin or agonist peptide renders the cell refractory to a second stimulus for 10-20 minutes after which a full recovery of peak cellular responses was evident at 45 minutes (Hoxie *et al.*, 1993, Hein *et al.*, 1994). As it takes at least 15 minutes to elapse before the transient MAP kinase signal in response to SLIGRL in RA smooth muscle cells to return to basal levels (Fig. 6.2.2C), a desensitizing stimulus of 20 minute exposure to 300 μM SLIGRL was chosen to check whether PAR-2 undergoes desensitization. Prior treatment of RA smooth muscle cells with SLIGRL prevented the subsequent activation of MEK and MAP kinase by a second exposure to the peptide (Fig. 6.4 & Table 6.2). Under these desensitization conditions, trypsin-stimulated kinase activities were attenuated by approximately 80 %. In contrast, over 85 % of the increase in MEK and MAP kinase activity evoked by thrombin was preserved after initial exposure to SLIGRL (Fig. 6.4 & Table 6.2).

6.4 Expression of PAR-2 mRNA in RA smooth muscle cells but not BPA fibroblasts

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was employed to investigate whether PAR-2 receptor mRNA was expressed in RA smooth muscle cells. RT-PCR analysis of total cellular RNA extracted from RA smooth muscle cells, using a primer pair specific to mouse PAR-2 (PF1/PR1) (Al-Ani *et al.*, 1995), yielded a single product of the predicted size (~526 bp) as assessed by agarose gel electrophoresis, which co-migrated with the PCR product derived from mouse PAR-2 cDNA (Fig. 6.5.1). Products of the same size were also detected in rat intestine and mouse intestine, a tissue that has been shown to highly express PAR-2 (Nystedt *et al.*, 1994). In control PCR experiments, no bands were detectable in the absence of cDNA or from reverse transcriptase reactions in which

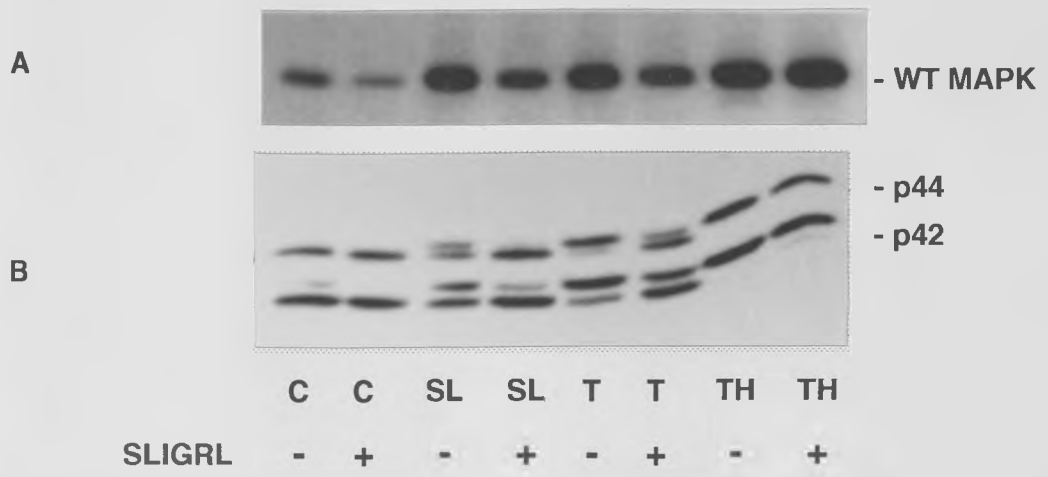


Figure 6.4 Desensitization of agonist-stimulated MAP kinase and MEK activation in RA smooth muscle cells by PAR-2-activating peptide pretreatment. Cells were treated with vehicle (-) or 300 μM SLIGRL (+) for 20 minutes prior to a second exposure to 300 μM SLIGRL (SL), trypsin 30 nM (T) or 30 nM thrombin (TH) for 5 minutes. Cell lysates were either assayed for MEK phosphotransferase activity by the incorporation of [^{32}P]phosphate into wild type MAP kinase (WT MAPK) (**A**) or resolved by SDS-PAGE and immunoblotted for MAP kinase (**B**) as described in Sections 2.3 & 2.4.1. Both the autoradiograph and the immunoblot are representative of three separate experiments.

Treatment	Agonist	% Desensitization MEK activity	% Desensitization MAPK activity
SLIGRL	SLIGRL	81.8±10.5	98.5±1.5
SLIGRL	trypsin	71.6±12.7	78.9±15.1
SLIGRL	thrombin	12.9±3.6	16.6±12.1

Table 6.2 Desensitization of agonist-stimulated MAP kinase and MEK activation in RA smooth muscle cells by PAR-2-activating peptide pretreatment. Cells were treated with vehicle or 300 μM SLIGRL for 20 minutes prior to a second exposure to SLIGRL (300 μM), trypsin (30 nM), thrombin (30 nM) or vehicle for 5 minutes. MAP kinase and MEK phosphotransferase activities were assayed as described in Section 2.4.1 & 2.4.2. Desensitization was determined by expressing agonist-evoked increases in kinase activity following SLIGRL pretreatment as percentage inhibition of kinase stimulation by the same agonist in cells pretreated with vehicle, and is presented as mean±s.e.m. from four separate experiments (MAP kinase activities: 181.3±25.1, 217.2±9.7 and 303.6±87.8 pmol Pi min^{-1} mg^{-1} protein for SLIGRL, trypsin and thrombin, respectively).

reverse transcriptase was omitted from the reaction mixture. To exclude the possibility that the RT-PCR signals were derived from amplification of contaminating genomic DNA, primer pairs PF2/PR2 encompassing a 710 bp region of mouse PAR-2 and spanning a 310 bp intron sequence were used. Using this primer pair, PCR analysis of the RT-product derived from RA smooth muscle cell RNA yielded a fragment of approximately 400 bp in length (Fig. 6.5.1) confirming that this signal was amplified from cDNA derived from an intronless mRNA sequence. In contrast to RA smooth muscle cells, no detectable signal was generated by RT-PCR when RNA extracted from BPA fibroblasts was probed with either primer pair PF1/PR1 or PF2/PR2 (Fig. 6.5.1). In concurrent analysis, however, the detection of a signal of the expected size (~243 bp) yielded by the actin primer pair AF1/AR1 from BPA fibroblasts, that was equally in strength to that from RA smooth muscle cells, confirmed that the failure to detect PAR-2 in BPA fibroblasts was not due to poor RNA yield.

Both products of PCR amplification using primer pairs PF1/PR1 and PF2/PR2 from RA smooth muscle were subjected to partial DNA sequencing performed in both the 5'- and 3'-directions. The order of nucleotides in 347 and 435 bp sequences within these fragments were found to show 89 % and 91 % identity, respectively, with the published sequence for the cloned mouse PAR-2 (Nystedt *et al.*, 1995b) and 90 % and 94 % homology, respectively, with the translated amino acid sequence (Fig. 6.5.2). Furthermore, the sequence encoding the putative receptor-activating amino acid sequence SLIGRL within the extracellular N-terminus was conserved.

6.5 Effect of trypsin and SLIGRL on DNA synthesis in RA smooth muscle cells and BPA fibroblasts

To investigate whether trypsin-responsive receptors were transducers of growth promoting signals as has been reported for the thrombin receptor (Hung *et al.*, 1992a, Hartmann *et al.*, 1994), the ability of trypsin and SLIGRL to stimulate [^{3}H]thymidine incorporation in both RA smooth muscle cells and BPA fibroblasts was investigated. Treatment of quiescent BPA fibroblasts with trypsin (30 nM) for 24 hours caused approximately 15 fold increase in [^{3}H]thymidine incorporation while SLIGRL, as with the kinase data, was ineffective (Table 6.3). This effect was completely blocked when the protease was treated with 1 mg/ml soybean trypsin inhibitor for 5 minutes prior to addition to cells. In RA smooth muscle cells, trypsin (30 nM) or SLIGRL (300 μM) stimulated much smaller

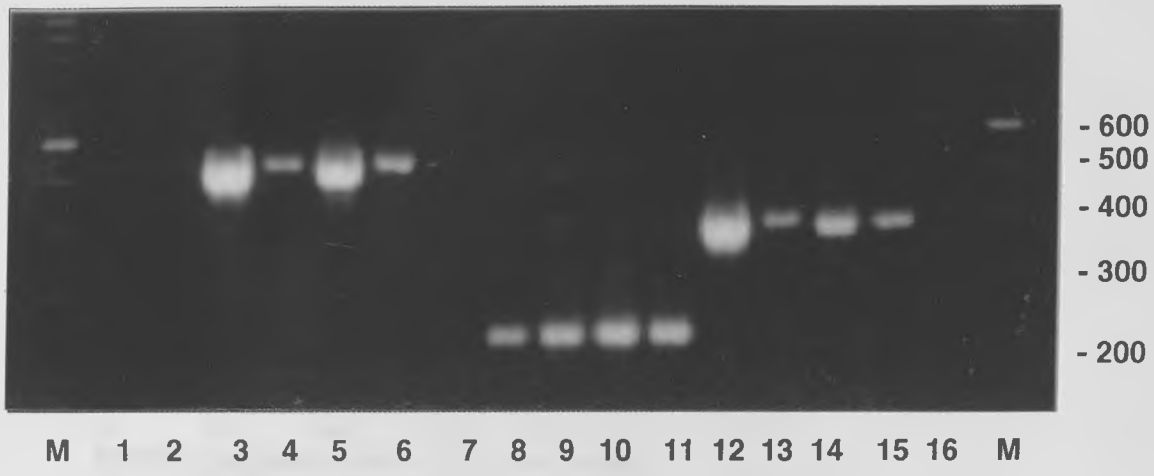
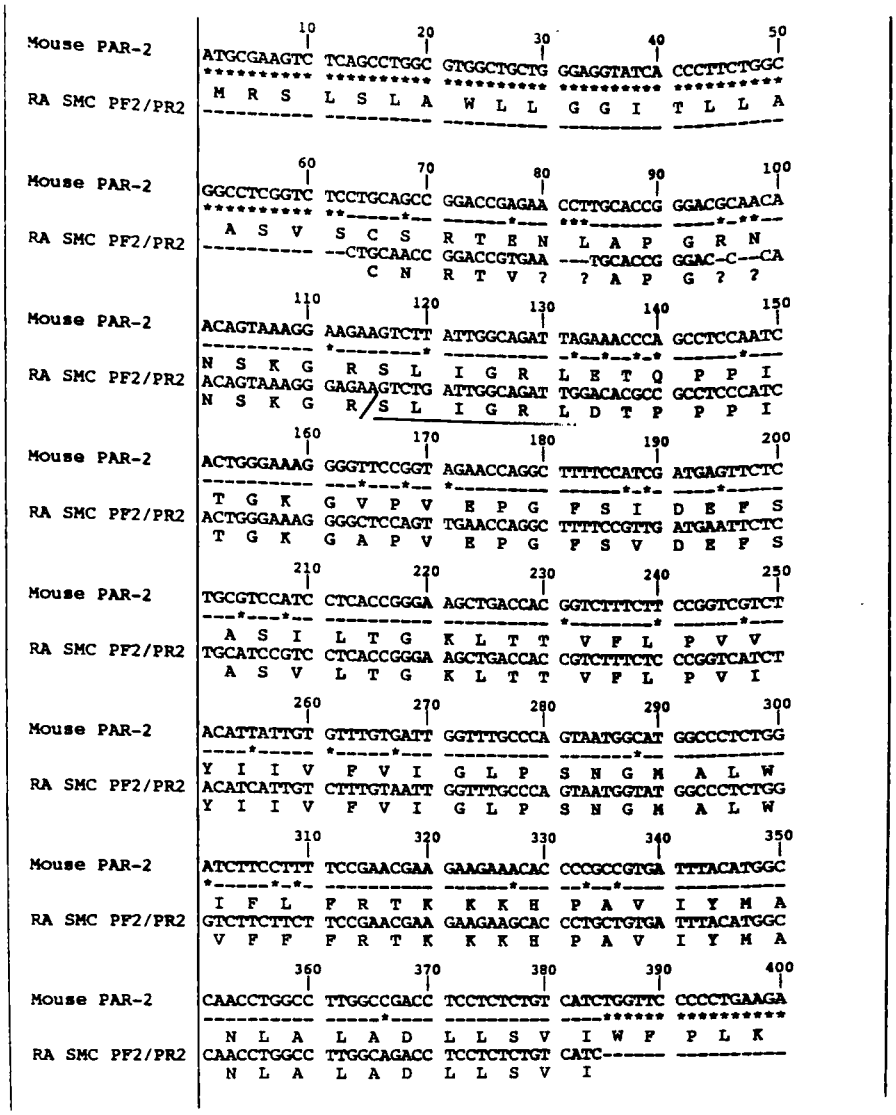


Figure 6.5.1 Detection of PAR-2 mRNA in RA smooth muscle cells by RT-PCR. Total RNA extracted from cells and tissues was analysed by RT-PCR using PAR-2 primer pair PF1/PR1 (lanes 1-7), actin primer pair AF1/AR1 (lanes 8-11) and PAR-2 primer pair PF2/PR2 (lanes 12-16) as outlined in Section 2.6. Products of PCR amplification were separated by electrophoresis through a 2 % agarose gel containing ethidium bromide and detected under UV illumination. Lane 1, PCR control (no cDNA); lane 2, RT control (no reverse transcriptase); lanes 3 & 12, mouse PAR-2 cDNA; lanes 4, 8 & 13, mouse intestine; lanes 5, 9, & 14, rat intestine; lanes 6, 10 & 15, RA smooth muscle cells and lanes 7, 11 & 16, BPA fibroblasts. DNA size markers were run on the outside lanes (M) and the positions of bands corresponding to 200, 300, 400, 500 and 600 bp indicated on the right. Figure depicts PCR products representative of at least three separate PCR reactions performed on three different RNA samples from each tissue or cell type.

A.


Mouse PAR-2
RA SMC PF2/PR2
ATGCGAAGTC TCAGCCTGGC GTGGCTGCTG GGAGGTATCA CCCTTCTGGC
M R S L S L A W L L G G I T L L A
GGCCTCGGTC TCCTGCAGCC GGACCGAGAA CCTTGCACCG GGACGCAACA
A S V S C S R T E N L A P G R N
ACAGTAAAGG AAGAAGTCTT ATTGGCAGAT TAGAAACCCA GCCTCCAATC
N S K G R S L I G R L E T Q P P I
ACAGTAAAGG GAGAAGTCTG ATTGGCAGAT TGGACACGCC GCCTCCCATC
N S K G R S L I G R L D T P P P I
ACTGGGAAAG GGGTTCCGGT AGAACCAGGC TTTTCCATCG ATGAGTTCTC
T G K G V P V E P G F S I D E F S
ACTGGGAAAG GGGCTCCAGT TGAACCAGGC TTTTCCGTTG ATGAATTCTC
T G K G A P V E P G F S V D E F S
TGCGTCCATC CTCACCGGGA AGCTGACCAC GGTCTTTCTT CCGGTCGTCT
A S I L T G K L T T V F L P V V
TGCATCCGTC CTCACCGGGA AGCTGACCAC CGTCTTTCTC CCGGTCATCT
A S V L T G K L T T V F L P V I
ACATTATTGT GTTTGTGATT GGTTTGCCCA GTAATGGCAT GGCCCTCTGG
Y I I V F V I G L P S N G M A L W
ACATCATTGT CTTTGTAATT GGTTTGCCCA GTAATGGTAT GGCCCTCTGG
Y I I V F V I G L P S N G M A L W
ATCTTCCTTT TCCGAACGAA GAAGAAACAC CCCGCCGTGA TTTACATGGC
I F L F R T K K K H P A V I Y M A
GTCTTCTTCT TCCGAACGAA GAAGAAGCAC CCTGCTGTGA TTTACATGGC
V F F F R T K K K H P A V I Y M A
CAACCTGGCC TTGGCCGACC TCCTCTCTGT CATCTGGTTC CCCCTGAAGA
N L A L A D L L S V I W F P L K
CAACCTGGCC TTGGCAGACC TCCTCTCTGT CATC------
N L A L A D L L S V I

B.

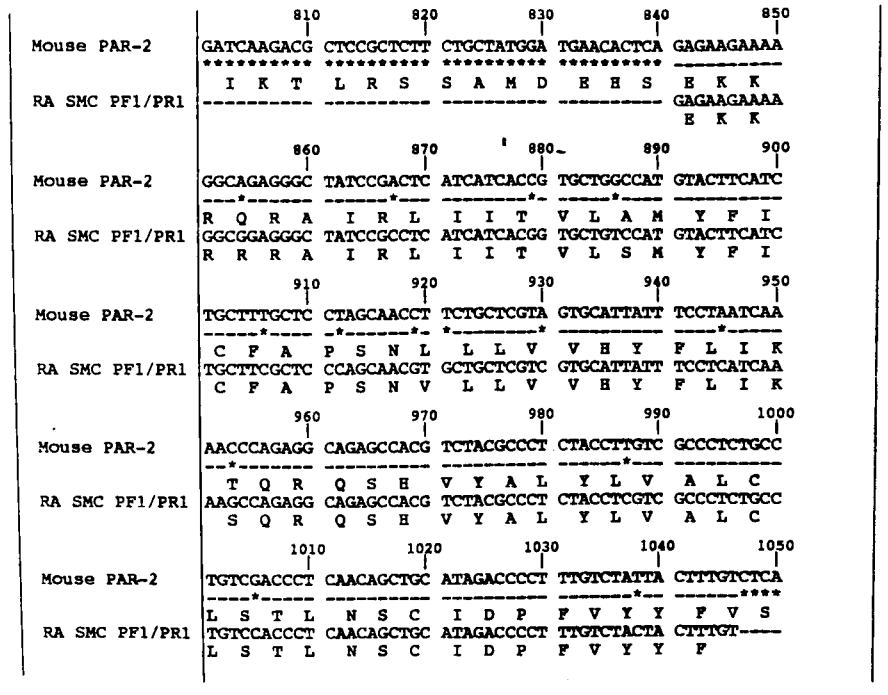

Mouse PAR-2
RA SMC PF1/PR1
GATCAAGACG CTCCGCTCTT CTGCTATGGA TGAACACTCA GAGAAGAAAA
I K T L R S S A M D E H S E K K
GAGAAGAAAA
E K K
GGCAGAGGGC TATCCGACTC ATCATCACCG TGCTGGCCAT GTACTTCATC
R Q R A I R L I I T V L A M Y F I
GGCGGAGGGC TATCCGCCTC ATCATCACGG TGCTGTCCAT GTACTTCATC
R R R A I R L I I T V L S M Y F I
TGCTTTGCTC CTAGCAACCT TCTGCTCGTA GTGCATTATT TCCTAATCAA
C F A P S N L L L V V H Y F L I K
TGCTTCGCTC CCAGCAACGT GCTGCTCGTC GTGCATTATT TCCTCATCAA
C F A P S N V L L V V H Y F L I K
AACCCAGAGG CAGAGCCACG TCTACGCCCT CTACCTTGTC GCCCTCTGCC
T Q R Q S H V Y A L Y L V A L C
AAGCCAGAGG CAGAGCCACG TCTACGCCCT CTACCTCGTC GCCCTCTGCC
S Q R Q S H V Y A L Y L V A L C
TGTCGACCCT CAACAGCTGC ATAGACCCCT TTGTCTATTA CTTTGTCTCA
L S T L N S C I D P F V Y Y F V S
TGTCCACCCT CAACAGCTGC ATAGACCCCT TTGTCTACTA CTTTGT-----
L S T L N S C I D P F V Y Y F

Figure 6.5.2 Comparison of oligonucleotide sequence of PCR products from RA smooth muscle cells with mouse PAR-2 sequence. Both strands of PCR products from RA smooth muscle cells were subjected to chain terminator cycle sequencing using fluorescent dye-labeled dideoxynucleotides and the order of nucleotides determined by an Applied Biosystem 373A DNA sequencer as described in Section 2.6.7. The partial oligonucleotide cDNA sequence from PCR products (bottom lane) using primer pairs PF2/PR2 **(A)** and PF1/PR1 **(B)** were compared with the mouse PAR-2 sequence obtained from GenBank™ accession number Z48043 (Nystedt *et al.*, 1995b)(top lane). Differences in nucleotides are indicated by *. The translated amino acid sequence is shown below the nucleotide sequence, amino acids represented by their single letter codes. Residues absent in the translated rat receptor sequence are indicated by ?. The region encoding the putative receptor-activating peptide sequence SLIGRL is underlined and the the putative site of trypsin cleavage shown by /. The numbering for nucleotides is shown above the mouse PAR-2 nucleotide sequence.

increases (3 fold) in [^{3}H]thymidine incorporation during the final 4 hours of agonist exposure (Table 6.3). The efficacy of trypsin as a mitogen for either cell type was also compared by normalizing the trypsin-evoked response against the extent of [^{3}H]thymidine incorporation stimulated by serum. Whereas trypsin was found to be over half as effective as 10 % FCS in BPA fibroblasts, the protease stimulated DNA synthesis to only approximately one tenth of the level observed in the presence of serum in RA smooth muscle cells.

6.6 DISCUSSION

6.6.1 PAR-2-independent activation of MAP kinase by trypsin in BPA fibroblasts

The purpose of this study was primarily to investigate activation of the MAP kinase signalling system in BPA fibroblasts via PAR-2 to determine whether this novel protease-sensitive receptor could account for the unusual features of the signalling events evoked by high concentrations of thrombin in this cell type. The serine protease trypsin has been identified as a relatively selective activator of PAR-2 at nanomolar concentrations and was used initially to screen BPA fibroblasts for possible PAR-2-dependent responses. Trypsin was found to stimulate activation of the Raf-1/MEK-1/2/MAP kinase signalling cascade in BPA fibroblasts within the concentration range reported to activate PAR-2 specifically (Nystedt *et al.*, 1994). To define the role of PAR-2 in the actions of trypsin, the hexapeptide sequence SLIGRL derived from the first six amino acids residues constituting the new amino terminus after the proposed cleavage of the cloned PAR-2 exodomain was employed. SLIGRL was unable to reproduce the effects of trypsin on MAP kinase activity in BPA fibroblasts indicating that the cellular responses to the protease in this cell type are unlikely to be mediated by PAR-2. It is conceivable that like TRAP-14 (see Section 3.8), the inability of the PAR-2 -derived peptide to be effective in BPA fibroblasts could reflect a species difference in the receptor-activating sequence for an equivalent bovine PAR-2 receptor. This is unlikely since it has been demonstrated that even in diverse species such as mouse and humans, where the putative regions of the cloned PAR-2 thought to be responsible for receptor activation have been identified and show differences (SLIGRL for murine and SLIGKV for human PAR-2 homologues), there is still a high degree of cross-species responsiveness of receptors to either synthetic peptide ligand (Blackheart *et al.*, 1996). Strong evidence to further negate a role for PAR-2 in the effects of trypsin in BPA fibroblasts was the inability to detect by RT-

Treatment	[^{3}H]thymidine incorporation mean±s.e.m. dpm	
	RA smooth muscle cells	BPA fibroblasts
C	213±14	437±25
FCS	3191±125	11360±540
trypsin	579±2	6388±125
SLIGRL	624±13	430±23
SBTI	N/D	581±73
trypsin +SBTI	N/D	514±28

Table 6.3 Agonist-stimulated [^{3}H]thymidine incorporation in RA smooth muscle cells and BPA fibroblasts. RA smooth muscle cells or BPA fibroblasts deprived of serum for 48 hours were exposed for 24 hours to trypsin (30 nM), SLIGRL (300 μM), FCS (10%) or vehicle (C). In some instances, trypsin or vehicle was incubated for 5 minutes at room temperature with 1 mg/ml soybean trypsin inhibitor (SBTI) prior to application to cells. Cells were incubated with 0.1 μCi/ml [^{3}H]thymidine for the remaining 4 hours of agonist treatment. After 24 hours, incorporation of [^{3}H]thymidine into DNA replicating cells was determined as described in Section 2.5. Data is expressed as mean±s.e.m. dpm for 3 independent experiments each performed in triplicate. (N/D, not determined).

PCR the expression of PAR-2-specific transcripts in BPA fibroblast RNA, as has been reported in other fibroblast types (Santulli *et al.*, 1995). This discovery indicated that PAR-2 cannot be responsible for mediating signalling events by thrombin in BPA fibroblasts, even at high concentrations. Furthermore, the effects of trypsin are also unlikely to be mediated by the same receptor activated by thrombin in this cell type since trypsin activates MAP kinase rapidly while it was evident in Section 3.2 that thrombin exhibits slower kinetics for kinase activation. Evidence for different receptors with distinct coupling mechanisms also comes from the finding that trypsin, unlike thrombin (see Section 5.3.1), was unable to activate p38 (not shown). Taken together, this evidence suggests that in BPA fibroblasts, trypsin does not appear to act via either of the two proteolytically-activated receptors described to date, raising the possibility that other cell-surface proteins present in this cell type may be substrates for this and other proteases. These targets could be novel members of the tethered-ligand receptor family with distinct peptide receptor-activating sequences. Although a previous publication has reported that trypsin can activate the insulin receptor by proteolytic truncation of the α-subunit releasing the β-subunit from inhibitory control, this effect was only evident at micromolar concentrations (Shoelson *et al.*, 1988). The actions of trypsin in BPA fibroblasts are unlikely to be insulin receptor-dependent since in additional experiments that were conducted in this cell type, it was not possible to detect by SDS-PAGE and immunoblotting the tyrosine phosphorylation of a protein migrating at a molecular weight corresponding to insulin receptor substrate-1 (IRS-1) on trypsin exposure, or comparable activation of MAP kinase in response to insulin (not shown).

6.6.2 PAR-2-dependent activation of MAP kinase by trypsin in RA smooth muscle cells

Despite PAR-2 not being involved in the actions of trypsin or thrombin in BPA fibroblast, it was of interest to find a cell type in which PAR-2 was present to characterize signalling events mediated by this novel receptor. The prediction that RA smooth muscle cells would serve as an appropriate model for studying PAR-2-mediated responses was made on the basis of the following previous observations: 1) Stimulation of the prototype PAR by thrombin activates MAP kinase in RA smooth muscle cells (Malarkey *et al.*, 1996) 2) Trypsin causes calcium mobilization in this cell type (Kable *et al.*, 1995) and 3) PAR-2 mRNA is highly expressed in denuded rat aorta (Saifeddine *et al.*, 1996). The ability of trypsin to

activate MAP kinase in BPA fibroblasts was reproduced in RA smooth muscle cells. In this case, however, SLIGRL closely mimicked the effect of trypsin, consistent with the possibility that functional PAR-2 may mediate the cellular effects of the protease in this cell type. The specificity of the response to this peptide was exemplified by the demonstration that the scrambled control LSIGRL was functionally inactive, substantiating the findings of another report showing that the serine residue in position 1 of the analogue is critically important for PAR-2 agonist activity (Blackheart *et al.*, 1996). The possible role of PAR-2 in trypsin and peptide -evoked signalling events in RA smooth muscle cells was further supported by the detection in this cell type of a RT-PCR product amplified from a PAR-2 -specific mRNA transcript. The cDNA sequence of this product revealed considerable sequence identity with the published sequence of the cloned murine receptor (Nystedt *et al.*, 1995b). Significantly, the deduced amino acid sequence of the putative tethered PAR-2-activating sequence in the rat was found to be identical to the mouse receptor, justifying the use of SLIGRL as a peptide mimetic of protease-mediated activation of PAR-2. Since this work was completed, the sequence of a cDNA clone isolated from a neonatal rat intestinal cDNA library λZAPII and identified as the rat homologue of the PAR-2 has been published by other authors (Saifeddine *et al.*, 1996). The PCR products derived from RA smooth muscle RNA in this study contained nucleotide sequences which corresponded precisely with the published λZAPII library clone sequence. Taken together, the data supports a role for functional PAR-2 in RA smooth muscle cells and is consistent with the proposed model for PAR-2 activation. In an analogous manner to thrombin-receptor activation, trypsin presumably cleaves the protease recognition site located in the extracellular N-terminal domain of PAR-2 revealing SLIGRL at the new N-terminus that functions as a tethered ligand to activate the receptor. In support of this mechanism for activation of PAR-2, it was confirmed using soybean trypsin inhibitor, that proteolytically active trypsin was required for these responses. Although the potency for trypsin was found to be very much greater than exogenously applied peptide, the simplest explanation for this is that a single enzyme molecule has the potential to activate many receptors while the peptide can only occupy one receptor at any given time. Furtherrnore, unlike the peptide ligand free in solution, the tethered ligand generated by proteolytic cleavage of receptors is automatically found close to the activation site and may favour the correct orientation to activate the receptor. While it was also found that the concentration range of SLIGRL required to activate the MAP kinase signalling pathway in RA smooth muscle cells was higher than that reported previously for other responses, to date, the potency of PAR-2-

derived peptides has only been characterized in evoking smooth muscle contraction in intact tissues (Al-Ani *et al.*, 1995, Saifeddine *et al*, 1996), or intracellular calcium mobilization (Nystedt *et al.*, 1995b, Santulli *et al.*, 1995, Böhm *et al.*, 1996), processes which generally become saturated at lower concentrations. Marked differences in sensitivity to any one PAR-2 -derived activating peptide in different tissues has been reported by other authors (Saifeddine *et al.* 1996) and in the present study, may reflect a lower expression of PAR-2 in RA smooth muscle cells. In addition, the hexapeptide used in this study contained a free carboxyl terminus which at physiological pH, will be ionized and may modify interaction with the region of the receptor affecting activation. Indeed, subsequent reports since this study was completed indicate that equivalent-length PAR-2 -activating peptides with a carboxyamide terminus, presumably reflecting the uncharged amide bond linking the sequence to the remainder of the extracellular portion of the receptor, show greater potency in evoking gastric muscle contraction than their carboxyl-terminated peptide counterparts (Saifeddine *et al.*, 1996).

In a manner consistent with propagation of signals leading to MAP kinase activation described for other agonists that activate authentic transmembrane receptors (Kyriakis *et al.*, 1992, Matsuda *et al.*, 1992), both trypsin and PAR-2-derived peptide also activated components of the cascade upstream of MAP kinase including MEK-1/2 and c-Raf-1. To establish whether these signalling molecules represent components of an integrated signalling pathway, the effect of modifying regulatory inputs to this pathway on the subsequent activation of each kinase by either protease or peptide was examined. As described in Section 3.9.2, PKC has been implicated in the activation of MAP kinase by a number of agonists in a variety of cell types, possibly by the ability of certain PKC isoforms to directly phosphorylate and activate c-Raf-1 (Sozeri *et al.*, 1992, Kolch *et al.*, 1993). In the present study, a phorbol ester pretreatment protocol which this laboratory has reported previously to effectively deplete RA smooth muscle cells of immunodetectable PKC α and ε isoforms (Malarkey *et al.*, 1996) was found to abrogate c-Raf-1 activity in response to trypsin and SLIGRL which was reflected downstream at the level of MEK-1/2 activity. This finding is consistent with the removal of a PKC-dependent component regulating c-Raf-1 activation by these two stimuli. Both angiotensin II and PDGF -stimulated MAP kinase activation in RA smooth muscle cells are also relient on PKC (Malarkey *et al.*, 1996) indicating that unlike BPA fibroblasts characterized in Chapter 3, RA smooth muscle cells may be a cell type in which PKC may be the major activator of the MAP kinase signalling pathway to the majority of agonists. Another phenomenon associated with the MAP kinase cascade is the sensitivity in a number of cell

types to an elevation in intracellular cyclic adenosine 3'5' -monophosphate (cAMP). Either, non-hydrolysable cAMP analogues or pharmacological interventions which raise cAMP levels artificially such as forskolin which directly activates the catalytic subunit of adenylyl cyclase (Seamon & Daly, 1981) or 3-isobutyl-1-methylxanthine (IBMX), which inhibits cAMP phosphodiesterase, have been shown to attenuate MAP kinase signalling in specific cell types in reponse to a range of agonists (Burgering *et al.*, 1993b, Cook & McCormick, 1993). Inhibition by cAMP may occur at a number of levels including the negative regulation of Raf-1 (Cook & McCormick, 1993, Wu *et al.*, 1993a). This may involve cyclic AMP-dependent protein kinase A (PKA) -mediated phosphorylation of c-Raf-1 within the regulatory domain, preventing binding of Raf-1 to Ras (Wu *et al.*, 1993a). Consistent with this possibility, in this investigation, forskolin was found to almost completely block trypsin and SLIGRL -stimulated c-Raf-1 and MEK-1/2 activities in RA smooth muscle cells. Taken together, this data indicates that Raf/MEK/MAP kinase constitutes a *bona fide* signalling pathway in RA smooth muscle cells, that is activated in response to trypsin and SLIGRL in an identical way that growth factors or G-protein coupled receptors activate this pathway.

Further evidence in support of a role for PAR-2 in trypsin evoked responses in RA smooth muscle cells was provided by receptor-desensitization studies. It has been recognized that the prototype PAR, the thrombin receptor, is desensitized very soon after stimulation with either thrombin or thrombin mimetic peptides. This desensitization process is believed to involve an inactivation step caused by rapid phosphorylation of serine or threonine residues in the cytoplasmic tail of the thrombin receptor probably by a G-protein -coupled receptor kinase (Ishii *et al.*, 1994). Depending on the cell type, a second mechanism for downregulation of activated thrombin receptors has been identified involving rapid internalization and targeting of receptors to endosomal vesicles where they are sorted for delivery to lysozomes for degradation (Hoxie *et al.*, 1993, Hein *et al.*, 1994). In cells of endothelial origin and fibroblasts, cell surface receptors are replenished within 60 min of thrombin removal from a protected intracellular pool, allowing the cell to respond to thrombin a second time (Hoxie *et al.*, 1993, Hein *et al.* 1994). As activation of proteinase-activated receptors by proteolysis is irreversible, rapid desensitization and receptor processing is likely to be a universal mechanism necessary for terminating signals generated by cleaved receptors which are potentially activated indefinitely, for recycling intact receptors to the cell surface, and allowing PARs to be activated in a concentration-dependent fashion. It was found in the present study that exposure to SLIGRL caused a subsequent loss of responsiveness of PAR-2-mediated

signalling events evoked by a second SLIGRL stimulus. While other papers published after completion of this work have also reported short term desensitization of PAR-2 peptide ligand responses using similar protocols entailing cumulative exposure to peptide at short time intervals (Mirza *et al.*, 1996 Saiffedine *et al.*, 1996) no studies to date have addressed whether the processes involved are analogous to desensitization of the thrombin receptor. The finding that trypsin-stimulated MAP kinase and MEK activation was also markedly inhibited under conditions where PAR-2-activating peptide responses were refractory strongly suggests that trypsin acts largely via the same mechanism. Although similar transient kinetics for activation of the MAP kinase cascade seen here with trypsin has been reported in this laboratory for thrombin in RA smooth muscle cells (Malarkey *et al.*, 1996), it is unlikely that the two serine proteases act via the same mechanism as both MAP kinase and MEK activities evoked by thrombin were only marginally affected by SLIGRL pretreatment in marked contrast to trypsin. The preservation of the thrombin signal confirmed that the decrease in MEK and MAP kinase activities that was observed following the second addition of SLIGRL or trypsin was not due to a downregulation of intracellular signalling processes. The finding that the thrombin response was not desensitized by prior SLIGRL treatment may reflect the inability of SLIGRL to interact with, and cause desensitization of the thrombin receptor, as might be predicted by peptide structure-activity studies showing that an aromatic residue at position 2, which is present in TRAPs (SFLLRN) but not PAR-2 -activating peptides (SLIGRL), is critical for activating the thrombin receptor in different cell systems (Scarborough *et al.*, 1992, Hollenberg *et al.*, 1993). Functional expression studies in *Xenopus laevis* oocytes and cells expressing thrombin receptor oligonucleotide antisense have confirmed that neither the murine or human PAR-2 peptide cross-reacts with the thrombin receptor (Blackheart *et al.*, 1996, Mirza *et al.*, 1996). In contrast to the present findings, however, other studies have demonstrated heterologous downregulation and internalization of the thrombin receptor in endothelial cells preactivated with SLIGRL (Mirza *et al.* 1996), suggesting the presence of functional coupling between the two receptors analogous to the cross-talk between muscarinic and adrenergic receptors (Lee & Frazer, 1993). Surprisingly, in a single experiment carried out to investigate whether trypsin could also desensitize PAR-2-dependent responses in RA smooth muscle cells, it was found that pretreatment with trypsin for 20 minutes, in contrast to SLIGRL, caused an inhibition of MAP kinase activation not only by trypsin and SLIGRL but also by thrombin (not shown). However, rather than heterologous desensitization of the thrombin receptor, which was clearly not observed when SLIGRL was used as the

desensitizing stimulus, the loss of the ability of thrombin to activate MAP kinase may reflect the proteolytic destruction of thrombin in the presence of trypsin that has been reported previously (Hartmann *et al.*, 1994), or an effect on the thrombin receptor impairing activation by thrombin. This might involve a similar action to cathepsin G, which has been shown to prevent thrombin-stimulated responses by cleaving the thrombin receptor at a second cleavage site on the N-terminus closer to the first transmembrane domain, removing the tethered ligand domain and rendering the receptor unresponsive to thrombin (Molino *et al.*, 1995). While an appropriate additional experiment to conduct would be to confirm using a complimentary desensitization strategy using TRAP peptides, that thrombin-receptor desensitization does not affect responses to SLIGRL or trypsin, results from other papers indicate that TRAP peptides do not show fidelity for the thrombin receptor (Santulli *et al.*, 1995, Blackheart *et al.*, 1996) and can efficiently activate PAR-2 expressed in *Xenopus* oocytes at similar concentrations to thrombin receptors. This cross-reactivity by TRAPs may result in desensitization of PAR-2 receptors and their associated responses to SLIGRL and trypsin as has been demonstrated in keratinocytes (Santulli *et al.*, 1995). Despite other studies arguing in favour of trypsin and thrombin acting at the same receptor (Kable *et al.*, 1995) this may be a consequence of the micromolar concentrations of trypsin used in such experiments. At the low nanomolar concentrations used in this study, trypsin was unable to activate the human thrombin receptor expressed in *Xenopus* oocytes (Vu *et al.*, 1991). Only at concentrations over fifty-fold higher than the EC_{50} for the trypsin-evoked responses determined here, was a significant response to the protease recorded. Thus, it is likely that the specificity of trypsin for PAR-2 depends largely on both the concentration and specific activity of trypsin used. Taken together this evidence indicates that the activation of the MAP kinase signalling cascade by trypsin and SLIGRL in RA smooth muscles cells is due to the activation of a receptor system separate from the thrombin receptor, most likely represented by PAR-2 itself. The coupling of this receptor appears to be distinct since activation of p42/p44 MAP by either trypsin or SLIGRL was not accompanied by a significant p38 or JNK signal (not shown).

6.6.3 Role of PAR-2 in vascular cell mitogenesis

Some groups have provided evidence to indicate that the mitogenic potential of thrombin in smooth muscle cells and fibroblasts is mediated exclusively by the cloned tethered-ligand thrombin receptor (Hung *et al.*, 1992a, van Corven *et al.*, 1993, Hartmann *et*

al., 1994), despite the data presented in Chapter 3 and by other authors (Vouret-Craviari *et al.* 1992) suggesting that an additional thrombin-sensitive receptor may be involved. Given the similarities between the cloned thrombin receptor and PAR-2 it was pertinent to investigate whether PAR-2 may serve a similar function. A rationale for testing the role of PAR-2 in RA smooth muscle cells mitogenesis was further reinforced by the finding that in this cell type, trypsin and SLIGRL activated p42 and p44 MAP kinases, which were demonstrated in Chapter 3 (Section 3.5.2) to play an obligatory role in agonist-stimulated DNA synthesis in BPA fibroblasts. Furthermore, very soon after completion of these studies, other investigators reported that PAR-2 mediates endothelial cell proliferation when activated by the PAR-2 derived peptide ligand (Mirza *et al.*, 1996). However, neither trypsin nor SLIGRL were found to be strong mitogens for RA smooth muscle cells relative to serum or compared to the effect of trypsin in BPA fibroblasts, suggesting in this cell type, PAR-2 is not involved in mitogenic signal transduction. This may reflect the transient nature of the MAP kinase signal which returns to unstimulated levels by 20 minutes. As outlined in Section 3.9.3 and demonstrated in a fibroblast cell line (Vouret-Craviari *et al.*, 1993), a persistent activation of p42 and p44 MAP kinases up to, and beyond 1 hour, may be required for RA smooth muscle cell mitogenesis, as was evident for stimuli which behaved as mitogens in BPA fibroblasts (Section 3.5.1). A previous publication from this laboratory has reported that thrombin also evokes transient activation of MAP kinase in RA smooth muscle cells (Malarkey *et al.*, 1996) and has a negligible effect on [^{3}H]thymidine incorporation (K. Malarkey, personal communication), contrary to data presented by other groups (McNamara *et al.*, 1993, Molloy *et al.*, 1996). The strong similarities between the functional responses evoked by trypsin and thrombin suggests that in RA smooth muscle cells, the two currently defined proteolytically-activated tethered ligand receptors may couple to the same signalling pathways. Trypsin was found to be a much more effective mitogen in BPA fibroblasts, which may reflect the ability of the protease to evoke a sustained MAP kinase signal in this cell type. While the mitogenic action of trypsin required proteolytic activity, the finding that SLIGRL was ineffective at stimulating DNA synthesis is consistent with the kinase and PCR data in implying that a proteolytically-activated receptor other than PAR-2 is involved in mediating the effects of trypsin in BPA fibroblasts.

6.6.4 Potential functions and mediators of PAR-2 dependent MAP kinase activation in RA smooth muscle cells

Although the present data does not support a major role for PAR-2 in RA smooth muscle cell mitogenesis, this finding does not invalidate a potential involvement of this receptor in mediating biological effects in this cell type. While the functional importance of transient MAP kinase activation is less well defined, one cellular consequence of MAP kinase activation in response to various stimuli is the phosphorylation and activation of $cPLA_2$ (Lin *et al.*, 1993). This enzyme releases fatty acid from the sn-2 position of phospholipids exhibiting a high selectivity toward arachidonic acid, the rate-limiting precursor for the synthesis of prostanoids and leukotrienes, two classes of vasoactive and inflammatory mediators. Thus, it is tempting to speculate that MAP kinase activation by PAR-2 may be involved in PLA_2-mediated production of agents which influence blood vessel tone. It is conceivable that the presence of PAR-2 mRNA in RA smooth muscle cells may indicate that like thrombin receptors, PAR-2 may regulate smooth muscle cell contractility directly. Activation of PAR-2 in a variety of cells has been shown to be coupled to an increase in intracellular Ca^{2+} concentration (Nystedt *et al.*, 1995b, Santulli *et al.*, 1995, Böhm *et al.*, 1996) which in vascular smooth muscle is required for excitation-contraction coupling. MAP kinase has also been implicated in the sustained phase of smooth muscle contraction (Childs *et al.*, 1992). Despite this causal relationship, a role for PAR-2 in directly regulating vascular tone is not supported by the work of Hollenburg's group who have shown that SLIGRL is unable to cause a change in tension of endothelium-denuded aortic rings (Al-Ani *et al.*, 1995, Saifeddine *et al.*, 1996).

While the functional significance of PAR-2-dependent activation of MAP kinase in vascular smooth muscle cells is uncertain, even less clear is the identity of the physiological activator(s) of this receptor in this cell type or of the trypsin-responsive receptor in BPA fibroblasts. Although trypsin can be used as a convenient tool for screening for PAR-2 - evoked responses, this protease is unlikely to be the activator of vascular PAR-2 *in vivo* as it is not normally present in the vascular space, usually being confined under physiological conditions to the pancreas and gastrointestinal tract. Like the highly specific interaction between thrombin and the hirudin-like recognition sequence of the tethered-ligand thrombin receptor, the specificity for activation of PAR-2 is likely to reside with the protease that is the physiological agonist for the receptor. The identity of this mediator remains elusive and could

be any one of a number of extracellular proteases exhibiting trypsin-like activity, especially those involved in the processes of blood coagulation and the compliment cascade or secreted from other cell types found in close proximity to the vasculature. It is now recognized that like thrombin, a number of these enzymes mediate cellular effects, although the molecular mechanisms of their action have remained undetermined. Prostacyclin synthesis has been shown to be stimulated in endothelial cells by trypsin, chymotrypsin, cathepsin G, a protease released from activated neutrophils, and plasmin, a key enzyme of the fibrinolytic system (LeRoy *et al.* 1984, Chang *et al.*, 1993) and in aortic smooth muscle cells by pancreatic elastase (Kawaguchi & Yasuda, 1988). Intriguingly, cathepsin G has also been shown to cause an increase in intracellular Ca^{2+} in platelets (Molino *et al.*, 1992, Selak, 1994) by a process involving a distinct receptor from the thrombin receptor (Selak, 1994). Whether or not these mediators interact with PAR-2 to evoke their cellular effects is not known at present. However, given that the release of prostacyclin and arachidonic acid is indicative of PLA_2 activation and that p42/p44 MAP kinase may play a role in the regulation of PLA_2, these proteases are attractive candidates to screen first for PAR-2-activating potential. Similarly, even though PAR-2 may not be a major player in RA smooth muscle cell mitogenesis, the physiological activator of the trypsin-responsive receptor in BPA fibroblasts may be found amongst the serine proteases that have been reported to diplay mitogenic potential for various cell types. These include the coagulation factors XII and XIIa which have been shown to exert mitogenic effects on Hep G_2 hepatocytes (Schmeidler-Sapiro *et al.*, 1991), granzyme A from T lymphocytes which induce proliferation of B-lymphocytes (Kramer *et al.*, 1986) and tryptase the major secretory granule protein of human mast cells, which has recently been shown to be a specific and potent growth factor for lung-derived hamster and human fibroblasts (Ruoss *et al.*, 1991, Hartmann *et al.*, 1992) and airway smooth muscle cells (Brown *et al.*, 1995b). This action of tryptase in fibroblasts may define more specifically the possible role of the other cleavable membrane protein(s) in mediating the effects of trypsin in BPA fibroblasts. Finding the endogenous activator of PAR-2 and other potential proteolytically-activated receptors will not be an easy task. Candidate enzymes would be selected on the basis of their proteolytic profile characterized by their ability to hydrolyze a range of substrates especially those resembling the PAR-2 extracellular domain, and susceptibility to inhibition by specific inhibitors, and screened against PAR-2 functionally expressed in cells which do not express any other protease receptor.

6.6.5 CONCLUDING REMARKS

In summary, the data presented demonstrates that while trypsin was found to activate components of the MAP kinase cascade in BPA fibroblasts, this effect does not appear to be a cellular consequence of PAR-2 or thrombin receptor activation. This finding indicates that the previously characterized signalling events evoked by high concentrations of thrombin cannot be explained by an involvement of PAR-2 and that in specific cell types, trypsin may act via a unique mechanism, perhaps involving novel receptor species. These receptors may mediate the strong growth-promoting effects of trypsin and other proteases on such cells. In RA smooth muscle cells, however, activation of the MAP kinase pathway by trypsin occurs in a manner consistent with an interaction with PAR-2. Although this receptor does not appear to be a major transducer of mitogenic signals in RA smooth muscle cells, it may play a functional role in cellular processes regulated by transient MAP kinase activation, which at present are not well characterized.

CHAPTER 7

GENERAL DISCUSSION

7.1 General Discussion

The data presented extends the repertoire of signalling molecules affected by thrombin to include elements of three distinct protein kinase pathways believed to play key roles in regulating specific cellular functions, highlighting the different mechanisms available to thrombin to evoke effects on cells. Firstly, thrombin activated the p42/p44 MAP kinases, confirming that in addition to a number of investigations conducted previously in fibroblast cell lines, these signalling molecules serve as intracellular targets for the protease in cells in primary culture. These studies were extended to address the upstream components of this pathway and revealed that activation of MAP kinase by thrombin in BPA fibroblasts is likely to occur via the well established protein kinase cascade involving MEK-1/2 and possibly Raf-1. Secondly, results are presented to demonstrate that thrombin stimulates the activation of $p70^{s6k}$ and identifies PKC, a heterotrimeric G protein of the $G_{i/o}$ family, and a PI 3-kinase different from p85/p110 as key regulators of this pathway. Finally, thrombin stimulates a MAPKAP kinase-2-associated kinase activity that may be attributable to the MAP kinase homologue p38, or a closely related protein.

The ability of specific pharmacological inhibitors of components of each of these pathways to either abolish or attenuate thrombin-stimulated DNA synthesis in growth arrested BPA fibroblasts implies that their biological function is to relay signals important for cells to re-enter the cell cycle. That a very effective mitogen such as thrombin evokes the activation of multiple signalling pathways emphasizes the intricate levels of control imposed on crucial cellular decisions such as those made prior to cell division. Ultimately, a complex process such as re-entry into the cell cycle will depend on the specific transcription factors which become activated by the terminal protein kinases of the pathways to which a receptor of a particular mitogen is coupled, and how these transcription factors combine in the nucleus to induce the co-ordinated expression of specific early genes critical for cell cycle progression. Research efforts in the past few years have been successful in identifying some of the transcription factors regulated by these kinases. For example, $p62^{TCF}$/Elk-1 is a common nuclear target for both the p42/p44 and p38 groups of MAP kinases (Marais *et al.*, 1993, Raingeaud *et al.*, 1996), while ATF-2 is a substrate for p38 *in vitro* and in transfected cells (Raingeaud *et al.*, 1996). In addition, specific nuclear targets for $p70^{s6k}$, MAPKAP kinase-2 and $p90^{rsk}$ are also coming to light (de Groot *et al.*, 1994). While all of these factors would be predicted to become activated on exposure of BPA fibroblasts to thrombin, the full array of

genes that they control, and the contribution that products of these genes make to the genetic program regulating the cell cycle is only at a rudimentary stage of comprehension. A better understanding of the transcriptional control of genes will be necessary to provide information concerning how various signalling inputs impinging upon the nucleus are co-ordinated to initiate complex genetic responses. In the meantime, compounds such as PD098059, rapamycin and SB203580 used in this study, will serve as valuable tools for identifying the specific nuclear apparatus by which transcriptional events are regulated.

Despite outlining the signal transduction pathways involved in thrombin-stimulated mitogenesis, a number of points remain unresolved regarding the upstream events involved in their activation, the possible interplay between these pathways, and the nature of the thrombin "receptor/s" that they are coupled to. These are all the more difficult to decipher in BPA fibroblasts due to the unique actions of the protease in this cell type. While signal transduction by thrombin has been reported to be mediated by G-protein interactions in a number of cell types, the most striking observation in BPA fibroblasts was the ostensible resemblance of thrombin mediated events to signalling characteristics of growth factors that activate receptor tyrosine kinases, such as PDGF. Thus, for example, activation of MAP kinase by thrombin followed a slow and gradual time course rather than the rapid kinetics reported in other cell types that are often maximal within 2-5 minutes. Furthermore, thrombin did not employ the established routes by which G-protein-coupled receptor agonists link to this pathway, namely PTX-sensitive G proteins or G_q/PLC-PKC. The relative importance of G-protein-coupled receptor and tyrosine kinase receptor -mediated signalling events triggered by thrombin and other G-protein-coupled receptor agonists, is currently a topic of considerable interest. Research within the past year has provided novel paradigms to support the concept of physiologically relevant cross-talk between G-protein-coupled receptors, including the thrombin receptor, and the tyrosine kinase receptor-linked routes for communication within the cell. How this occurs is not entirely clear but in some instances, thrombin may transactivate a tyrosine kinase receptor itself. For example, thrombin, TRAP, endothelin-1 and LPA stimulate tyrosine phosphorylation of the EGF receptor in Rat 1 fibroblasts (Daub *et al.*, 1996). Specific inhibition of this receptor by a dominant-negative EGF receptor mutant or by pharmacological intervention with the tyrphostin AG1478 has been shown to inhibit MAP kinase activation, c-*fos* induction and DNA synthesis by these agonists, confirming that a functional EGF receptor is required for these mitogenic responses. Similarly, in vascular smooth muscle cells, thrombin and angiotensin II cause the tyrosine phosphorylation of the β-

chain of the IGF-1 receptor and IRS-1 (Rao *et al.*, 1995, Du *et al.*, 1996), and the PDGF β receptor (Linseman *et al.*, 1995), respectively. Cross-activation of tyrosine kinase receptors by G-proteins may promote the recruitment and activation of proteins that are well established upstream regulators of Ras in tyrosine kinase receptor-mediated signalling (see Section 1.6.5.1). In support of this notion, thrombin, TRAP and angiotensin II have also recently been shown to phosphorylate isoforms of the adaptor protein Shc resulting in their association with Grb2 (Linseman *et al.*, 1995, Chen *et al.*, 1996, Daub *et al.*, 1996). Furthermore, this effect occurred by a PTX-insensitive and PKC-independent route, with kinetics that resembled the time course for the sustained activation of MAP kinase seen in these cells, satisfying all the criteria of thrombin activation of MAP kinase in BPA fibroblasts (Chen *et al.*, 1996). Thus, the utilization by G-protein-coupled receptors of signal-transducing elements intrinsic to receptor tyrosine kinase pathways reveals a new dimension in signalling diversity and is one attractive model to explain the growth factor-like behaviour of thrombin in BPA fibroblasts. Moreover, this model directly challenges current paradigms for G-protein-coupled receptor linkage to protein kinase cascades such as the regulation of the Ras-p42/p44 MAP kinase cascade by pertussis toxin-sensitive G_i proteins and βγ subunit (see Section 1.6.5.2).

An additional complication in interpreting signalling events upstream of the kinases described is the likelihood that at the relatively high concentrations required to evoke their activation in BPA fibroblasts, thrombin elicits different effects at the surface of the cell, acting on at least one other receptor than the currently defined tethered ligand thrombin receptor. Thus, it is uncertain whether the mitogenic signalling pathways that become activated in response to thrombin represent parallel independent axis that are engaged by a single receptor or different thrombin receptors, or arise from a bifurcation further down after initiation of the signal at a point that is a common upstream component of more than one pathway. Of the available evidence, however, $p70^{s6k}$, p42/p44 MAP kinases and p38 lie on discrete axes and mechanisms for crossregulation are so far unknown, suggesting that the latter possibility is unlikely. Nevertheless, recent evidence has indicated that FGF stimulates gene transcription via a pathway involving p38 kinase and requiring Ras (Tan *et al.*, 1996), indicating that in some instances, perhaps unique to certain agonists, signals may bifurcate at the level of Ras to activate both p42 /p44 MAP kinases and p38. On the other hand, $p70^{s6k}$ is activated in a Ras-independent manner (Ming *et al.*, 1994). Despite knowledge of the events upstream of this kinase being sketchy, and likely to be the topic of intense research in the immediate future, a recent advance in this area is the discovery that $p70^{s6k}$ can complex with, and become

activated *in vivo* by the Rho family G-proteins Cdc42 and Rac (Chou & Blenis, 1996) which are also thought to be efficient activators of the signalling cascade affecting p38 activation (Bagrodia *et al.*, 1995, Minden *et al.*, 1995, Zhang *et al.*, 1995). Whether or not different monomeric G proteins serve as junctions at which signals generated by thrombin diverge to activate MAP kinase, p38 and $p70^{s6k}$ pathways in BPA fibroblasts is an important question that further research might attempt to resolve.

While downstream events may arise from shared upstream signalling components, recent evidence has also come to light, however, to suggest that different receptor mechanisms independently regulate distinct signalling pathways in response to thrombin. The platelet has been found to be another cell in which thrombin stimulates both p42/p44 MAP kinases and p38, yet in this case, the thrombin receptor agonist peptide SFLLRN solely stimulates the signalling pathway causing p38 activation (Kramer *et al.*, 1995a, 1995b). While TRAP-14 was not tested in BPA fibroblasts for activation of the MAPKAP kinase-2 -associated kinase activity, these findings suggest that p38 may be an integral part of a signalling pathway utilized by the currently defined thrombin receptor, while MAP kinase is an element of a parallel cascade activated by another thrombin sensor.

Identifying and characterizing other cell surface proteins which respond to thrombin at the cell surface is likely to be an important research priority to fully understand how thrombin initiates those signalling events which can be either completely or partially dissociated from responses evoked by TRAPs, such as activation of MAP kinase in BPA fibroblasts. This is especially pertinent for two reasons. Firstly, the recent discovery that TRAPs cross-react with the recently cloned PAR-2 (Mirza *et al.* 1996), indicates that previous deductions made regarding the number of effects of thrombin attributable to the tethered ligand thrombin receptor may actually be overestimated. Secondly, an additional site for thrombin interaction may prove to be a more appropriate target for blockade by novel pharmaceuticals developed with the aim of combating disease states where thrombin has been implicated. A reappraisal of the role of the cloned thrombin receptor in mediating the effects of thrombin is needed but up until now, the tools to do this have not been available since receptor-based peptides and monoclonal antibodies that have been produced have generally proved to be of little success as thrombin receptor antagonists. Two state of the art approaches have been employed to specifically eliminate the contribution made by the thrombin receptor to screen for other thrombin-evoked responses. Antisense oligonucleotide for the thrombin receptor has been shown to selectively decrease thrombin receptor expression

and inhibit the mitogenic responses to α-thrombin and SFFLRN in rat vascular smooth muscle cells (Chaikof *et al.*, 1995). In addition, "knockout" mice have been engineered that are homozygous for the thrombin receptor gene deletion ($tr^{-/-}$). Thrombin and TRAP-stimulated DNA synthesis and MAP kinase activation have been shown to be selectively lost in lung fibroblasts derived from $tr^{-/-}$ mice (Trejo *et al.*, 1996). While both of these reports indicate that activation of the cloned thrombin receptor, and not a second receptor, is necessary and sufficient for these responses, platelets from $tr^{-/-}$ mice have been shown to respond normally to thrombin indicating that a distinct thrombin receptor does exist in certain cell types (Connolly *et al.*, 1996). Further application of these techniques to a number of other cell systems will be required to make definitive deductions regarding thrombin-receptor-mediated signals and identify a suitable model for studying those events evoked by activation of additional thrombin binding sites.

In addition to raising the possibility that disparate receptors mediate the effects of thrombin, these studies have revealed that vascular cells also possess other distinct protease-sensitive cell surface proteins that initiate cellular signalling events. Rat aortic smooth muscle cells were found to express the novel PAR-2 which functionally coupled to the MAP kinase pathway while a different receptor mediated the same response to trypsin in BPA fibroblasts. It is highly likely that in the not too distant future, other members of the receptor family activated by proteases will come to light. These PARs may turn out to be physiologically significant target substrates for protease mediators found to be present in the extracellular milieu under physiological or pathological states, and mediate functional responses in vascular and other cell types as far reaching as those proposed for the thrombin receptor. The role that these receptor systems play *in vivo* will only be understood fully when the specific protease activators are identified, or when strategies similar to those used to disable the thrombin receptor are employed to block a particular physiological function.

Despite several questions that still need to be resolved, these studies have outlined a number of novel cellular consequences of thrombin action on cells, further defining the possible mechanisms by which the protease is able to influence their behaviour. One corollary of the findings is a clearer understanding of the cellular processes that underlie the ability of thrombin to act as a strong mitogen for diverse cell types. In particular, the studies highlight the pathways that are likely to operate during vascular disease states where hyperplasia of cell types constituting the blood vessel wall is a hallmark, and where thrombin has been proposed as a possible mediator, such as atherosclerosis, restenosis and possibly pulmonary

hypertension. Whether or not the same pathways are involved in other aspects of thrombin's hormone-like activity either during physiological or pathological settings will become apparent in the forthcoming literature.

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