Isolation and Characterisation of a Uniquely Regulated Threonine, Tyrosine Phosphatase (TYP 1) which Inactivates Mitogen-activated Protein Kinases.

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To my parents

for all their love, continual support and encouragement

-and my Grandfather, for always believing in me.

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Abbreviations

A	Adenine
ATP	Adenosine triphosphate
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
^{60}Co	Cobalt source
CTP	Cytidine triphosphate
dNTP	deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxynuclease
G	Guanine
Gi	inhibitory G protein
Ig	Immunoglobulin
mRNA	messenger ribonucleic acid
RNase	Ribonuclease
SV40	simian virus 40
T	Thymidine
UV	Ultraviolet
^{32}P	32 phosphorous
^{35}S	35 sulphur

Units

bp	base pair
Ci	Curies
cpm	counts per minute
cm	centimetres
cm^2	centimetres squared
g	gram
Gy	Grays
h	hour
kb	kilobases
kDa	kilodaltons
l	litre
µ	micro
'	Minutes
m	milli

M	Molar
n	nano
^{o}C	degrees Celsius
OD	Optical density (absorbance)
rpm	revolutions per minute
V	Volts
v/v	volume for volume
w/v	weight for volume

Single letter code for amino acids

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
E	Glutamic acid
Q	Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

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Abstract

The recent discovery of the vaccinia virus protein phosphatase VH1, and its mammalian counterparts has highlighted a novel subfamily of protein phosphatases that exhibit dual specificity towards phosphotyrosine- and phosphoserine/threonine-residues. The aim of this thesis was to isolate and characterise further members of this phosphatase subfamily.

Employing the technique of polymerase chain reaction (PCR), degenerate PCR primers, designed from amino acid sequences conserved between two members of this dual specificity phosphatase family, were used to amplify related sequences from poly (A)+ RNA isolated from the human squamous cell line A431. Screening cDNA libraries with probes derived from the PCR analysis resulted in the isolation of five novel genes, which show significant homology to the catalytic domain of the dual specificity phosphatases, such as the human phosphatase CL100. Two of these, TYP 1 and TYP 2 (threonine-tyrosine phosphatase), have been analysed further. Interestingly, whilst TYP 2 expression is regulated with similar kinetics to CL100, TYP 1 differs dramatically in its regulation. The TYP 1 gene is not expressed in human fibroblasts unlike other CL100-like genes. Furthermore, northern analysis has demonstrated that following mitogenic stimulation of squamous cells, induction of TYP 1 mRNA reaches its maximal levels after four hours, in contrast to the immediate-early CL100-like genes. Both TYP 1 and CL100 mRNAs are induced upon transforming growth factor-beta treatment of squamous cell lines sensitive to the growth factors antiproliferative effects.

Investigations were undertaken to determine if TYP 1 demonstrated substrate specificity towards the mitogen-activated protein kinases, a function peculiar to the other CL100-like phosphatases. Indeed, transient transfection of TYP 1 into COS-1 cells resulted in the inhibition of both ERK2 and p54JNK MAP kinase isoforms. In addition, purified TYP 1 protein efficiently inactivates recombinant ERK2 *in vitro* by the concomitant dephosphorylation of both the phosphothreonine and -tyrosine residues. The subcellular localisation of TYP 1 was investigated, and shown to encode

a nuclear protein. Interestingly, the mitogenic induction of TYP 1 results in an increase in the half-life of the protein.

The results presented in this thesis suggest that the dual specificity phosphatases are an expanding family, some members of which are differentially regulated, therefore providing alternative mechanisms for the control of cellular responses to different ligands in different cell types. As discussed, TYP 1 is particularly exciting as its kinetics of induction and cell specificity of expression make it unique in terms of its possible roles.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Signal transduction- an overview

The biochemical process through which a cell responds to an extracellular stimulus involves the interplay of multiple intracellular signalling pathways that operate in a stimulus- and cell type-dependent manner. A critical aspect that is not yet fully understood is how cells integrate the complex array of positive and negative signals they receive in such a way that each cell assumes and maintains its proper identity and function in an appropriate spatial and temporal context. Many of these signalling events initiate at the cell surface via specific receptor-ligand interactions and are relayed through the cytoplasm to the nucleus, where transcription factors, thought to be among the ultimate targets of such signalling pathways, elicit a response by modulating gene expression. Thus, co-ordinated regulation of transcription factor activity represents a logical integration point for the various inductive and suppressive signals received by each cell.

1.1.1. Mitogens and the cell cycle

Mitogenic signal transduction is the result of a series of events initiated by the binding of growth factors to their cognate receptors at the cell surface (reviewed in Aaronson 1991; Baserga *et al.*, 1993). Growth factors produced by a cell stimulate a vast network of cellular interactions, integrating cell proliferation (thus referred to as mitogens), growth inhibition and differentiation. It is becoming clear that *in vivo*, the majority of soluble growth factors are effective locally, exerted via autocrine, paracrine and intracrine mechanisms, (Sporn and Todara, 1980; Re, 1989), whilst cell surface growth factors are brought into juxtaposition (juxtacrine) by cell-cell contact (Massague, 1990). Mitogenic growth factors, such as epidermal growth factor (EGF), insulin, and platelet-derived growth factor (PDGF) stimulate cells in the quiescent state (G0) to enter and proceed through the cell cycle, via a number of "control points" which require both competence and progression factors (Muller *et al.*, 1993).

Cell cycle transitions are orchestrated by the sequential assembly and activation of cyclin-dependent kinases, whose formation depends on the cell cycle-regulated expression of cyclins (the regulatory subunit), associating with pre-existing catalytic subunits known as cyclin dependent kinases (Cdks), reviewed in Pines, 1995. In vertebrate cells, the main cyclin-Cdks are cyclin D-Cdk4, Cyclin E-Cdk2, Cyclin A-Cdk2, and Cyclin B-Cdc2, acting in G1, at G1/S, in S, and at G2/M, respectively. Cdks act as integrators of positive and negative signals that determine progression towards a point in late G1 beyond which the cell cycle proceeds autonomously, until further checkpoints in G2. According to this paradigm, cell cycle stage is determined by the constellation of proteins activated or inactivated by phosphorylation as a result of the activity of Cdks. The Cdk subunits are themselves subject to modification by phosphorylation and dephosphorylation (Pines, 1995). To be fully activated, the Cdk component needs to be phosphorylated on a conserved threonine (T160 in Cdk2). Interestingly, the analogous residue is phosphorylated in most, if not all, other families of protein kinases. There are also two inhibitory phosphorylation sites in mammalian Cdc2 and Cdk2, threonine 14 and tyrosine 15 (in Cdk4 the threonine is replaced by an alanine). These sites lie within the ATP-binding region, and the onset of mitosis is determined by the dephosphorylation of these two residues. Negative controls on cell cycle progression are exerted during development, differentiation, senescence, and cell death (apoptosis), and consequently may play an important role in preventing tumourigenesis.

1.1.2. Antimitogenic signals and cell cycle arrest

In many mammalian cell types, transforming growth factor-beta (TGFβ) promotes growth arrest in G1 that is thought to be mediated through elements of the cell cycle machinery (reviewed in Massague and Polyak, 1995). These antiproliferative effects are initiated through the activation of the TGFβ receptor (Wrana *et al.*, 1994), and are particularly pronounced in epithelial, endothelial, and hematopoietic cells. It is now widely accepted that the Cdk complexes are needed during G1 to phosphorylate and thus inactivate the retinoblastoma susceptibility gene (pRb), which inhibits cell

cycle progression in its underphosphorylated state (Weinberg, 1995). It is thought that TGFβ causes G1 arrest by inhibiting G1 cyclin-Cdk activities, thereby suppressing pRb phosphorylation. Consistent with this model, it has been demonstrated that TGFβ can inhibit cyclin E-Cdk2 kinase activity (Koff *et al.*, 1993). In addition, it has been reported that TGFβ treatment inhibits the synthesis of Cdk4 and that overexpression of the Cdk4 gene precludes the TGFβ cytoinhibitory effect in a sensitive cell line (Ewen *et al.*, 1993). Interestingly, these effects are concomitant with inhibition of phosphorylation of pRb. These studies are indicative that Cdk-inhibitors are involved in TGFβ cell cycle arrest, and led to the recent discovery of several mammalian Cdk inhibitors which physically associate with cyclin-Cdk complexes (reviewed in Peter and Herskowitz, 1994; Peters, 1994). These have been implicated as effectors of TGFβ-induced cell cycle arrest, whereby TGFβ causes a rapid and significant increase in levels of the inhibitors p21 (also called Cip1 and WAF1) and $p15^{INK4B}$, which leads to an increased association with, and consequent inhibition of the Cdk complexes (Datto *et al.*, 1995; Li *et al.*, 1995; Hannon and Beach, 1994). An alternative model has been suggested in the case of the inhibitor $p27^{kip1}$, which in untreated cells is bound to cyclin D-Cdk4. Upon stimulation with TGFβ Cdk4 levels are decreased (Ewen *et al.*, 1993), which subsequently lowers the levels of cyclin D-Cdk4, thus releasing p27. This effectively elevates the level of free p27 allowing it to associate with and inhibit cyclin E and A complexes (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). However, these two models should not be conceived as mutually separate mechanisms. Recent data would suggest that TGFβ imparts antimitogenic signals through the co-operative action of $p15^{INK4B}$ and p27, or p21, and then, subsequent cell cycle arrest leads to a decline in Cdk2 and Cdk4 levels, possibly as a measure of the cell adapting to the quiescent state (Reynisdottir *et al.*, 1995). Further understanding of the various cell cycle regulators will greatly facilitate our understanding of the complex pathways constituting cellular sensitivity to TGFβ.

1.1.3. The growth factor connection to cancer

Present knowledge indicates that the constitutive activation of growth factor signalling pathways, through multiple genetic alterations affecting their genes, inextricably contributes to the development and progression of most cancers, as well as a variety of chronic diseases (Aaronson and Tronick 1991). However, cancer cells do not simply arise from uncontrolled cellular growth but rather from a process of cellular evolution accelerated by genetic instability (Nowell, 1986). Oncogenes activated by a variety of mechanisms (Bishop 1991), have frequently been shown to encode growth factors such as Sis, receptor tyrosine kinases such as ErbB/Neu, and other intracellular enzymes including Myc, Ras and Src, that participate in mitogenic signalling, some of which have therefore been identified as potential targets for drug design (Levitzki and Gazit, 1995; Dudley *et al.*, 1995).

1.1.4. The significance of protein phosphorylation

Protein phosphorylation is an important cellular regulatory modification, and is recognised as a fundamental mechanism for the transduction of external and internal stimuli. Regulatory protein phosphorylation occurs most frequently on serine (Ser) and threonine (Thr) residues (Roach, 1991) and until 1980, only phosphoserine and phosphothreonine had been identified as naturally occurring phosphoamino acids. However, tyrosine (Tyr) phosphorylation began to receive greater attention when it was determined that v-Src, the transforming principle of the Rous sarcoma virus, was a unique protein tyrosine kinase (Hunter and Sefton, 1980). These observations, coincident with the discovery that the epidermal growth factor receptor also possesses intrinsic tyrosyl kinase activity (Ushiro and Cohen, 1980), led to the suggestion that tyrosine kinases may be linked to the removal of growth restraint from cells, and stimulated a great deal of biochemical and genetic experimentation on the role of tyrosine kinase oncogenes and proto-oncogenes in normal and malignant cells.

Tyrosine phosphorylation may be the primary, or even the exclusive, initiator of signal transduction in multicellular organisms critical for key biologic functions (for extensive reviews on receptor tyrosine kinase signalling see Ullrich and Schlessinger,

1990; van der Geer and Hunter, 1994). Within the intricate signalling network, intermediary serine/threonine and tyrosine protein kinases propagate signals initiated from cell surface receptors with tyrosine kinase activity to modulate events leading up to and including gene expression. To date, the focus of much research has centered around the mitogen-activated protein kinase (MAPK) pathway, which is firmly established as the paradigm for signal transduction cascades. The mechanisms involved at the different steps of this cascade are, however, not completely clear yet and accumulating evidence indicates complex regulatory relationships between the various components.

1.2. The mitogen-activated protein kinases

The mitogen-activated protein kinase cascade is a sophisticated signal transduction pathway involved in diverse responses in eukaryotic cells. MAPKs are one element in a series of kinases which have remained highly conserved throughout evolution, being present in various species, including yeasts, *Xenopus*, *C. elegans*, *Drosophila* and mammals (see reviews in Herskowitz, 1995; Gotoh *et al.*, 1995; Selfors and Stern, 1994; Perrimon, 1993; Cano and Mahadevan, 1995 respectively). It has become apparent that signals from receptor tyrosine kinases, cytokine receptors, seven spanning heterotrimeric G protein coupled receptors, and presumptively some receptors linked to protein kinase C, result in the activation of the MAPK family: proteins which have served as a point to trace signal transduction back through a kinase cascade to the plasma membrane, and forward to transcription factors in the nucleus. Subversion of this cascade can lead to, and is critical for, cellular transformation and oncogenesis (Cowley *et al.*, 1994). The components of this pathway shall be outlined below.

1.2.1. The MAP kinase family

Historically, the MAPKs were originally described as a rapidly stimulated insulin-sensitive serine/threonine protein kinase activity in 3T3-LI preadipocytes by

Ray and Sturgill (1987); and subsequently named MAP2 kinases for their ability to phosphorylate microtubule-associated protein-2. The discovery of the kinase was a fortuitous event, since at the same time it became clear that the ribosomal protein S6 was controlled through phosphorylation. Consequently, Sturgill and co-workers (1988) demonstrated that S6 kinase was phosphorylated by partially purified insulin-stimulated MAPK2, the first evidence implicating a signal transduction phosphorylation cascade. This was recognised independently in other laboratories, and the MAPKs became known by a variety of designations reflecting the protein substrates that were originally used for their detection, MBP kinase (after myelin basic protein), RSK (after ribosomal S6 kinase) and MAPK2. Other names such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) point to the vast array of agents that stimulate the activation of the MAPK family (for reviews see L'Allemain, 1994; Robbins *et al.*, 1994).

Currently three MAPK pathways are known in mammalian cells, the best understood of these involves the ERKs. These archetypal members of the MAPK family have many isoforms: ERK1/p44mapk, ERK2/p42mapk, ERK3/p63- and p97mapk, ERK4/p46mapk, and p57mapk (Gonzalez *et al.*, 1992; Robbins *et al.*, 1994; Zhu *et al.*, 1994). ERK1 and ERK2 are the best characterised, ubiquitously expressed in all tissues and cell lines, although their subcellular distributions are less certain. Both of these MAPKs have been reported to be activated biphasically in fibroblasts treated with mitogens, with the first peak occurring between 5 and 20 minutes, and the second occurring between 1 and 4 hours. Activation of ERK1 and -2 during the second phase is correlated with both nuclear translocation and initiation of DNA synthesis (Seth *et al.*, 1992). A particular member of the MAPK family is ERK3, which has the highest expression level in early development, a restricted expression pattern (mainly neuronal cells and muscle), and a different substrate specificity (L'Allemain, 1994). Furthermore, ERK3 proteins possess a completely different regulatory site sequence, discussed below.

Distinct from the above classical MAPK proteins, recent work has identified a multifamily of MAPK proteins, which include the JNK/SAPK subfamily. JNK1 and -2

were identified as novel protein kinase activities that bind to the oncogene c-Jun within the transactivation domain and phosphorylate two serine residues, hence c-Jun NH2-terminal kinases (JNK), (Hibi *et al.*, 1993; Derijard *et al.*, 1994). Simultaneous studies identified three further MAPK-related proteins through the screening of a rat cDNA library (Kyriakis *et al.*, 1994). These kinases (α, β and γ) are strongly activated by stress-inducing stimuli, including translational inhibitors, heat shock and tumour necrosis factor (TNF), and were subsequently designated stress-activated protein kinases (SAPKs). The third type of mammalian MAPK to be identified is p38, which is activated by heat shock and hyperosmolar medium (Han *et al.*, 1994). It is interesting that p38 is most closely related to HOG1, an enzyme required for adaption to osmotic stress in *Saccharomyces cerevisiae.*

There are several other recently discovered mammalian kinases that are probably of the MAPK family, although how these proteins fall within the scheme of the three known cascades discussed above is presently not known. These include Fos-regulating kinase (FRK), which augments the transcriptional activity of c-Fos through phosphorylation at Thr-232, the homologue of Ser-73 of c-Jun (Deng and Karin, 1994). The other is reactivating kinase, RK, which both stimulates MAPK-activated protein kinase-2 and phosphorylation of the small heat shock proteins, and appears to be identical or at least closely related to p38 (Rouse *et al.*, 1994).

1.2.2. Selective activation of ERKs, JNK/SAPKs and p38

Transactivation of the MAPKs is through an ordered phosphorylation of both a threonine and a tyrosine residue, which form part of the dual-phosphorylation tripeptide motif, the sequence of which distinguishes the three MAPK subgroups. Phosphorylation on *TEY* is the defining property of the ERK subfamily, whilst the JNK/SAPKs and p38 MAPKs are phosphorylated on *TPY* and *TGY* respectively (Cano and Mahadevan, 1995). However, as previously mentioned, ERK3 is the anomaly being phosphorylated on *SEG*. Preceding the tripeptide motif, which is located between subdomain VII and VIII of the MAPK gene, is an autophosphorylation site. Whilst there is evidence that ERK1 and -2 can

autophosphorylate at the tyrosine and threonine within the motif, the basal rate of autophosphorylation is too slow to account for the rapid activation of MAPK activity upon mitogen stimulation (Seger *et al.*, 1991). It has become apparent that these phosphorylations are in fact mediated by upstream components called MAP kinase kinases (MKKs or MEKs, for continuity I shall use the designation MKK). A crucial question is how specificity of signalling through the MAPK subtypes is organised? There is strong evidence which implicates that the TEY, TPY and TGY motifs are targeted by different dual specificity kinases, which are selectively activated by various agonists. Present data argues for a complex interconnected system of upstream components that have access to separate, but co-ordinately regulated cascades controlling the distinct, dual specific MKKs.

Although hyperosmolarity can potentially activate all three MAPK subtypes (Matsuda *et al.*, 1995), the relative activation of the MAPKs is characteristic of the stimulus used (reviewed in Cano and Mahadevan, 1995). ERKs are potently activated by EGF (e.g. growth factors) and phorbol esters (TPA); whilst protein synthesis inhibitors, inflammatory cytokines (tumour necrosis factor α and interleukin-1), heat shock and ultraviolet irradiation strongly activate the JNK/SAPKs. Conversely, TPA has no effect on p38, which appears to be activated in the inflammatory response to lipopolysaccharides or to inflammatory mediators such as interleukin-1. However, the important caveat is that the comparative activation of the MAPKs subtypes in response to the above stimuli is specifically cell-type dependent.

1.2.3. MAP kinase activator: A dual-specific kinase

A 45 kDa protein factor that can induce phosphorylation and activation of inactive MAPK *in vitro* was first purified from *Xenopus* unfertilised eggs (Matsuda *et al.*, 1992) and subsequently two isoforms (MKK1 and MKK2) were isolated and cloned from mammalian cells (Seger *et al.*, 1992; Crews *et al.*, 1992; Ashworth *et al.*, 1992; Wu *et al.*, 1993). These dual specific kinases show a high degree of homology, and are themselves phosphorylated by Ser/Thr kinases at two sites located in the catalytic subdomain VIII. A distinctive feature of the vertebrate MKKs is a

proline-rich region inserted between kinase subdomain IX and X. The function of this domain is not clear, but it is interesting that in some isoforms (MKK1) this region contains phosphorylation sites and negative regulatory sequences (Wu *et al.*, 1993), implicating differences in feedback controls for MKKs.

Thus, as previously mentioned, recent evidence has established that the molecular basis for the separate identity of the MAPK signal transduction pathways is that each subtype is activated by a specific MKK in response to a certain stimuli, see figure 1.1, p13. The identification of further MKKs, namely MKK3 and MKK4 (Derijard *et al.*, 1995), has led to the demonstration that MKK1 and MKK2 exclusively activate the ERKs, whilst MKK3 is a specific activator of p38. In contrast, MKK4 activates both p38 and JNK. Sanchez and co-workers (1994) have also identified a novel protein kinase called SAPK/ERK kinase-1 (SEK1), which is the murine homolog of human MKK4; and a distinct upstream RK kinase (RKK) has been isolated by Rouse *et al.* (1994). It seems only plausible that as novel MAPK isoforms are being identified, the MKKs will follow suit, and in keeping with this Zhou *et al.* (1995) have recently identified an additional MKK5 along with its partner ERK5. Their results show that ERK5 does not interact with MKK1 or -2, and its predicted size is twice that of all known ERKs due to additional carboxy-terminal sequences, which may target the enzyme to cytoskeletal components of the cell. MKK5 is distinct from all other MKKs in that it contains a long amino terminal sequence which shows homology to the *S. cerevisiae* gene CDC24. The CDC24 encoded protein has GDP releasing activity and binds to the GTPase CDC42, the mammalian equivalent of which is Cdc42Hs, a member of the Rho subfamily. This may provide a mechanism for coupling GTPases to downstream protein kinase signalling cascades, a mechanism referred to later.

1.2.4. The MAP kinase kinase kinase family

The initial experiments that demonstrated that MKK was inactivated by Ser/Thr-specific but not Tyr-specific phosphatases led to the existence of a MAP kinase kinase kinase. The first to be identified was p74Raf-1 the product of the

c-Raf-1 proto-oncogene. Immunoprecipitates of oncogenically activated Raf, or of p74Raf-1 from growth factor stimulated cells, phosphorylate and reactivate dephosphorylated MKK (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Further studies utilising PCR with degenerate oligonucleotides designed against the yeast MKKKs identified the mammalian MKK kinase, MEKK (Lange-Carter *et al.*, 1993). Finally, work from Nebreda and Hunt (1993) has demonstrated that Mos may also act as a MKK kinase. Thus, three distinct protein kinases appear to activate MKKs, increasing the complexity of the MAP kinase cascade.

1.3. Is the MAP kinase cascade redundant?
- Evidence for the differential activation of the MAPK subfamilies

Intracellular signalling has been dominated by the MAPKs, more specifically the ERKs, which are activated through a kinase cascade which links the MKK kinase Raf to p21Ras. This is recognised as the classical transduction pathway and is discussed in the next section. However, the recent discovery of two other MAPK subtypes, the JNK/SAPKs and p38/RK isoforms has revealed the existence of parallel MAPK cascades that can be activated simultaneously. The concept of multiple MAP kinase cascades is by no means new, and presently are best characterised in *S. cerevisiae*, which has at least six identified pathways that contain MAPKs or their presumed upstream regulators (Herskowitz, 1995). The important distinction between these and mammalian MAPKs is that the various yeast pathways regulate separate physiological functions; they seem not to be activated in parallel in response to the same stimulus. Hence, we now need to reassess our understanding of the mammalian MAPKs. The balance between functional separation and crosstalk between these pathways is an interesting problem. However, it is anticipated that any crosstalk will promote the existence of a physiologically flexible and complex network.

The evidence for parallel cascades has been demonstrated by numerous studies. The expression of constitutively active oncogenic forms of Ras has been shown to potently induce the ERK subtypes (reviewed in Blenis, 1993), however, Ras fails to

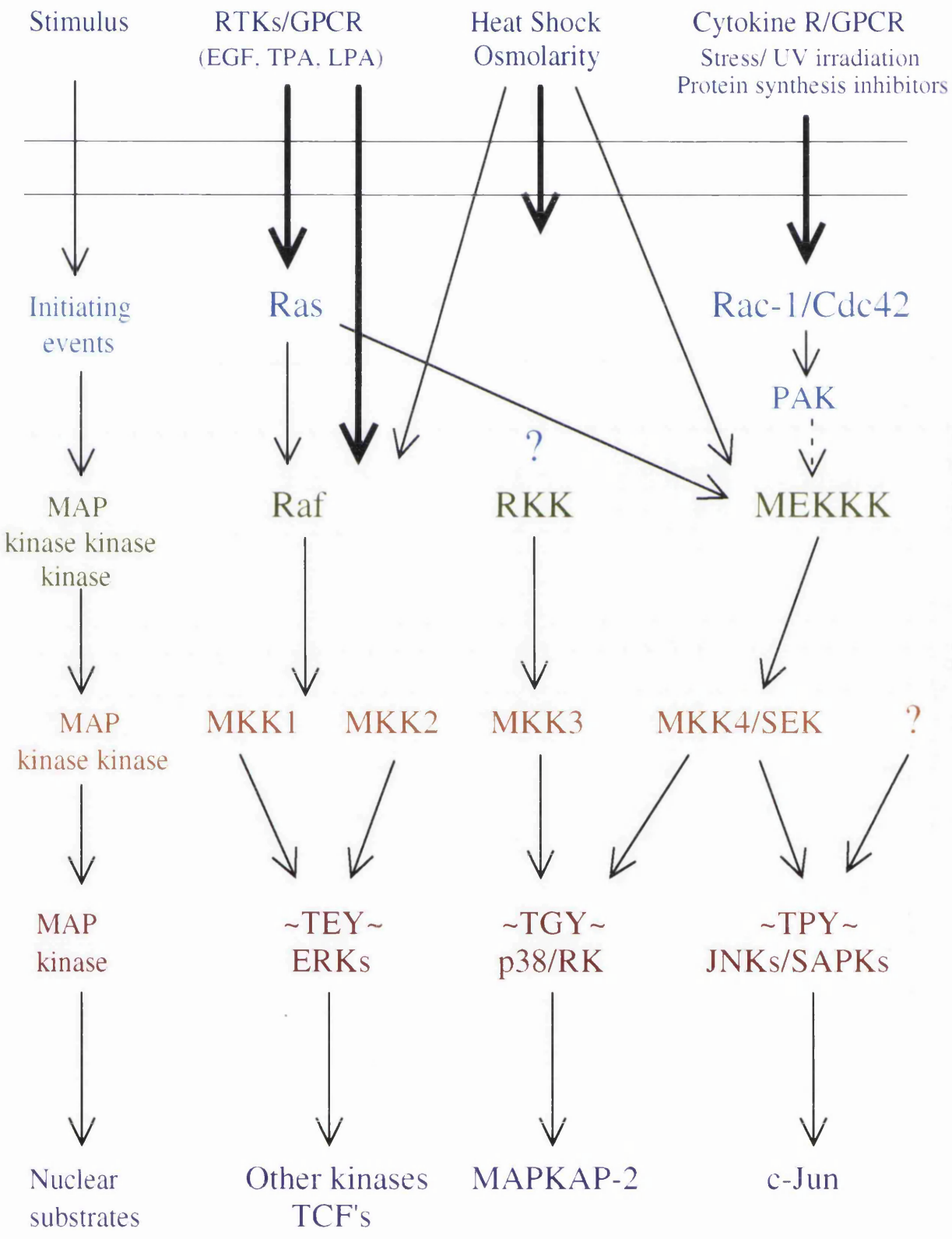
fully activate JNK (Ras is necessary only for growth factor activation of JNK), (Derijard *et al.*, 1994; Minden *et al.*, 1994). Furthermore, a division between the ERK and JNK cascades is implied by the evidence that Raf is not directly involved in JNK activation (Minden *et al.*, 1994). Instead the JNK cascade is strongly activated by the MAPKK kinase MEKK/SEK, which, unless overexpressed, does not lead to ERK activation (Sanchez *et al.*, 1994; Yan *et al.*, 1994). Although MEKK activity is stimulated by growth factors in a Ras-dependent manner (Lange-Carter *et al.*, 1993), the mechanism of its activation is not clear, and has been linked to G protein-coupled receptors as opposed to receptor tyrosine kinase receptors. These results support the contention that at least two signal transduction cascades exist in mammals with distinct functions: the MEKK/SEK/SAPK-mediated stress response and the Raf/MKK/MAPK-mediated mitogenic response, (see figure 1.1, p13). Hence, the specificity of SEKs and MKKs defines the segregation of the stress and mitogenic pathways. Intriguingly, two recent studies have implicated that the GTPase proteins Rac and Cdc42Hs, members of the Ras subfamily Rho, are specific activators of the JNK pathway (Minden *et al.*, 1995, Coso *et al.*, 1995a). The GTP-bound forms of Rac1 and Cdc42 can specifically associate and activate a novel Ser/Thr kinase, the p21-activated kinase (PAK65). This situation is highly analogous to the Ras GTPase-Raf interaction, suggesting that the Rho family of GTP-binding proteins might also initiate activity of a kinase cascade. The rationale is that either PAK65 or related kinases link Rac1 and Cdc42 to the MEKK/SEK/JNK module. As previously mentioned, it is interesting that a newly identified MKK5 contains a region homologous to the yeast protein CDC24 (Zhou *et al.*, 1995). CDC24 acts as a guanine nucleotide-exchange factor for the GTPase CDC42, which has been demonstrated to have a direct signalling role in the mating-pheromone response between the G protein and the downstream protein kinase cascade (Simon *et al.*, 1995). Thus, Cdc42Hs may provide the link between G proteins and the MEKK/SEK cascade, leading to the activation of JNK/SAP kinases. These studies suggest the divergence of signal transduction pathways at the levels of Ras and Raf, as opposed to the MAPKs as originally thought. This will prove to be an interesting area for future research.

Figure 1.1.

Schematic representation of the protein kinase cascades leading to the activation of distinct members of the MAP kinase family.

Accumulating evidence suggests that parallel kinase cascades control the activity of the MAPK isoforms. In mammalian cells, three hierarchical levels have been identified, represented by MAPK, MKK, and Raf-1/MEKK. The small GTP-binding protein Ras activates the classical ERK cascade, whereby binding of Ras to Raf-1 localises Raf to the plasma membrane, upon which a second (undefined) event takes place to activate the Raf kinase activity. In the JNK/SAPK signalling route, two members of the Rho family, Rac-1 and Cdc42, activate PAK65 which has been postulated to activate MEKK, however this connection may not be direct (broken arrow). Although MEKK activation can be Ras dependent, there is no evidence for a direct interaction. Both a distinct MKK isoform that is specific for the JNK/SAPK proteins, and the upstream kinases for the p38/RK pathway remain to be established (question marks). The thick arrows indicate the major response to the stimulus, note that not all stimuli leading to Raf-1 activation may be mediated by Ras. The precise pathways connecting cell surface receptors to the GTP-binding proteins are yet to be elucidated. There also exist cell line differences in the way these cascades are organised (for details see text).

Figure 1.1.


Stimulus
RTKs/GPCR
(EGF, TPA, LPA)
Heat Shock
Osmolarity
Cytokine R/GPCR
Stress/ UV irradiation
Protein synthesis inhibitors
Initiating events
Ras
Rac-1/Cdc42
PAK
?
MAP kinase kinase kinase
Raf
RKK
MEKKK
MAP kinase kinase
MKK1
MKK2
MKK3
MKK4/SEK
?
MAP kinase
~TEY~ ERKs
~TGY~ p38/RK
~TPY~ JNKs/SAPKs
Nuclear substrates
Other kinases TCF's
MAPKAP-2
c-Jun

1.4. Protein interactions that regulate the MAP kinase cascade

1.4.1. Coupling to the receptor

Two major mechanisms exist for transmembrane signalling in intracellular communication, mediated respectively by receptor tyrosine kinases (RTKs) and by G-protein-coupled receptors (GPCRs). While traditionally, these two pathways have been considered to be totally independent, recent evidence has shown that these pathways can converge on the same effectors, namely the Ras-MAPK cascade.

1.4.2. Receptor tyrosine kinase mediated activation of Ras

A variety of extracellular ligands (growth factors or cytokines) have been shown to cause an activation of p21Ras, among these are EGF, insulin, PDGF and macrophage-colony stimulating factor. Other tyrosine kinases such as Src, Abl and Neu also transduce signals which stimulate Ras (reviewed in Maruta and Burgess, 1994; Medema and Bos, 1993). Considerable recent attention has focused on developing a detailed model for explaining how EGF activates Ras, and it is this pathway that will be discussed here, although undoubtedly, aspects of this intracellular signalling mechanism will be common to all extracellular stimuli and RTKs.

The epidermal growth factor receptor is a 170-kDa transmembrane glycoprotein with an extracellular domain that binds EGF and a cytoplasmic domain with intrinsic tyrosine kinase activity (reviewed in Prigent and Lemoine, 1992). The binding of EGF causes an increase in the receptor tyrosine kinase activity by an allosteric mechanism that involves receptor dimerisation (Schlessinger, 1988; Lemmon and Schlessinger, 1994). The activated EGF receptor then undergoes both autophosphorylation of the carboxy-terminal domain, and phosphorylation of substrate proteins such as RasGAP, phospholipase C-γ, and phosphatidyl inositol 3-kinase. With the exception of the insulin receptor family, all known receptors with tyrosine kinase activity undergo dimerisation upon binding of their cognate ligand, and multiplicity of tyrosine autophosphorylation sites is thought to underlie the pleiotropic cellular response to growth factors. These autophosphorylation sites represent recognition structures for specific target proteins

containing Src homology 2 (SH2) domains. SH2 domains are conserved sequences of approximately 100 amino acids found in various signalling molecules and oncogenic proteins (reviewed in Margolis, 1992; Cohen *et al.*, 1995), and they provide a mechanism whereby growth factor stimulation can couple to multiple intracellular signalling pathways. Different SH2s bind to distinct phosphotyrosine containing regions, the specificity of which are determined by the residues immediately adjacent to the phosphotyrosine residue. SH2 domains are often accompanied by a stretch of conserved sequences termed SH3 domains, which are proline-rich binding sites, indicative of a role in the intracellular compartmentalisation of proteins. Some molecules with SH2 or SH3 domains have intrinsic enzymatic activity, for example Src and RasGAP. Others, often with multiple SH2 and/or SH3 domains, serve as bivalent or multibivalent adaptors that mediate interactions among signalling molecules.

Two proteins that appear to mediate EGF receptor-induced Ras activation include Grb2 and Sos. Grb2 (Growth factor receptor-bound protein 2) is a cytosolic protein, whose entire sequence is composed of a single SH2 domain flanked by two SH3 domains. Identification of Grb2 was through the high affinity binding of the SH2 domain to autophosphorylated EGF receptors (Lowenstein *et al.*, 1992). Grb2 binds to a second protein, mSos (mammalian homolog to the *Drosophila* Ras activator Son of sevenless), which is also cytosolic and functions as a guanine nucleotide-releasing factor (GNRF), facilitating the activation of Ras (Bonfini *et al.*, 1992; Bowtell *et al.*, 1992). In most cells, prior to receptor stimulation, Grb2 and Sos are already associated forming a stable interaction through the carboxy terminal proline-rich domain of Sos and an SH3 domain of Grb2. Following receptor stimulation, autophosphorylated receptor binds the Grb2/Sos complex through the SH2 domain of Grb2, thereby recruiting the cytosolic complex to the plasma membrane where Ras is localised, see figure 1.2, p17 (Buday and Downward, 1993; Rozakis-Adcock *et al.*, 1993). The binding of Sos to Grb2 is not essential for Ras activation since truncated 5'-Sos, which no longer binds Grb2, is an efficient activator of Ras provided it is targeted to the plasma membrane by means of a myristoylation signal (Aronheim *et al.*, 1994). In these experiments the truncated Sos derivatives were considerably more

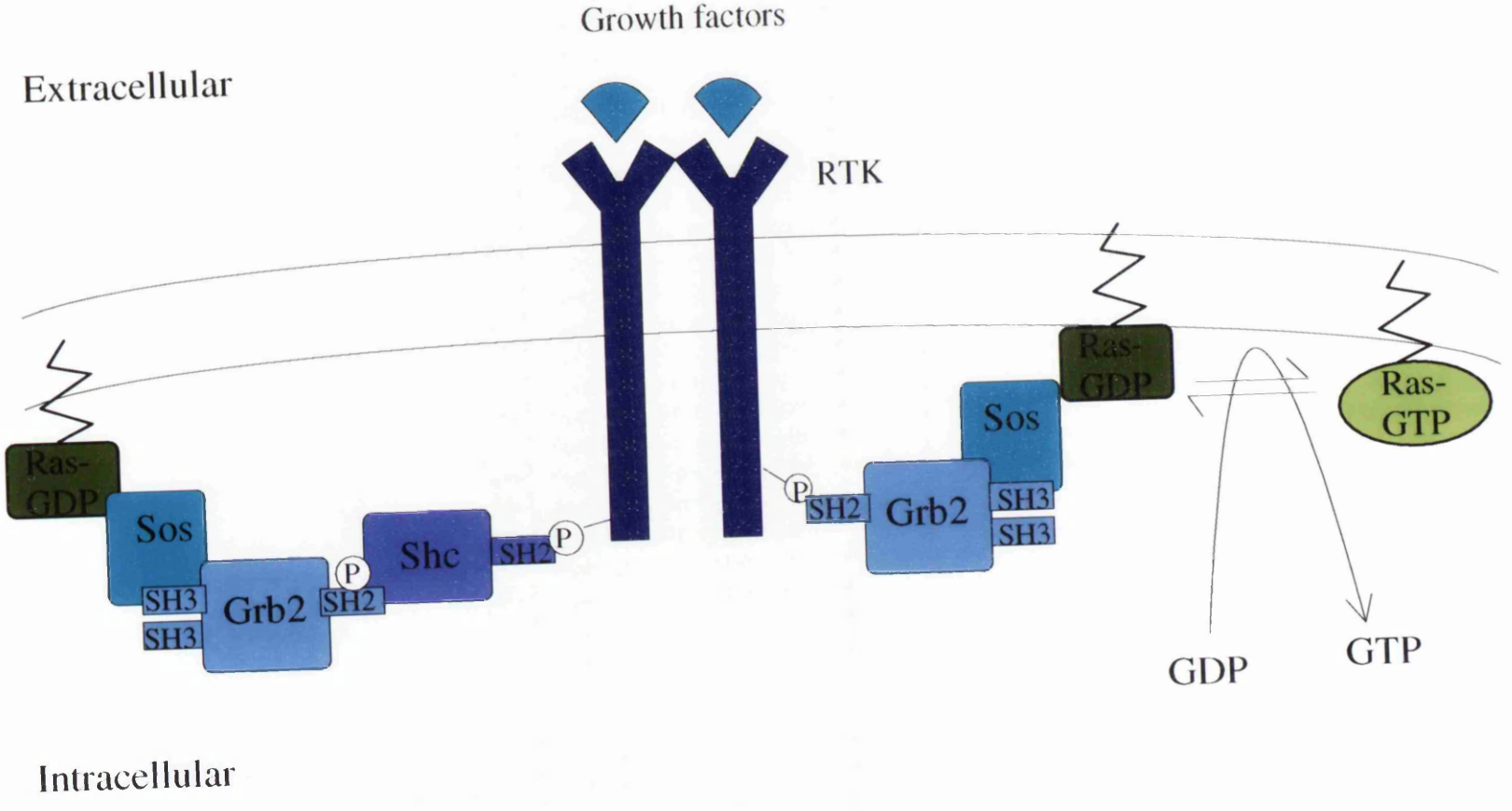
active, suggesting that a further mechanism may relieve the inhibitory effect of the Sos carboxy-terminus, such as allosteric changes induced upon Grb2 binding to the receptor, or indeed phosphorylation of Sos. It is possible that kinases downstream of Ras act in a positive feedback loop by phosphorylating the mammalian homolog of Sos. It is becoming apparent that Grb2 can be "plugged in" to many other receptor systems. Indeed, Schlaepfer *et al.* (1994) demonstrate that integrin-stimulated signal transduction leads to the subsequent phosphorylation of FAK (focal adhesion protein-tyrosine kinase) by c-Src or related kinases. This creates an SH2-binding site for Grb2 which may link integrin engagement to the activation of the Ras/MAPK signal transduction pathway. Grb2 represents a newly emerging class of adaptor proteins, whose common feature is signal transmission to Ras. One such protein Crk, consists of an SH2 and an SH3 domain, but unlike Sos and Grb2 is highly oncogenic, suggesting that Crk oncogenicity is not totally dependent on Sos. In accordance with this, a recently identified GNRF for Ras, C3G, has been demonstrated to bind to the SH3 domain of Crk (Tanaka *et al.*, 1994). A third adaptor protein is Shc, consisting of only an SH2 domain, which also binds autophosphorylated EGF receptor. Once bound, Shc is itself tyrosine phosphorylated and consequently able to bind the Grb2/Sos complex, see figure 1.2, p17 (Pelicci *et al.*, 1992, Sasaoka *et al.*, 1994). Free Shc·Grb2/Sos complexes are known to exist, consistent with either dissociation from the receptor, or a parallel pathway containing an intermediary tyrosine kinase, such as Src, which phosphorylates Shc such that it binds Grb2. Hence, it is clear that many molecules with different surface interfaces are capable of transmitting extracellular stimuli to activate the p21Ras pathway.

Figure 1.2.

A model depicting possible interactions between receptor tyrosine kinases and Ras.

Tyrosine autophosphorylated EGF receptor binds to the SH2 domain of Shc, which is itself tyrosine phosphorylated enabling the binding of the SH2 domain of Grb2. Grb2 binds with the guanine nucleotide-exchange factor Sos, via one of its SH3 domains, thus forming a multimeric unit which enhances the formation and stimulation of p21Ras-GTP. Alternatively, the EGF receptor can bind directly to the Grb2 SH2 domain forming an EGFR•Grb2/Sos complex. It is possible that a single EGF receptor can concomitantly associate with both Grb2/Sos and Shc•Grb2/Sos complexes. Finally, following EGF stimulation, Shc•Grb2/Sos complexes could exist, independent of the EGF receptor.

Figure 1.2.


Growth factors
Extracellular
RTK
Ras-GDP
Sos
SH3
SH3
Grb2
P
SH2
Shc
SH2
P
P
SH2
Grb2
SH3
SH3
Sos
Ras-GDP
Ras-GTP
GDP
GTP
Intracellular

1.4.3. G protein coupled-receptor activation of Ras

In addition to the widely studied ligands of RTKs, certain agonists of GPCRs are also capable of stimulating DNA synthesis in responsive cells. Such mitogens include the lipid lysophosphatidic acid (LPA), a platelet-derived serum factor, although preliminary analysis suggests that LPA is also released by growth factor stimulated fibroblasts (Fukami and Takenawa, 1992). LPA has long been known as a critical precursor in glycerolipid biosynthesis, however, its function as a "first messenger" has only recently been recognised (for review see Moolenaar, 1995). A major signalling event induced by LPA is the rapid and transient activation of the proto-oncogene product p21Ras in quiescent fibroblasts. There is compelling evidence to indicate that LPA-induced mitogenesis is through a pertussis toxin (PTX)-sensitive pathway, assigning a central role for a heterotrimeric G protein of the G_i subfamily; yet it appears that G_i-mediated p21Ras activation is independent of known PTX-senstive effector routes (Van Corven *et al.*, 1993). One such pathway is the LPA stimulated inhibition of adenylyl cyclase (Van Corven *et al.*, 1989), however, raising cAMP levels does not affect Ras activation. G_i-mediated activation of the Ras-MAPK pathway is not unique for LPA: it is also observed after stimulation of the endogenous thrombin receptor in hamster fibroblasts (Van Corven *et al.*, 1993), and transfected muscarinic M_2 or α_2- adrenoceptors in Rat-1 cells (Winitz *et al.*, 1993; Alblas *et al.*, 1993).

At present, relatively little is understood about the intermediary signals that couple G_i to Ras-MAPK activation. However, increasing evidence implicates that it is the $\beta\gamma$-subunits of the G proteins which are specifically involved, see figure 1.3, p20 (Faure *et al.*, 1994; Crespo *et al.*, 1994). Further studies also demonstrate that dominant-negative versions of the Ras and Raf kinases inhibit LPA-induced activation of MAPK (Cook *et al.*, 1993; Howe and Marshall, 1993). It has been proposed that the effector pathway comprises a non-receptor tyrosine kinase, since tyrosine kinase inhibitors such as staurosporine, selectively inhibit LPA-induced Ras-GTP accumulation at doses that do not effect EGF-induced Ras activation (Hordijk *et al.*, 1994). Obvious candidates are the members of the Src family of tyrosine kinases, since Ras acts downstream of activated $p60^{src}$ in proliferation and transformation of

fibroblasts (Smith *et al.*, 1986), and LPA and thrombin rapidly activate Src in their target cells (Jalink *et al.*, 1993; Chen *et al.*, 1994a), although it remains to be seen whether this activation is G_i mediated. However, the use of Src-neutralising antibodies suggests that Src family kinases are not critically involved in LPA-induced mitogenesis (Roche *et al.*, 1995). Additional studies by Tsukada and colleagues (1994), have demonstrated a direct interaction between the βγ-subunits of G_i protein and the pleckstrin homology (PH) domain of Bruton tyrosine kinase, Btk. Btk is a non-receptor tyrosine kinase linked to B-cell deficiencies in humans and mice, and defective proteins are the result of mutations in the PH domain. The PH domain is a region of approximately 100 amino acids found in a wide variety of signalling and cytoskeletal proteins. The function of PH domains is unknown, but it is clear that they provide an interface through which kinases can couple to membrane-anchored $G_{\beta\gamma}$ subunits in a stimulus-dependent manner (for a review on protein binding domains see Cohen *et al.*, 1995). Transmission of the signal from $G_{\beta\gamma}$ to Ras could then be realised through binding of beta/gamma dimers to the PH domain present in several Ras interacting molecules as in GRF, the GDP releasing factor; in SOS, the GDP to GTP exchange factor; or in GAP, the GTPase activating protein, or indeed through the interaction with non-receptor tyrosine kinases such as Btk. It is interesting that both the RTK and GPCR pathways recruit cytoplasmic intermediates to the membrane by lipid-modified G proteins such as Ras and $G_{\beta\gamma}$. Whether the PH domains serve as a general mechanism for protein-protein interactions analogous to those mediated by SH2- and SH3 domains is currently speculative. Blesen and colleagues (1995), have demonstrated that activation of G_i-coupled receptors by LPA leads to the tyrosine phosphorylation of Shc, mediated entirely through the $G_{\beta\gamma}$ subunits. Concurrent with Shc phosphorylation is the functional recruitment of the Grb2/Sos complex, which subsequently leads to Ras-dependent MAPK activation. It is feasible that $G_{\beta\gamma}$ subunits might activate Ras by a Sos PH domain-directed translocation event, however, disruption of the Shc•Grb2/Sos interaction blocks MAPK activation, demonstrating that both Shc and Grb2 are necessary to transmit the $G_{\beta\gamma}$ signal to MAPK.

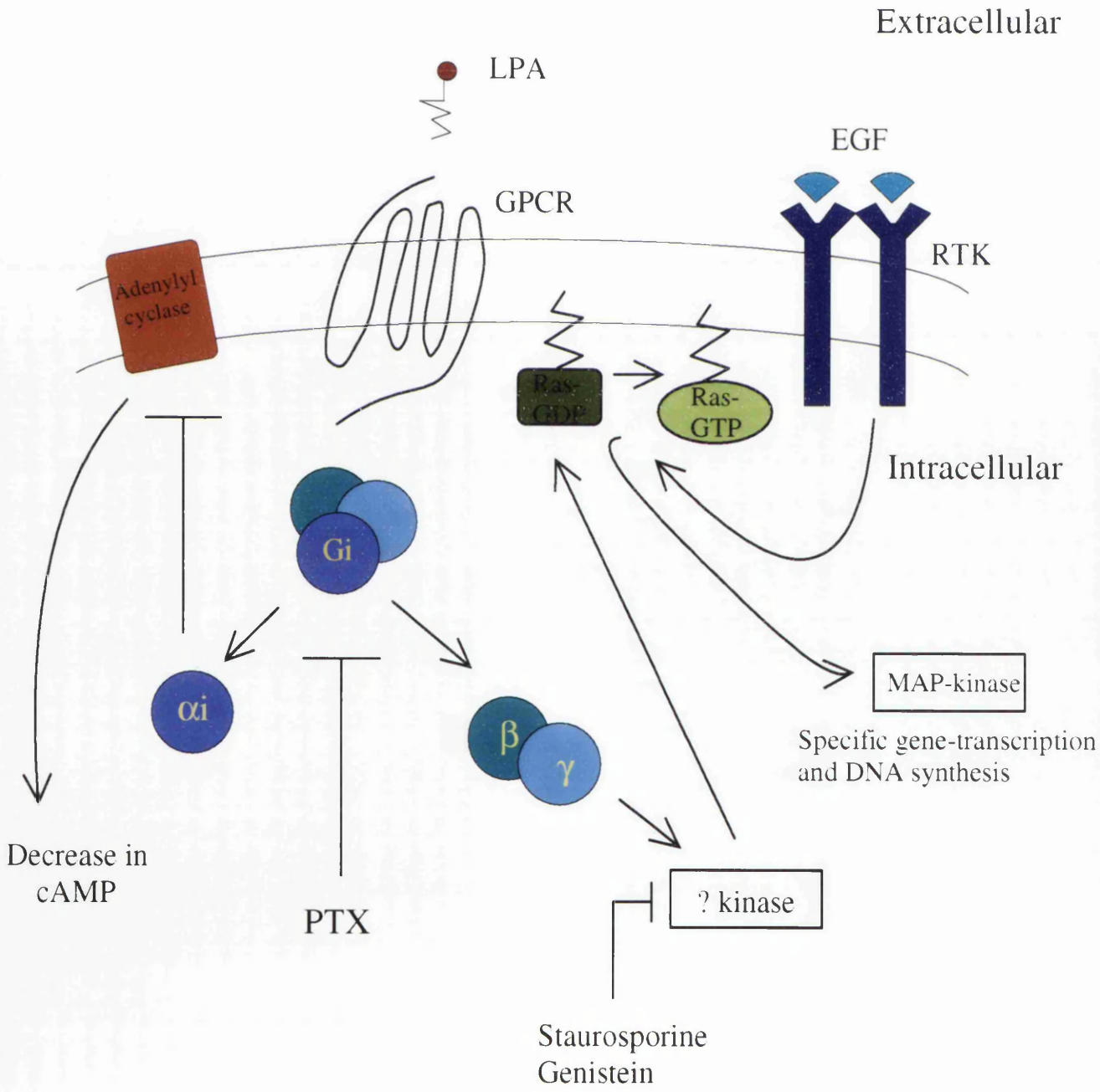
It is worth mentioning at this point that a recent study by Coso *et al.* (1995b) demonstrated that G protein-coupled receptors can lead to the activation of JNK, which is temporally distinct from the activation of p21Ras and ERKs. Whilst evidence presented by Vara Prasad *et al.*, (1995) describe a Ras-dependent signal transduction pathway involving the α subunit of the G proteins regulating the JNK/SAPKs. These findings are corroborative with previous studies that parallel pathways evidently exist leading to the activation of either ERKs or JNKs.

Figure 1.3.

Proposed model of early LPA signalling pathway triggering G_i-mediated Ras activation.

LPA activates its cognate G protein-coupled receptor to trigger inhibition of adenylate cyclase (flat headed arrow), and activation of p21Ras which subsequently leads to activation of the MAP kinase pathway. Both these events are mediated through the pertussis toxin sensitive G_i protein. Ligand binding to the G protein-coupled receptor leads to the dissociation of Gβγ from Gα upon GTP-GDP exchange. Gα-subunits inhibit adenylyl cyclase, resulting in decreased cAMP levels, while free Gβγ-subunits activate p21Ras via a putative staurosporine- and genistein-sensitive protein tyrosine kinase. For further details, see text.

Figure 1.3.


Extracellular
LPA
EGF
GPCR
RTK
Adenylyl cyclase
Ras-GDP
Ras-GTP
Intracellular
Gi
αi
β
γ
MAP-kinase
Specific gene-transcription and DNA synthesis
Decrease in cAMP
PTX
? kinase
Staurosporine
Genistein

1.4.4. The role of Ras in signal transduction

The requirement for *ras* activity in RTK signalling was proposed as early as 1986 on the basis of experiments with neutralising antibodies against p21Ras (Smith *et al.*, 1986). In all eukaryotic organisms examined, Ras homologues have been identified which act as key, pivotal signal transducers in receptor-activated pathways. Significantly, mutations of Ras are prevalent in many different forms of human cancers, accounting for approximately 30% of carcinomas. Consequently their role in signal transduction and transformation has been studied extensively, (for comprehensive reviews see Maruta and Burgess, 1994; Boguski and McCormick, 1993; Medema and Bos, 1993).

Mammalian cells express four "true" Ras proteins (encoded by H-*ras*, N-*ras*, K-*ras*A, K-*ras*B), each of which can function as an oncogene following mutational activation. These isoforms are plasma membrane-associated guanine nucleotide-binding proteins which function as binary switches, cycling between active and inactive states bound to GTP and GDP respectively. The GTP-bound form is slowly converted to the GDP-bound form by the protein's intrinsic capacity to hydrolyse GTP, a process accelerated by GTPase-activating proteins (GAPs), of which there are two in mammalian cells: p120-GAP and neurofibromin, which thereby negatively regulate Ras function (Trahey and McCormick, 1987; Xu *et al.*, 1990; Buchberg *et al.*, 1990). Activation in turn involves the replacement of GDP for GTP, an event mediated by guanine nucleotide-exchange proteins (or guanine nucleotide-release factors), such as SOS and Ras-GRF (Downward, 1992; Buday and Downward, 1993).

The elucidation of the identities of the critical cellular targets, or "effectors" through which Ras proteins exert their biological effects has long been an area of intense research. The domain of Ras that interacts with the effectors has been delineated by a set of mutations in activated mutant Ras that ablates its biological activity, without reducing GTP binding. This region, between residues 32-40, is defined as the effector domain (reviewed in Marshall, 1993). The first protein for which evidence for a possible effector role was found was p120-GAP (Cales *et al.*, 1988). This protein interacts only with GTP-bound Ras and fails to interact with most

effector region mutants. This is also true for the GAP-related protein neurofibromin (Downward, 1992). Because both these proteins negatively regulate Ras, this activity could be incorporated in an effector as a negative feedback mechanism. Strong evidence indicates that p120-GAP can have an influence downstream of Ras in various signalling pathways (Yatani *et al.*, 1990; Schweighoffer *et al.*, 1992; Duchesne *et al.*, 1993). Studies by Medema and co-workers (1992), suggest that GAP SH2-SH3 domains serve to induce gene expression by p21Ras, but that additional signals coming from p21Ras are required for full functioning. However, contrasting data demonstrates only a negative regulatory function for p120-GAP (Zhang *et al.*, 1990; Gaul *et al.*, 1992). Therefore, although GAP can mediate Ras activity in some situations, it seems unlikely that it can account for all the signals emerging from Ras.

Significant evidence suggests that GTP-Ras is an essential component of the pathway leading to the activation of MAPK (reviewed in Blenis, 1993). The introduction of oncogenic forms of *ras* into cells activates MAPK in the absence of growth factor stimulation (Leevers and Marshall, 1992), while transfection with dominant negative mutants of *ras*, overexpressed from a dexamethasone-inducible promoter, blocks the activation of MAPK in response to growth factors (Thomas *et al.*, 1992; Wood *et al.*, 1992). Furthermore, microinjection of oncogenic Ras into *Xenopus* oocytes or its addition to cell-free extracts leads to MAPK activation (Hattori *et al.*, 1992). The effectors discovered so far that are most likely to mediate the growth stimulatory signals from Ras are the cytoplasmic serine/threonine Raf kinases. These function downstream of Ras and activate the MAPK, and possibly other, pathways.

1.4.5. Ras-Raf interactions

That p21Ras and Raf-1 kinase function in the same signalling pathway has been demonstrated by several laboratories: expression of oncogenic forms of p21Ras leads to enhanced phosphorylation of *raf-1* kinase in NIH3T3 and PC12 cells (Wood *et al.*, 1992), whilst transformation by *v-raf* does not require the function of p21Ras (Smith

et al., 1986). Recently, a number of independent studies have converged to clearly establish the direct Ras-Raf association.

The structure of p74Raf-1 contains three regions (CR1, CR2 and CR3) conserved among all Raf family members (Daum *et al.*, 1994). CR3 in the carboxy-terminal half of the protein is the catalytic domain, while CR1 and CR2 comprise the amino-terminal domain. The Ras-Raf association has been demonstrated through the use of fusion-binding proteins *in vitro* (Warne *et al.*, 1993; Vojtek *et al.*, 1993), as well as the yeast two hybrid system that detects protein-protein interactions (Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993). Ras-Raf binding requires the effector domain of transformation-competent Ras and the CR1 of Raf, where the putative zinc finger motif is necessary for optimal binding to the lipid bilayer. Activation of the transforming potential of Raf-1 has been associated with amino-terminal truncation and/or fusion of Raf-1 to other proteins, suggesting that the amino-terminal half harbours a negative regulatory domain. However, recent findings demonstrate that the amino-terminal deletions in Raf-1 do not necessarily result in constitutive kinase activity (Chow *et al.*, 1995).

Recent work shows that the role of p21Ras in Raf-1 activation is for p21Ras-GTP to translocate cytosolic p74Raf-1 to the plasma membrane, where Raf then becomes activated (Traverse *et al.*, 1993). Whilst this association can occur in the absence of GTP, interaction between Ras and Raf is reported to be much stronger in the presence of GTP, perhaps a GTP-induced conformational change occurs which enables Raf to become stabilised in the plasma membrane. (Warne *et al.*, 1993). However, activation of purified Raf by Ras-GTP has not yet been demonstrated, implicating that additional components or modifications may be required, and several lines of evidence suggest that the Ras-Raf complex must contain some other protein(s) (Maruta and Burgess, 1994; Wartmann and Davis, 1994).

1.4.6. Components of the Raf-binding multicomplex

Physiological activation of Raf-1 is paralleled by its hyperphosphorylation, which occurs mainly on serine residues, but is also present on threonine and tyrosine

residues (Daum *et al.*, 1994). Previous work in which Raf-1 and oncogenic Src are overexpressed in insect cells indicates a role for tyrosine phosphorylation in the activation of Raf-1 (Fabian *et al.*, 1993). Marais and co-workers (1995), have demonstrated that in mammalian cells, the maximal level of activation of Raf-1 is observed when it is co-expressed with oncogenic Ras and Src, thereby it is the combination of the synergy between Ras and Src in promoting both membrane-bound activation and tyrosine phosphorylation of p74Raf-1. In vertebrate cells, the phosphorylation of tyrosine appears to be an additional level of control imposed on Raf-1, since Raf proteins of *D. melanogaster* and *C. elegans* have negatively charged amino acids in place of the modified tyrosine residues. However, this supports the idea that there must be additional membrane-localised events that can activate Raf-1, since a negative charge can be achieved through an acidic amino acid, as is the case with mammalian B-Raf, or by phosphorylation of tyrosine residues.

The highly conserved 14-3-3 family of proteins also function as potential co-activators of Raf-1. Several experimental approaches have identified the 14-3-3 proteins complexed with Raf-1, both in the cytosol and the plasma membrane (Yamamori *et al.*, 1995; Fu *et al.*, 1994; Freed *et al.*, 1994). However, while these studies suggest that 14-3-3 proteins can stimulate Raf-1 activity, no direct activation of recombinant Raf-1 by 14-3-3 proteins *in vitro* has been demonstrated. In fact, their interaction with inactive, cytosolic Raf-1 would suggest, therefore, that 14-3-3 acts as a co-factor that is necessary, but not sufficient for Raf-1 activation. It has been recognised for some time that the 14-3-3 proteins interact with many different kinases (reviewed in Burbelo and Hall, 1995; Aitken, 1995). One possible function is that cells have recruited these proteins to regulate key kinases such as protein kinase C, Raf and Bcr, so that multiple signal transduction pathways can be simultaneously co-ordinated. Alternatively, 14-3-3 proteins may act independently in these transduction pathways, as co-factors or scaffolds, allowing multiple kinases to function in an ordered fashion.

1.4.7. The linearity of the Ras/Raf pathway

In the eye development of *Drosophila* and the vulval induction of *C. elegans*, Ras, Raf-1 and MAPK have been suggested to form a linear pathway (reviewed in Perrimon, 1993; Selfors and Stern, 1994). It is clear however, that coupling of Ras/Raf with the MAPK signal transduction pathway has many branches, and is subject to regulation by other signalling proteins, depending on the cellular background (Gallego *et al.*, 1992). Ras is now implicated not only in the activation of Raf-1 and MEKK, but has other effectors including phosphatidylinositol-3-OH kinase and p120-GAP (Rodriguez-Viciana *et al.*, 1994; Duchesne *et al.*, 1993). In line with this, Raf-1 has substrates other than MKK, such as IκB, a component of the NF-κB transcription factor (Li and Sedivy, 1993); and interestingly, Raf-1 activation is not always accompanied by MAPK activation (Porras *et al.*, 1994). There also exist Ras-independent pathways for Raf-1 activation, protein kinase C is one candidate (Sozeri *et al.*, 1992; Kolch *et al.*, 1993). Significantly, the activation of protein kinase A by cyclic AMP levels suppresses the activity of Raf-1 (Cook and McCormick, 1993). Furthermore, as previously mentioned in section 1.3, some subtypes of MAPK can be activated without activation of Ras or Raf-1, in a MEKK/SEK-dependent manner. It is also clear that ERK2 activation can be mediated by a protein kinase C cascade independent of Ras (Burgering *et al.*, 1993), presumably this pathway converges at the level of Raf-1 activation. The cloning of an intracellular receptor for protein kinase C which shows homology to the β subunit of heterotrimeric G proteins, defines a way that protein kinase C could be activated itself from the growth factor receptor (Ron *et al.*, 1994). It would seem that independent pathways to regulate MAPKs co-exist within one cell type, but the nature of these pathways is cell type specific by virtue of the agonist employed by that cell, although a stimulus can exert an effect through different combinations of these pathways.

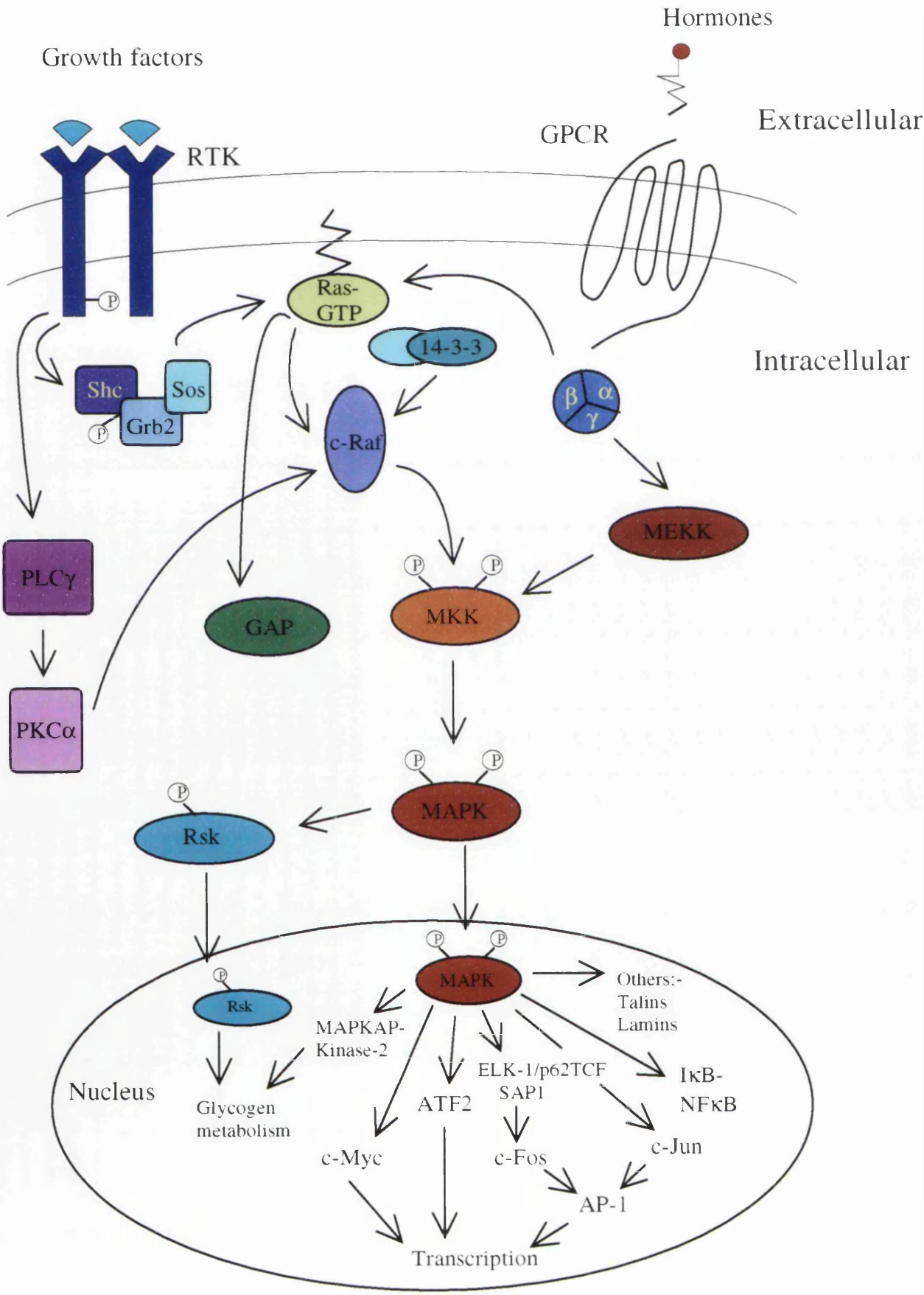
A model for the signal transduction pathway regulating the MAPKs, and their substrates, is illustrated in figure 1.4, overleaf.

Figure 1.4.

Schematic representation of the signal transduction pathways regulating the MAP kinase family and their substrates.

Greater detail for this model is provided in the text. For simplicity, JAK/STAT-mediated signalling, and some of the regulatory feedback loops described in the text are omitted. Briefly, the SH2-adaptor protein Shc is tyrosine phosphorylated in response to growth factor-activated tyrosine kinases. The tyrosine phosphorylated Shc then interacts with Grb2 via its SH2 domain. Alternatively, Grb2 may directly interact with activated receptors. Grb2 then recruits the Ras effector molecule Sos to the plasma membrane to activate p21Ras. Ras then activates Raf-1 by an as yet unknown mechanism, but which involves the 14-3-3 proteins, and then subsequently activates a phosphorylation cascade within which reside the MKKs, MAPKs and RSK. Raf is also activated by PKCα, although precisely how PKC participates in this phosphorylation cascade is not known. This pathway may also be regulated by the heterotrimeric G proteins via activated MEKK protein. MAP kinase and RSK have been shown to be both cytoplasmic and nuclear, and possess the potential for directly regulating gene expression by transcription factor phosphorylation. (Diagram adapted from Blenis, 1993).

Figure 1.4.


Hormones
Growth factors
Extracellular
GPCR
RTK
Ras-GTP
14-3-3
Intracellular
Shc
Sos
Grb2
c-Raf
β
α
γ
MEKK
PLCγ
GAP
MKK
PKCα
MAPK
Rsk
Nucleus
Rsk
MAPK
Others:-
Talins
Lamins
MAPKAP-Kinase-2
ELK-1/p62TCF
SAP1
IκB-NFκB
ATF2
Glycogen metabolism
c-Myc
c-Fos
c-Jun
AP-1
Transcription

1.5. The substrates of MAP kinase

The sequence of the MAPK phosphorylation site in MBP led investigators to realise the specificity of the amino acid environment around the phosphorylated residues. A MAP kinase consensus sequence was thus established as being P-X-S/T-P, where X is a basic or neutral amino acid, (see reviews in L'Allemain, 1994; Robbins *et al.*, 1994). Using this consensus phosphorylation site for MAPK targets, a series of putative *in vivo* substrates have been characterised.

1.5.1. Cytosolic targets

The initial interest in MAPK arose after *in vitro* studies demonstrated that an insulin-stimulated protein kinase activity was able to phosphorylate and activate the ribosomal protein S6 (Sturgill *et al.*, 1988). The physiological relevance of activated S6 kinase in mitogenicity is unclear, however, phosphorylated p90Rsk does translocate into the nucleus (as does MAPK), where there is the possibility of interaction with proteins involved in the regulation of transcription. Further cytosolic substrates include MBP, MAPKAP Kinase 2 (MAP Kinase-Activated Protein Kinase), and phospholipase A_2 (PLA_2). PLA_2 causes hormone-dependent release of arachidonic acid, the precursor of biosynthesis pathways involved in the inflammatory response. Whilst MAPK signalling is not the only way to activate PLA_2, its potential as an *in vivo* substrate implies that the MAPKs lie at a pivotal point in a pathway that controls numerous second messengers derived from arachidonic acid. A number of other substrates that have been identified *in vitro* are part of the MAPK cascade, and therefore have the capability of functioning within a regulatory feedback loop. These proteins include the EGF receptor, Raf/MKK and Sos. The phosphorylation of the EGF receptor by MAPK at Thr 669 was reported to regulate receptor internalisation, however, presently there is no evidence for the phosphorylation affecting tyrosine kinase activity of the receptor. MAPK phosphorylation sites are also present at the carboxy-terminus of the MKK proteins. Recent evidence from a number of laboratories suggests that phosphorylation of the two sites in MKK1 is not

accompanied by activation of the protein, and indeed, mutation of the threonine residues within these two MAPK consensus sites suggests that MKK1 protein is negatively regulated by MAPK-directed threonine phosphorylation (L'Allemain, 1994). The phosphorylation pattern of Raf is exceedingly complex, and as with Sos, the MKKs and the EGF receptor, more research is necessary to accurately decipher how these MAPK-catalysed phosphorylations are involved, if at all, in a regulatory feedback loop on the MAPK cascade.

1.5.2. Nuclear targets- Transcription factors

Primary gene induction or repression of "immediate-early" genes in eukaryotes does not require *de novo* protein synthesis, suggesting the involvement of post-translational modifications. Since stimuli that affect gene expression also lead to the activation of protein kinases, it is not surprising that transcription factors are directly regulated by protein phosphorylation (Meek and Street, 1992). Transcriptional targets for the MAPKs comprise the components of the AP-1 activator, the proto-oncogene c-Myc, and NF-IL6, whilst other nuclear substrates include the anti-oncogene p53 and the nuclear lamins. This introduction will briefly outline the phosphorylation of a selected number of nuclear proteins involved in the AP-1 complex, for a more comprehensive review of nuclear targets see L'Allemain (1994). Phosphorylation of transcription factors by MAPKs is made attractive by the observation that activated MAPKs can enter the nucleus (Seth *et al.*, 1992). However, this process is independent of the activation state of MAPK since the mutation of one or two of the regulatory sites of MAPK do not alter the kinetics of nuclear localisation (Lenormand *et al.*, 1993). It is possible that nuclear translocation of MAPK requires a chaperone, as the MAPK sequence does not contain a nuclear localisation signal.

1.5.2.1. The regulation of AP-1 activity by MAP kinases

The activator protein-1 (AP-1) transcription factor modulates the expression of genes implicated in growth regulation, differentiation, and neoplastic transformation by binding to consensus DNA sequences, the TRE (TPA Responsive Element), within gene promoter regions. Activation of AP-1 is postulated to involve a network of kinases which may be classified as those that increase the abundance of AP-1 components, i.e. their transcription, and those that stimulate their activity, i.e. transactivation.

Most of the genes that encode AP-1 components behave as "immediate-early" genes, and among these the regulation of c-*fos* and c-*jun* transcription is best understood. Several *cis* elements mediate c-Fos induction: a cAMP response element, a serum response element, and a Sis-inducible enhancer that mediates induction by stimuli that activate the JAK (Janus) group of protein kinases. In this situation, a "direct effector" model has been proposed which involves the activation of cytoplasmic transcription factors by tyrosine phosphorylation. For example, the ligands interferon α and γ employ a pathway that leads to the phosphorylation of two polypeptides, p113 and p91 or p84, by a membrane receptor-associated tyrosine kinase(s). These activated polypeptides, termed STATs (signal transducers and activators of transcription), then translocate to the nucleus to associate with p48, thus forming the ISGF-3 (IFN-stimulated gene factor-3) complex, which binds to the SIE to initiate transcription of the c-Fos gene (Fu and Zhang, 1993 and references therein). Further evidence has suggested that p91, through its SH2 domain, can directly interact with the EGF receptor in a ligand-dependent manner, and subsequently mediate the induction of c-Fos (Fu and Zhang, 1993; Silvennoinen *et al.*, 1993). Although p113 and p84 seem not to be involved in EGF signalling, the association of a different combination of p91 and other related protein(s) is a possibility, and might be a way to differentiate and maintain specificity between various ligands. The serum response element mediates c-Fos induction through stimuli that activate the MAPKs, and is recognised by the serum response factor. This is a ubiquitous transcription factor that forms a dimer and binds to the SRE promoting the recruitment of the ternary complex

factor (TCF). The activity defined as TCF includes the proteins of the Ets-related gene family: $p62^{TCF}$, Elk-1 and Sap-1. Following mitogenic stimulation, the candidate TCF's are rapidly phosphorylated within their carboxy-terminal region by specific MAPK subtypes, the ERKs (Zinck *et al.*, 1993; Gille *et al.*, 1995; Janknecht *et al.*, 1995). Although recent evidence would suggest that Elk-1 provides a potential convergence point for the ERK and SAPK signalling pathways, since SAPKα also efficiently phosphorylates the Elk-1 carboxy-terminal domain (Zinck *et al.*, 1995). The MAPK-induced phosphorylation increases the transcriptional activity of the TCF, thereby resulting in increased c-Fos synthesis (reviewed in Karin, 1995). Interestingly, Rao and Reddy (1994) have introduce a novel concept that MAP kinase activators can also be non-kinase proteins, through their demonstration that Elk-1, apart from being a MAPK substrate, can also interact with MAPK to selectively enhance MAPK autophosphorylation and activation.

By comparison with c-Fos, the c-Jun promoter is much simpler, with its inducers operating through one major *cis* element, the c-Jun TRE. This differs from the AP-1 consensus TRE sequence by 1-base pair insertion, and due to this subtle change it is more efficiently recognised by c-Jun•ATF2 heterodimers than by conventional AP-1 complexes. Following exposure to stimuli that activate the JNK group of MAPKs, both c-Jun, discussed below, and ATF2 (Gupta *et al.*, 1995) are rapidly phosphorylated, which stimulates their ability to activate transcription of c-Jun (reviewed in Karin, 1995).

The biologic function of c-Jun as a transcription factor is regulated by two distinct phosphorylation systems. The transcriptional potential of c-Jun is augmented by serine phosphorylations (Ser-63 and -73) in the amino-terminal transactivation domain, in response to signalling pathways that culminate in the activation of the MAPKs, all of which potentially phosphorylate c-Jun, albeit with varying efficacy (Minden *et al.*, 1994; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). So far, the JNK/SAPKs are the only kinases found to efficiently phosphorylate these amino-terminal sites in c-Jun, and the mechanism underlying this high degree of substrate specificity most likely involves the interaction of the docking-site, located

upstream of the Ser phosphorylations in c-Jun, with a region near the catalytic pocket of the MAPKs. The amino acids within this region are variable amongst all MAPKs, and the docking-site in c-Jun shows substrate specificity towards MAPKs of the JNK subtype, with JNK2 displaying a higher affinity for c-Jun than JNK1 (Kallunki *et al.*, 1994). In contrast, phosphorylation of three residues within the carboxy-terminus of c-Jun precludes DNA binding activity. The regulation of these phosphorylations is not certain, however, ERK1 and -2 have been shown to phosphorylate c-Jun at a carboxy Ser residue (Minden *et al.*, 1994); and recent evidence suggests that phosphorylation of the Ser residues in the amino-terminus causes a conformational change in the c-Jun protein, stimulating the dephosphorylation of the carboxy-terminal sites, and consequently increasing DNA-binding (Papavassiliou *et al.*, 1995).

Hence, the transcriptional regulation of AP-1 activity is through the induction of both *c-fos* and *c-jun* genes. The newly synthesised c-Fos and c-Jun proteins combine to form stable heterodimers, and a further increase in AP-1 activity is brought about by post-translational regulation of both c-Fos and c-Jun. Increased transactivation of c-Jun is through the phosphorylation of the amino-terminal serines by JNKs, in a similar manner to the phosphorylation of c-Jun when it is complexed with ATF2. The sequence surrounding the amino-terminal phosphoacceptors of c-Jun are also conserved in the carboxy-terminal activation domain of c-Fos, suggesting that c-Fos also undergoes phosphorylation. This presumably is brought about by the newly identified FRK protein (Deng and Karin, 1994). In the context of c-Jun•c-Fos heterodimers, phosphorylation of each protein makes a similar contribution to stimulation of transcription via AP-1. Thus, specificity is brought about by the formation of complexes involving members of the MAPK cascade and their substrates: ERKs to TCF proteins, JNK/SAPKs to c-Jun, and FRK to c-Fos, see figure 1.5, p34. There is evidence of another TGY containing MAPK, Mxi2, capable of dimerising with and phosphorylating the transcription factor binding Max (Cano and Mahadevan, 1995).

In addition to the differences in substrate specificities, the types of MAPKs that affect AP-1 activity differ in their responses to extracellular stimuli, discussed in

section 1.2.2. The induction of the two components of the AP-1 complex through different and distinctly regulated members of the MAPK subtypes could ensure that AP-1 activity responds to a large spectrum of extracellular stimuli. It is also possible that through the divergent activation of ERKs and JNKs, different extracellular stimuli induce different types of AP-1 complexes (e.g. Jun•Jun homodimers versus Jun•Fos heterodimers) and thereby lead to activation of overlapping yet distinct sets of target genes.

1.6. Cellular differentiation versus proliferation.

Receptor tyrosine kinases are involved in signalling both cell proliferation and differentiation (van der Geer and Hunter, 1994). Numerous studies show that depending on the cellular context, the same receptor can signal proliferation or differentiation (reviewed in Marshall, 1995). For example, the fibroblast growth factor receptor signals differentiation in PC12 neuronal cells, whilst in fibroblasts stimulates proliferation. The response of PC12 cells to receptor tyrosine kinase activation has been extensively used as an experimental system to study proliferation versus differentiation. Treatment of PC12 cells with fibroblast growth factor (FGF), or nerve growth factor (NGF), leads to the differentiation of neurites, whereas treatment with EGF leads to a proliferative signal. Notable quantitative differences were found between these two pathways, namely that ERK activation is sustained for several hours following NGF stimulation, but it is short lived after EGF stimulation (Traverse *et al.*, 1992). This may reflect differences in the down-regulation of the EGF receptor and the NGF receptor, TrkA, or the fact that the EGF receptor utilise both the Grb2-Sos complex and the Shc•Grb2-Sos complex to activate p21Ras, whereas TrkA stimulates Shc phosphorylation and the formation of Shc•Grb2-Sos complexes (Buday and Downward, 1993). However, there is much evidence to suggest that there is no specific pathway for differentiation (Marshall, 1995, and references therein). Whilst stimulation of the endogenous EGF receptor in PC12 cells leads to proliferation, overexpression of the EGF receptor induces differentiation and sustained ERK activation (Traverse *et al.*, 1992). Furthermore, the appropriate mutations in the Raf

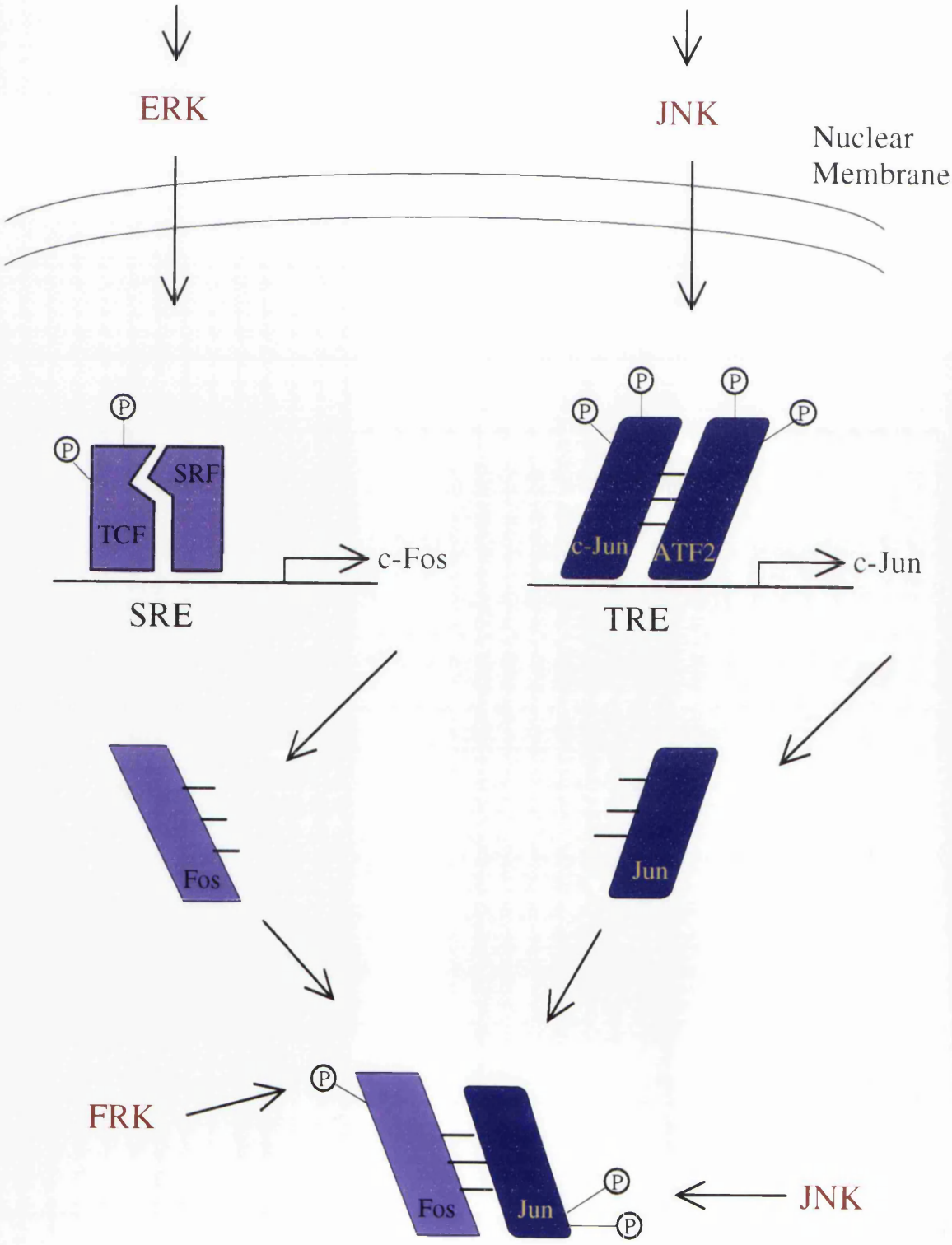
phosphorylation sites of MKK1 demonstrate that interfering mutants block ligand activation of ERK and growth factor-induced PC12 differentiation, while the activating mutants induce neuronal differentiation in the absence of differentiating factors (Cowley *et al.*, 1994). This has led to a model whereby the temporal activation of ERKs is sufficient for cells to enact proliferative or differentiation responses. This is also linked to the spatial distribution of the ERKs, since only sustained ERK activation in PC12 cells is associated with nuclear translocation. Transient activation will therefore have very different consequences for gene expression. However, it is not a requirement of this model that sustained activation of ERKs invariably leads to differentiation. In other cell types the converse might be true, so that receptor tyrosine kinase signalling depends both on the duration of MAPK activation and the cell context.

Figure 1.5.

Three distinct MAP kinases contribute to induction of AP-1 activity.

The serum response element in the c-Fos promoter binds both the SRF and the TCF (which includes Elk-1, $p62^{TCF}$ and Sap-1). The TCF is phosphorylated by the ERKs, which consequently stimulates transcriptional activity, thus mediating c-Fos induction. JNK-mediates phosphorylation of ATF2 and c-Jun bound to the TPA responsive element in the c-Jun promoter, leading to c-Jun induction. The newly synthesised c-Fos and c-Jun proteins combine to form stable AP-1 heterodimers. A further increase in AP-1 activity is brought about by the JNKs and FRK, which phophorylate c-Jun and c-Fos respectively, on sites that augment their transcriptional activities. c-Fos is phosphorylated on many sites, although the number of sites attributable to FRK is presently unknown. Refer to text for details, Diagram taken from Karin, (1995).

Figure 1.5.


ERK
JNK
Nuclear
Membrane
P
SRF
TCF
c-Fos
SRE
c-Jun
ATF2
c-Jun
TRE
Fos
Jun
FRK
Fos
Jun
JNK

1.7. Protein phosphatases- regulators of signal transduction

As mentioned at the onset, receptors for a variety of hormones and growth factors, as well as the transforming proteins of certain acutely oncogenic retroviruses, possess intrinsic kinase activity, consistent with protein phosphorylation playing a major role in signal transduction. However, the sequence of events through which a signal is initially activated at the receptor tyrosine kinase and then amplified via a cascade of intracellular kinases to the nucleus, is only one dimension of the signalling response. This system must be tightly regulated if a cell is to co-ordinate functions such as proliferation and differentiation, and the modulation of the protein phosphorylation state is realised through an interplay between protein kinases and protein phosphatases, two antagonistic, but integrated enzymes. Approximately two hundred protein kinases have been identified (Hunter, 1987). By comparison, the number of protein phosphatases is limited and does not equal the diversity of the protein kinases. Indeed, until recently, it was generally assumed that the kinase reaction was the key regulatory process for determining the level of phosphorylated proteins, and that the dephosphorylation reaction was an unregulated event: a "house keeping" function. There is now strong evidence that this is not the case, and protein phosphatases are recognised as playing a central role in regulating many cellular activities. Although, compared to the protein kinases, the role of phosphatases in transformation has remained obscure. At present, there are no known oncogenes that encode protein phosphatases, although dephosphorylation of an inhibitory tyrosine residue in the proto-oncogene c-Src is required for its functioning.

Protein phosphatases represent a diverse family of biological catalysts, and have been broadly classified according to amino acid specificity: the non-specific phosphatases, the serine/threonine (Ser/Thr) phosphatases, and the tyrosine (Tyr) phosphatases. However, this initial distinction is now complicated by the recent discovery of protein phosphatases with dual specificity towards both phosphoserine/threonine and phosphotyrosine. For purposes of introduction, each of the classes of protein phosphatase will be described together with their putative

functions in signal transduction, specifically their interplay with the MAPK cascade, illustrated in figure 1.6, p58.

1.7.1. The non-specific phosphatases

This class includes proteins such as the alkaline and acid phosphatases, enzymes capable of hydrolysing both proteinaceous and non-proteinaceous (e.g. alcohols) phosphate monoesters. The non-specific phosphatases typically function in degradative or catabolic pathways and due to their wide distribution are believed to serve as "phosphate scavengers". In contrast to the other classes of phosphatase, and indeed the kinases, these diverse enzymes do not share amino acid sequence similarities with one another. Mechanistically, the alkaline phosphatases function through the formation of a covalent phosphoseryl intermediate, while phosphate ester hydrolysis by at least some acid phosphatases proceeds through a phosphohistidine intermediate (reviewed in Coleman, 1992).

1.7. 2. Classification of serine/threonine phosphatases

In mammalian cells, biochemical characterisation has identified four major classes of Ser/Thr phosphatase, which are presently classified into two categories (reviewed in DePalaoli-Roach *et al.*, 1994; Walter and Mumby, 1993; Cohen, 1989). The type 1 phosphatases (PP1), preferentially dephosphorylate the β subunit of phosphorylase kinase, and are defined as containing a catalytic subunit that is inhibited by two small heat- and acid-stable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2). While I-1 is active as an inhibitor only after phosphorylation by the cAMP-dependent protein kinase; I-2 is spontaneously active, with phosphorylation at Thr-72 inactivating I-2. The type 2 phosphatases (PP2), specifically dephosphorylate the α subunit of phosphorylase kinase, and are insensitive to I-1 and I-2. These enzymes are further subdivided into three distinct classes, PP2A, 2B, and 2C in a number of ways, but most simply by their dependence on divalent cations. Based on new insights into the molecular and biochemical properties of these enzymes, revision of the above scheme is now necessary. For example, the catalytic subunits of PP2A

and PP2B share considerable sequence similarity with PP1, but not with PP2C. Moreover, recent studies have revealed an increasing number of novel phosphatases which structurally do not fit into any of the above four categories. These include mammalian PP4, also termed PPX (da Cruz e. Silva *et al.*, 1988; Brewis *et al.*, 1993), which is localised at centrosomes, and shows 45% and 65% identity to the PP1 and PP2A catalytic subunits, respectively. A further nuclear phosphatase, PP5, shows less than 40% similarity to any known member (Chen *et al.*, 1994b), and a novel membrane-anchored phosphatase, designated PP3, has also been described (Honkanen *et al.*, 1991).

1.7.2.1. Serine/threonine phosphatase catalytic subunits

Molecular cloning has defined the primary structures of the phosphatase catalytic subunits which belong to a single gene family, except for PP2C. Four PP1 and two PP2A, PP2B and PP2C catalytic subunit isoforms have been predicted from cDNAs isolated from mammalian sources. There is a high degree of structural information conserved not only among isoforms, but between different organisms; for example, the PP1 and PP2A catalytic subunits are more than 80% identical from yeast to mammals. This argues for a fundamental role of these enzymes in essential cell functions, and in support of this, these two classes of phosphatase represent more than 90% of the activity present in most tissues examined. However, at present there are approximately twelve known Ser/Thr phosphatase catalytic subunits to counteract the vast numbers of Ser/Thr kinases so far identified (Hunter, 1987). Furthermore, protein phosphatases exhibit relatively broad specificity *in vitro* and the use of synthetic peptides as substrates has revealed little about the determinants for phosphatase recognition. Do protein phosphatases simply display extremely broad specificity or do other factors impose a greater selectivity of function? A key point is that all Ser/Thr phosphatases characterised biochemically, with the exception of PP2C, are in fact multi-subunit enzymes. Increasing evidence has established that the non-catalytic subunits serve to regulate or specify the function of the catalytic moiety. The structural components of the four classes of Ser/Thr phosphatases are outlined briefly below.

•**PP1** There are four isoforms of the catalytic subunit of PP1 which are generated by alternative splicing. The free catalytic subunits have not been detected in cells, but are identified as parts of larger molecular complexes. Hence, heterogeneity of PP1 is generated by the combination of a similar catalytic component, the holoenzyme, with various accessory proteins which are responsible for regulating both the enzymatic activity and targeting of PP1 to specific subcellular locations. The catalytic isoforms are 90% identical and differ mainly at the carboxy-terminus of the protein, suggesting that this region may be involved in the interaction with the different regulatory proteins. At least four molecular forms of PP1 have been identified: (a) the ATP-Mg-dependent phosphatase for which I-2 is the regulatory component, (b) the glycogen/sarcoplasmic reticulum-associated phosphatase, (c) the myosin-associated phosphatase and (d) a nuclear phosphatase which is associated with chromatin. This compartmentalisation of the holoenzymes means that specific association of the regulatory subunits with distinct isoforms cannot be ruled out. Beullens and co-workers (1992), isolated a nuclear inhibitor of PP1, NIPP1, which associates with the catalytic subunit to form a heterodimeric holoenzyme. Phosphorylation of NIPP1 by the cAMP-dependent protein kinase renders the protein less effective and decreases its affinity for the catalytic subunit (Beullens *et al.*, 1993). This effect of phosphorylation is opposite to that seen with I-1. In the cytosol PP1 interacts with I-1 and I-2, preventing illicit dephosphorylation events; however, the function of I-2 is not simply that of an inhibitor and may be a regulatory subunit acting as a chaperone to fold PP1 into its native conformation (Alessi *et al.*, 1993b).

•**PP2A** The enzyme PP2A is mainly cytosolic, and has been isolated in a trimeric form composed of a catalytic subunit (C), an accessory subunit (A) and one of three regulatory subunits (B). Dimeric A/C forms of PP2A have frequently been reported but there is increasing evidence that these represent artifacts generated from the trimeric form due to dissociation and/or proteolysis of the B-subunit (Cohen, 1989). Heterogeneity of PP2A phosphatases is accomplished by the binding of one of the three B-subunits to the AC core, and by the existence of multiple isoforms of all three of these components. PP2A substrate specificity is influenced by the subunit complex,

and experimental approaches point to a critical role for the B-subunits in determining PP2A function. Similar to PP1, a nuclear, chromatin-associated form of PP2A has been identified (Jakes *et al.*, 1986).

•**PP2B** This enzyme was initially isolated as an activity that dephosphorylated the α subunit of phosphorylase kinase, and was subsequently shown to be a calcium-dependent calmodulin-binding protein showing striking resemblance to that of calcineurin (Stewart *et al.*, 1982). Further experiments confirmed that calcineurin was a calcium/calmodulin-regulated protein phosphatase. PP2B is found predominantly in nervous tissue, and as is the case with PP1 and PP2A, there are multiple isoforms of the catalytic subunit-A, which contains the calmodulin-binding domain, whilst the regulatory B-subunit is the calcium-binding component.

•**PP2C** PP2C is unique in that it is monomeric comprising of only a catalytic subunit, which requires magnesium for half-maximal activity, and is insensitive to many phosphatase inhibitors (Cohen, 1989). It is located mainly in the cytosol, and genes encoding two isozymes of PP2C have been identified in mammals, the sequences of which are unrelated to the catalytic subunits of other types of phosphatases.

1.7.3. Involvement of serine/threonine phosphatases in growth control

Although tyrosine phosphorylation has a special significance for the events proximal to the receptor, much of the intracellular signalling is mediated by Ser/Thr phosphorylation. Hence, the Ser/Thr phosphatases are important candidates for major regulatory roles in the control of signal transduction. However, PP1, PP2A, and PP2C have broad and overlapping specificities *in vitro*, and account for virtually all measurable activity in tissue extracts towards a variety of phosphoproteins. Their precise functions *in vivo* have consequently remained enigmatic, although evidence implicating that the Ser/Thr phosphatases must be directly involved in growth control is provided by the demonstration that okadaic acid, a potent inhibitor of PP1 and PP2A (Bialojan and Takai, 1988), behaves as a tumour promoting agent (Suganuma *et al.*, 1988). Tentative roles for Ser/Thr phosphatases in the MAPK signal transduction cascade have been postulated, since several of the enzymes in this

pathway are themselves regulated by phosphorylation. In this regard, PP2A has been shown to dephosphorylate and inactivate both MKK and MAPK *in vitro* (Dent *et al.*, 1992; Kyriakis *et al.*, 1992), whilst Raf-1 and p90Rsk are also dephosphorylated by PP2A *in vitro* (Kovacina *et al.*, 1990; Sturgill *et al.*, 1988), refer to figure 1.6, p58. Phosphatases may also influence the final targets of signal transduction pathways: the transcription factors, whereby changes in phosphorylation could alter their nuclear translocation, their DNA binding activity, or their transactivation properties. Evidence that PP2A might be involved in the control of c-Jun phosphorylation was provided by Alberts *et al.* (1993), who demonstrated that gene expression controlled by the TRE was stimulated upon microinjection of the catalytic subunit and AC form of PP2A. This is suggestive that PP2A may dephosphorylate the inhibitory carboxy-terminal residues of c-Jun, thus promoting DNA binding to the TRE sequence. Biochemical studies have suggested that Ser/Thr phosphatases are involved in cell cycle regulation through the control of Cdc2 kinase activity (Walter and Mumby, 1993). The identification of a PP2A-like activity, INH, in extracts of *Xenopus* oocytes, has led to the demonstration that INH appears to target Thr-161, thus inhibiting the formation of active cyclin-Cdc2 complexes (Lee *et al.*, 1991). So, whilst there is evidence for many substrates *in vitro*, it is extremely difficult to prove that the same proteins are substrates *in vivo*. A future direction will be to isolate or develop inhibitors that are specific for individual phosphatases. Presumably, the subcellular localisation of the phosphatases will confer important regulatory properties upon them, and this concept is best established for PP1. The holoenzyme of PP1 associates with regulatory subunits that target the phosphatase to particular locations, enhancing its activity towards certain substrates, for example the glycogen-bound enzyme (PP1G), and the myofibrillar form (PP1M), found in skeletal muscle. The activities of the Ser/Thr phosphatases are also controlled by second messengers, which adds a further temporal dimension to their regulation and potential substrates. As previously mentioned, PP2B is dependent on calcium and calmodulin, while PP1 is controlled in a variety of ways that depend on the form and the tissue. PP1 can be inhibited by cAMP through the PKA-catalysed phosphorylation of I-1. Furthermore, phosphorylation of PP1G by

PKA promotes translocation of the catalytic subunit from glycogen particles to the cytosol, thus inhibiting the dephosphorylation of glycogen-metabolising enzymes. PP1 can also be activated indirectly by calcium through the ability of PP2B to dephosphorylate I-1. Recent studies by Wang and co-workers (1995) have demonstrated that the phosphorylation of I-2, which results in activation of the ATP-Mg-dependent PP1, is mediated by MAPK. These findings suggest that MAPK may be the physiological kinase that activates cytosolic PP1 in growth factor stimulated cells. This is particularly interesting when it is considered that glycogen synthase kinase-3 (GSK-3), which is presently assumed to be the kinase responsible for activating the ATP-Mg-dependent PP1, is inactivated following insulin and growth factor stimulation, when PP1 is rapidly and transiently activated. In summary, it is believed that the regulatory subunits will play the important role in determining the properties and substrates of the Ser/Thr phosphatases, and hence their physiological functions.

1.7.4. Protein tyrosine phosphatases

Protein phosphorylation on tyrosine residues is the initial stimulus at the plasma membrane in eukaryotic signalling, and plays a pivotal role in a range of cellular pathways. The protein tyrosine kinases constitute a well characterised family of both cytoplasmically localised as well as transmembrane enzymes, and their function in signal transduction has been extensively studied. Whilst the increase in number and structural diversity of the protein tyrosine phosphatases (PTPs) is well recognised, in comparison, their precise involvement in signal transduction cascades is less well understood. In spite of the fact that the majority of phosphorylation in the cell is associated with serine/threonine residues, the greatest number of protein phosphatases identified so far belong to the phosphotyrosine-specific class. The first evidence for the existence of the phosphotyrosyl dephosphorylation system came from *in vitro* studies with the A431 human epidermoid carcinoma cells, which are high in EGF receptor phosphotyrosyl kinase activity (Ushiro and Cohen, 1980). These experiments demonstrated that when A431 cell membrane proteins were labelled at tyrosine

residues with [γ-^{32}P]ATP in the presence of EGF, a slow and time-dependent release of [^{32}P]Pi from the labelled proteins could be observed, suggesting that an activity in the membrane preparation was responsible for dephosphorylating phosphotyrosyl proteins (Brautigan *et al.*, 1981). The presence of PTPs in intact cells was deduced from experiments using tumour viruses with temperature-sensitive transforming proteins. At the permissive temperature, cells infected with Rous sarcoma virus rapidly incorporate phosphate into tyrosine residues of proteins. However, when the transformed cells are shifted to nonpermissive temperatures, the degree of cellular Tyr(P) declines to the basal (untransformed) level, presumably due to the activities of cellular PTPs (Sefton *et al.*, 1980). These important observations were followed by numerous attempts to identify and characterise the PTPs, which now consist of over 40 enzymes, most of which have been cloned using recombinant techniques based on the sequence homology of the catalytic domain (for extensive reviews see Walton and Dixon, 1993; Charbonneau and Tonks, 1992; Brautigan, 1992). Without exception, all PTPases contain an active site signature motif, (I/V)HCXAGXGR(S/T)G, located within the catalytic domain. This sequence has an absolutely conserved nucleophilic cysteine residue that is essential for PTP activity, specifically involved in the formation of a phosphoenzyme reaction intermediate. PTPase catalysis has been clarified through the elucidation of the crystal structures of the *Yersinia* PTPase and the mammalian PTP1B (Stuckey *et al.*, 1994; Barford *et al.*, 1994). Although these two enzymes show only 20% sequence identity, it is clear from crystalline studies that these molecules share close similarities in secondary and tertiary structure. Biochemical characterisation of PTPases has broadly classified them into two families: transmembrane or cytosolic.

Most transmembrane PTPs contain two cytoplasmic catalytic PTPase domains, the most proximal to the membrane exhibiting the majority of the PTP activity. However, in some cases the second domain also possesses detectable, but low PTP activity (Wang and Pallen, 1991), and it is possible that the presence of two catalytic domains may allow for differential substrate specificity within the same enzyme. Alternatively, the second domain may purely function to regulate the activity of the

first domain, or possibly associate with second tyrosine-phosphorylated proteins in a manner analogous to an SH2 domain. Whether the extracellular domain of transmembrane PTPs can bind ligands, which potentially modulate PTP activity, perhaps by inducing oligomerisation of the PTP-receptors, remains to be determined. A subset of receptor-like PTPases have extracellular domains that share sequence similarities with the cell adhesion molecules (CAMs), with the implication that these PTPases may participate in cell-cell interactions. The best characterised of these are the mammalian phosphatases PTPμ and PTPκ, which both display a single extracellular immunoglobulin-like domain and four fibronectin type III repeats. Expression of these phosphatases in insect cells mediates cell-cell aggregation via "homophilic" interactions (Sap *et al.*, 1994; Gebbink *et al.*, 1993). Since cellular proliferation is frequently associated with tyrosine phosphorylation, it is interesting that cell confluence often results in cell quiescence, with an increase in membrane PTPase activity (Pallen and Tong, 1991).

The cytosolic PTPs are further divided into subfamilies, based on their structural features in the non-catalytic regions which serve to localise these enzymes within the cell, which, as for all enzymes, offers a powerful means of controlling activity by limiting available substrates. Examples include PTP1B and the T-cell PTPase, which have intracellular localisation signals at their carboxy-terminus. This targets PTP1B to the endoplasmic reticulum (Frangioni *et al.*, 1992), whilst expression of the carboxyl-truncated form of the T-cell PTPase produces multinucleated cells, presumably through a failure in cytokinesis, concomitant with a reduced growth rate (Cool *et al.*, 1992). An intimate association between PTPs and focal adhesion is supported by the finding that the related PTPs: PTPH1, PTPMEG1, and PTPD1 contain regions of homology to cytoskeletal proteins, such as erzin and talin (Møller *et al.*, 1994 and references therein). A further motif identified in cytosolic PTPases is the SH2 domain, which has great implications for PTPase activity towards growth factor signalling pathways, discussed below. In line with this, a PTP has been identified which shows homology with the basic domains of the transcription factors Fos and Jun (Swarup *et al.*, 1991), implicating a nuclear signalling function for this phosphatase. A

full accounting of the protein tyrosine phosphatases is beyond the scope of this introduction, and consequently only examples implicated in RTK pathways will be discussed below.

1.7.5. Physiological roles of tyrosine phosphatases in signal transduction

Since de-regulated PTKs have been found to be encoded by oncogenes, then conceptually, PTPs are potential tumour supressors or anti-oncogenes. Indeed, inactivating mutations in genes encoding PTPs may contribute to the aberrant tyrosine phosphorylation associated with certain neoplastic conditions. Considerable progress has been made in determining the chromosomal localisation of genes encoding PTPs, with the aim of correlating map positions with sites of abnormality in human disease. Most notable is the gene for PTPγ on chromosome 3p21, a loci frequently altered in renal and small-cell lung carcinomas (Laforgia *et al.*, 1991). Several studies have reported the inhibitory effects of PTPs on cellular transformation. Overexpression of PTP1B within a tissue culture system has been demonstrated to revert the transformed phenotype of v-*src* and suppress subsequent transformation by both the oncogenes *neu* and v-*erbB2* (Woodford-Thomas *et al.*, 1992; Brown-Shimer *et al.*, 1992). Evidence that PTPs function as negative-regulators of cellular proliferation is supported by the observations that PTPase inhibitors, such as sodium vanadate, are able to transiently substitute for growth factors and induce mitogenic responses (Klarlund *et al.*, 1985). However, it is apparent that some PTPs are able to promote signalling responses through the activation of members of the Src family of PTKs. These kinases have an inhibitory tyrosyl phosphorylation in their carboxyl termini, dephosphorylation of which promotes kinase activity. A specific example is illustrated in T-cell receptor signalling, which is mediated by the receptor PTP, CD45, also known as leukocyte common antigen (Trowbridge and Thomas, 1994). The Src-related PTKs in the hematopoietic system, $p56^{lck}$ and $p59^{fyn}$, are associated with the T-cell receptor, and have been implicated as the targets of CD45 in this signalling response.

The interplay between PTKs and PTPs has been strikingly illustrated by the discovery of a subfamily of cytoplasmic PTPs containing SH2 domains. PTP1C (Shen

et al., 1991), also termed SH-PTP1 (Plutzky *et al.*, 1992), HCP (Yi *et al.*, 1992) or SHP (Matthews *et al.*, 1992), is a hematopoietic cell PTPase characterised by two amino-terminal SH2 domains, however its expression has also been observed in breast cancer epithelial cell lines. This phosphatase has been shown to interact with autophosphorylated EGF receptor *in vitro* (Shen *et al.*, 1991), mediated via the most amino-terminal SH2 domain. Consistent with PTP1C being preferentially expressed in the hematopoietic system, is the finding that this phosphatase transiently associates with the activated Kit and IL-3 receptors in myeloid cells. Homozygously carried defects in the murine homolog of PTP1C lead to the *motheaten* phenotype, whereby mice develop systemic autoimmune disease, suggestive that PTP1C protein normally acts to restrict the proliferation of hematopoietic cells (Tsui and Tsui, 1994). Perhaps this is through the phosphatase antagonising the effects of growth factors which stimulate the Kit and IL-3 receptors. A second phosphatase to be cloned that contains SH2 domains is Syp (Feng *et al.*, 1993), also variously called SH-PTP2 (Freeman *et al.*, 1992), PTP1D (Vogel *et al.*, 1993), and PTP2C (Ahmad *et al.*, 1993). This phosphatase is expressed ubiquitously, and growing evidence implicates that it is involved with growth factor signalling and in MAPK activation, playing a positive regulatory role as opposed to that of PTP1C. Syp stably associates through its SH2 domains to the activated PDGF- and EGF receptors (Kazlauskas *et al.*, 1993; Vogel *et al.*, 1993). In addition, Syp also binds to the insulin receptor substrate IRS-1 (Noguchi *et al.*, 1994), and overexpression of a catalytically inactive Syp blocks both the EGF and insulin-induced MAPK induction and c-Fos transcription (Zhao *et al.*, 1995; Noguchi *et al.*, 1994). The SH2 domains appear to not only mediate the targeting of Syp, but also regulate its activity, since unoccupied SH2 domains maintain phosphatase activity in a repressed state. It has been demonstrated that Syp becomes tyrosine phosphorylated within the carboxy-terminus following stimulation with PDGF and EGF, although this is not dependent upon Syp binding with the receptor. As regards the PDGF receptor, this induced phosphorylation creates a binding site for the SH2 domain of Grb2, see figure 1.6, p58 (Li *et al.*, 1994; Bennett *et al.*, 1994). This indicates that Syp can potentially act as an adaptor between activated PDGF receptor

and the Grb2/Sos complex, thereby stimulating Ras and the MAPK pathway. It would seem that while the EGF receptor can directly bind Grb2, or indeed function through Shc•Grb2 (Buday and Downward, 1993), the PDGF receptor binds Grb2 through complexing with Syp, indicating that different growth factor receptors can interact with the same downstream signalling molecule by different mechanisms. However, Syp may not function as an adaptor protein that couples IRS-1 to the Grb2/Sos complex, since Syp is not phosphorylated on tyrosine residues following insulin treatment (Noguchi *et al.*, 1994). In this system, it is possible that the phosphatase functions to down-regulate GAP activity; thus the inactivation of GAP would result in a relative increase in Ras activity. The importance of SH2-containing PTPases is further underscored by recent developmental studies. The *Drosophila* gene, *corkscrew* (csw), is the homolog of Syp, and is required maternally as a positive signal transducer in concert with D-*raf*, downstream from the Torso receptor tyrosine kinase (Perkins *et al.*, 1992). Additionally, Syp is expressed maternally in *Xenopus* during embryogenesis, acting as a positive component in FGF signalling downstream of the FGF receptor and upstream of MAPK (Tang *et al.*, 1995). However, not all the phosphatases which are implicated in regulating components of the MAPK cascade necessarily possess SH2 domains. RPTPα is a transmembrane PTP which is tyrosine phosphorylated *in vivo* within the carboxy-terminus of the protein, which subsequently creates a binding site for Grb2. den Hertog and colleagues (1994) have demonstrated that RPTPα associates with Grb2 in immunoprecipitates from fibroblast cells, however, Sos is not detected in these complexes. The absence of Sos means that Grb2 cannot activate the Ras pathway, suggesting that RPTPα may play a negative role in Ras signalling by sequestering Grb2. The mechanism by which RPTPα is constitutively phosphorylated on tyrosine is at present, not fully understood. Interestingly, RPTPα can show oncogenic potential, as demonstrated by its ability to catalyse the activation of c-Src, which in the same cell system has the effect of inducing the activation of MAPK and c-Jun transcription, illustrated in figure 1.6, (Zheng and Pallen, 1994). The corollary is that PTPs can apparently function as both negative and positive growth

stimulators, thus a critical balance must exist in the cell between these activities, and those of protein kinases, if a cell is to maintain essential growth control.

1.7.6 A novel subfamily: The dual specific protein phosphatases

A increasing number of proteins have recently been identified which are able to dephosphorylate both phospho-serine/threonine and -tyrosine residues (reviewed in Keyse, 1995). The prototype for these "dual specific" phosphatases is encoded by the H1 gene of the vaccinia virus, which expresses a protein during the late stage of viral infection. VH1 is a small, 20 kDa PTP, which apart from the presence of the PTPase active site signature motif, is only distantly related to the eukaryotic tyrosine phosphatases. Whilst the precise physiological function of VH1 is unknown, it does however efficiently dephosphorylate both phosphotyrosine- and phosphoserine-containing substrates *in vitro* (Guan *et al.*, 1991). At the present time the rapidly expanding VH1-like family of phosphatases includes genes isolated from the budding yeast *S. cerevisiae*, such as YVH1; a gene dramatically induced upon nitrogen starvation, and the inactivation of which correlates with a decrease in growth rate (Guan *et al.*, 1992). Doi and colleagues, (1994), have also identified *MSG5*, a dual specific phosphatase required for the yeast mating pheromone pathway. Genetic studies have placed MSG5 downstream of *STE7*, a MAPKK homolog, and have demonstrated that it directly inactivates the MAP kinase, FUS3. Further VH1-like phosphatases have additionally been identified in baculo- and orthopox-viruses (Hakes *et al.*, 1993; Sheng and Charbonneau, 1993), and importantly, a number of genes have been isolated from mammalian tissues, including VHR, the VH1-related human Ser/Tyr phosphatase (Ishibashi *et al.*, 1992). These are discussed more fully below in context with the MAPKs. Since the dual specific phosphatases display amino acid sequence identity to the PTPases across the active site, it is not surprising that they also utilise a thiol phosphate intermediate during the catalytic reaction (Zhou *et al.*, 1994); however, outside of this region the dual specific phosphatases show limited sequence identity with the PTPs.

A further protein demonstrated to dephosphorylate both tyrosine and threonine residues is Cdc25, which is involved in controlling cell entry into mitosis. As previously mentioned, passage through successive phases of the cell cycle is controlled by Cdks, whose activity is regulated foremost by the association with the ancillary proteins, the cyclins, and also by physical association with Cdk inhibitors. Cdk activity is additionally regulated by phosphorylation, and Cdc25 phosphatases, although weakly related to the main family of protein phosphatases, have been shown to specifically dephosphorylate Thr-14 and Tyr-15 of Cdc2/cyclin B (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991). Cdc25 is otherwise rather inefficient against phosphotyrosine and -threonine substrates *in vitro*. Mammalian cells have three related Cdc25 genes: Cdc25A, -B, and -C, which appear to interact with different cyclin-Cdk complexes (Galaktionov *et al.*, 1995a). Cdc25B and -C are predominantly expressed in G2, whilst Cdc25A appears not to be a mitotic regulator, functioning at the G1/S transition of the cell cycle. The catalytic activity of Cdc25C is stimulated 4- to 5-fold in the presence of cyclin B at the onset of mitosis, due to an auto-amplification loop. Cyclin B possesses a region known as the P box, which is a necessary requirement for hypophosphorylated Cdc25C to recognise and bind cyclin B and dephosphorylate Cdc2, albeit at a low efficiency (Zheng and Rudderman, 1993). The activated cyclin B/Cdc2 complex then phosphorylates Cdc25C, which increases Cdc25C activity further activating Cdc2 kinase. Such positive feedback loops are essential to create sharp, rapid boundaries between cell cycle phases. Hoffman and co-workers, (1994), have demonstrated that Cdc25A plays a crucial role early in the cell cycle, and is phosphorylated by cyclin E/Cdk2, however, in this instance, the involvement of a positive feedback loop is as yet unknown. An interesting possibility is that activated Cdc25A is the initial phosphatase that activates cyclin B/Cdc2, which then subsequently phosphorylates Cdc25C to establish the hyperactivation of cyclin B/Cdc2. It has recently been shown that the carboxyl-terminus of Raf-1 forms a tight association with Cdc25A at the plasma membrane, phosphorylating and stimulating the phosphatase activity both in somatic cells, and in *Xenopus* oocytes (Galaktionov *et al.*, 1995a). Cdc25 phosphatases also show oncogenic co-operation with either oncogenic

Ras mutants or pRb deletion mutants, indicating that Cdc25 phosphatases may promote transformation (Galaktionov *et al.*, 1995b). In support of this, Cdc25B is found to be highly expressed in human primary breast cancers. This provides a strong link between mitogenic signalling and the cell cycle, which is further substantiated by the demonstration that the 14-3-3 proteins, which function as potential co-activators of Raf-1, also physically associate with Cdc25A both *in vivo* and *in vitro* (Conklin *et al.*, 1995). Withdrawal of growth factors in G1 preceding the "control" point could therefore abort the cell cycle via the rapid inactivation of Cdc25A, whilst DNA damage and inhibitory factors, such as TGFβ, inhibit the cell cycle through the induction of the Cdk inhibitors. Studies from two individual groups have identified an additional dual specificity phosphatase by virtue of its association with certain Cdks, referred to as Cdi1 (Gyruris *et al.*, 1993) or KAP (Hannon *et al.*, 1994). This enzyme contains a single copy of the PTPase active site motif, but does not share sequence identity with any known phosphatase beyond this catalytic core, a property shared by Cdc25. Presumably this phosphatase will play a direct role in cell cycle regulation, and may represent yet another subfamily of the dual-specific phosphatases.

1.7.6.1. The mammalian MAP kinase phosphatases

The first mammalian dual specific phosphatase was isolated from skin fibroblasts, as part of a study to identify cDNAs corresponding to genes induced by oxidative stress. This led to the characterisation of a novel complementary DNA (CL100), which corresponds to a mRNA of approximately 2.4 kb, and is highly inducible by both oxidative stress (including hydrogen peroxide, menadione and ultraviolet A radiation), and heat shock (Keyse and Emslie, 1992). CL100 shows analogous structural features to the non-receptor PTPs, significantly VH1, and indeed, recombinant protein expressed in bacteria rapidly hydrolyses *p*-nitrophenol phosphate (pNPP), a chromogenic molecule related to phosphotyrosine. This enzyme activity is ablated in the presence of the phosphotyrosine inhibitor sodium vanadate. The similarity between CL100 and VH1 is across the catalytic core only, however, a database search demonstrated that the amino acid sequence of CL100 is 96.5%

identical to that of 3CH134, an immediate early gene isolated from the mouse. Immediate early genes constitute the primary genomic response to mitogenic stimulation, and are rapidly induced even in the presence of protein synthesis inhibitors. This superinduction by protein synthesis inhibitors is manifested in at least three ways: i) mRNA stabilisation, ii) activation of intracellular signalling cascades, iii) interference with transcriptional down-regulation (Mahadevan and Edwards, 1991). The mitogenic stimulation of 3CH134 in fibroblasts is rapid and transient, with transcription reaching a peak level at 20 minutes, accumulating during the G0/G1 transition. Consistent with these kinetics, synthesis of 3CH134 protein peaks at 1 hour following serum stimulation and is barely detectable by 3 hours, with an estimated half-life of 40-60 minutes (Charles *et al.*, 1992). Subsequently, 3CH134 was identified as the mouse homolog of CL100, a gene which implicated for the first time the involvement of tyrosine phosphorylation in the cellular response to stress. Its isolation as an immediate early gene led to the proposal that mitogenic signalling may be linked to the stress response in mammalian cells. Further groups also identified the murine homolog, and it is now known variously as 3CH134, ERP (externally-regulated phosphatase, Noguchi *et al.*, 1993), and MKP-1 (MAP kinase phosphatase, Sun *et al.*, 1993). In contrast to VH1, CL100 protein shows no measurable catalytic activity towards several model proteins modified on either tyrosine or serine/threonine (Alessi *et al.*, 1993a), whilst 3CH134 protein demonstrates only a weak intrinsic PTPase activity towards phosphotyrosine substrates, with no detectable dephosphorylation of phosphoserine and -threonine residues (Charles *et al.*, 1993). However, several studies confirm that CL100/MKP-1 potently, and specifically inactivates recombinant ERK2 *in vitro* by the concomitant dephosphorylation of both the activating threonine and tyrosine residues (Alessi *et al.*, 1993a; Zheng and Guan, 1993; Sun *et al.*, 1993). CL100 is capable of dephosphorylating either phosphorylated residue in the absence of the other, and is active towards both the ERK isoforms, -1 and -2, isolated from NGF-stimulated PC12 cells. In addition, CL100 suppresses the oncogenic Ras-induced activation of MAPK in *Xenopus* oocytes, a cell-free system where signalling pathways are intact. All of the above activities are abolished by mutagenesis of the highly

conserved cysteine within the phosphatase active site (Alessi *et al.*, 1993a). Sun and co-workers (1993), additionally demonstrated that the transient transfection of MKP-1 in Cos cells leads to the selective dephosphorylation of ERK2, and blocks ERK2 activation by serum, oncogenic Ras, and activated Raf. In this regard, the kinetics of inactivation of ERK2 coincide with the appearance of newly synthesised MKP-1, and the protein synthesis inhibitor cycloheximide leads to the persistent activation of ERK2. Interestingly, a catalytically inactive mutant of the phosphatase, Cys-258-Ser, physically complexes with activated ERK2 and leads to a sustained serum-induced phosphorylation of the MAPK. Presumably such a specific association of the MAPK with the dominant, negative phosphatase blocks any further dephosphorylation by endogenous phosphatases. Additional experiments using actinomycin D and antisense oligonucleotides targeted to MKP-1, also demonstrate that MKP-1 regulates ERK activity in vascular smooth muscle cells (Duff *et al.*, 1995). These studies suggest that the cell maintains control over RTK signalling cascades by the ultimate induction of an immediate early gene, CL100, which attenuates the signalling response by dephosphorylating MAPK. Promoter analysis of the CL100 gene indicates that an 800 bp region flanking the transcriptional initiation site is sufficient to confer transcriptional response to serum and TPA stimulation, and located within this region are two CREs (cAMP response element, binding CREB/AFT proteins), and one TRE, binding AP-1 dimers, (Kwak *et al.*, 1994). In contrast to other immediate early genes, an SRE site has not yet been identified. The link between CL100 and cellular proliferation is reinforced by the observation that de-regulated expression of ERP in NIH 3T3 cells has a negative effect on cell growth (Noguchi *et al.*, 1993).

Following these observations, a focus of research has identified an increasing number of dual specific phosphatases (see Table 1, p54), demonstrating absolute substrate specificity towards the MAP kinases both *in vitro*, and *in vivo*. At present it is not known whether each of these phosphatases shows specificity for particular MAPK isoforms, however, considering that there are distinct MAPK kinases, it is likely that the phosphatases will in turn, be specific for the MAPKs. The dual specificity phosphatase family now includes PAC-1, identified as a mitogen-induced

tyrosine phosphatase isolated from T-cells, which is predominantly expressed in hematopoietic tissues (Rohan *et al.*, 1993). PAC-1 is localised to the nucleus, and has subsequently been shown to be a physiologically relevant MAPK phosphatase, specific for Thr and Tyr residues (Ward *et al.*, 1994). Other more recently identified dual specific phosphatases include HVH3 isolated from human placenta (Kwak and Dixon, 1995), B23 isolated from human epithelial cells (Ishibashi *et al.*, 1994), HVH2 also isolated from human placenta (Guan and Butch, 1995), and MKP-2 cloned from rat PC12 cells (Misra-Press *et al.*, 1995). These multiple dual specific phosphatases exhibit some similar features: that is they are specific Thr/Tyr phosphatases for MAPK, and their expression is induced by both mitogenic and stress related pathways. However, these phosphatases have distinct tissue distribution profiles, which may dictate unique roles for the members of this family in the regulation of MAPKs, and might reflect their co-expression with certain MAPK isoforms. For example, PAC-1 is predominantly expressed in hematopoietic tissue. Furthermore, MKP-2 has been demonstrated to co-localise with ERK1 in specific areas of the brain where MKP-1 is not expressed (Misra-Press *et al.*, 1995; Kwak *et al.*, 1994). The precise function of MAP kinase in post-mitotic neuronal cells is unclear, however, *in situ* hybridisation has demonstrated that in the adult brain, *erp* (MKP-1) transcripts are also primarily localised in postmitotic, terminally differentiated cells (Carrasco and Bravo, 1993). Thus, it would appear that there is a strong correlation for *erp* expression in cellular differentiation. The subcellular compartmentalisation of the MAPK phosphatases will also determine their substrate specificity, and immunofluorescence studies have demonstrated that CL100, PAC-1, HVH2 and HVH3 all localise to the nucleus (Sneddon and Keyse, unpublished; Rohan *et al.*, 1993; Guan and Butch, 1995; Kwak and Dixon, 1995). In addition, MAPK also translocates to the nucleus under certain conditions (Seth *et al.*, 1992), and it has been suggested that the duration of MAPK activation serves to determine the biological response to growth factor stimulation, discussed in section 1.6. Perhaps then, it is not so coincident that the MAPK phosphatases are differentially regulated in response to mitogens and cellular stress, also demonstrating different kinetics of induction. Although all the dual specific

phosphatases identified to date behave as immediate early genes, HVH3 is up-regulated for 6 hours, in contrast to the more transient activation of CL100 (Kwak and Dixon, 1995). Misra-Press and co-workers (1995), have also observed that serum stimulation of MKP-2 is biphasic, whilst NGF, which sustains the level of active MAPK for a longer duration than EGF, results in a prolonged, elevated induction of both MKP-2 and -1.

The strict substrate specificity of the CL100-like phosphatases is reminiscent to that of the Cdc25 genes. Besides the PTPase catalytic motif shared by these two families, the dual specific phosphatases also contain the CH2 domains (Cdc25 homology 2), which are regions that flank the Cdc25 catalytic domain (Keyse and Ginsburg, 1993). However, in the dual specific phosphatases, the catalytic domain is situated at the C-terminus and not at the N-terminus where the CH2 domains are found. Whether these CH2 are functional has yet to be proven, but they have been speculated to either increase substrate selectivity or to be involved in localising proteins to nuclear or cytoskeletal locations (Kwak *et al.*, 1994).

Table 1.

Properties of mammalian dual specific protein phosphatases

Gene and References	Source	Size (amino acid kilobases)	Remarks (↑ = highest expression)
CL100 Keyse and Emslie, '92 Alessi *et al.*, 1993a Zheng and Guan, 1993	Human	367 aa 2.4 kb mRNA	Inducible by cellular stress and growth factors, expression fairly ubiquitous and independent of protein synthesis. Substrate specificity for MAPK both *in vivo* and *in vitro*.
3CH134/MKP-1/Erp Charles *et al.*, 1993 Sun *et al.*, 1993 Noguchi *et al.*, 1993	Mouse	367 aa 2.4 kb mRNA	Mouse CL100 homolog. Substrate specificity towards MAP kinases both *in vitro* and *in vivo*.
PAC-1 Rohan *et al.*, 1993 Ward *et al.*, 1994	Human and Mouse	314 aa 2.4 kb mRNA	Mitogen inducible, localised in the nucleus. Hematopoietic cell specific, with activity towards MAPK both *in vitro* and *in vivo*. Closely related to CL100.
VHR Ishibashi *et al.*, 1992	Human	185 aa	Activity towards Tyr and Ser phosphorylated substrates *in vitro*. MAPK specificity unknown.
B23 Ishibashi *et al.*, 1994	Human	397 aa 2.5 kb mRNA	Induced by serum and heat shock, with similar kinetics to CL100. *In vitro* specificity towards MAPK.
HVH2 Guan and Butch, 1995	Human	394 aa 2.5- and 6.0-kb mRNAs	Localised in nucleus and induced by phorbol esters. Specificity towards MAPK *in vitro* and *in vivo*. ↑ expression in placenta and pancreas
HVH3 Kwak and Dixon, 1995	Human	384 aa 2.4 kb mRNA	Nuclear localisation, serum induction elevated for 3 hours, dependent on protein synthesis. ↑ expression in liver and placenta. Specificity towards MAPK *in vitro*.
MKP-2 Misra-Press *et al.*, 1995	Rat	393 aa 6.0 kb mRNA	Serum induced bi-phasic stimulation, sustained ~6Hrs. ↑ expression in heart and lung. Also induced by stress in CNS. Specificity for MAPK *in vitro* and *in vivo*.
Cdc25A, B, C Gautier *et al.*, 1991 Galaktionov *et al.*, '95	Human	~ 417 aa	-C and -A specifically dephosphorylate Cdc2-cyclin B and Cdk2-cyclin E, regulating entry into mitosis and G1/S transition respectively.
KAP/Cdi1 Gyruis *et al.*, 1993 Hannon *et al.*, 1994	Human	212 aa	Like Cdc25, shows homology only across PTPase domain. Activity towards Ser and Tyr *in vitro*. Complexes with certain Cdks

1.8. Switching off MAP Kinases

Only recently has the complexity of the MAP kinase signalling been understood, which involves the integration of at least three parallel pathways which can be stimulated simultaneously. In contrast to the well characterised activation of MAP kinases, much less is understood regarding their inactivation. This can be accomplished by dephosphorylation of either the regulatory phosphothreonine or phosphotyrosine residue, or both. Evidence from *in vitro* studies show that ERK1 and -2 can be inactivated completely by treatment with either the protein phosphatase 2A, specific for Ser/Thr residues, or CD45, a tyrosine phosphatase (Anderson *et al.*, 1990). PP2A is also strongly implicated in regulating the MAPK pathway *in vivo* by the finding that expression of SV40 small t antigen in CV1 cells up-regulates MKK and ERK1 and -2 activity, without any effect on Raf-1 activity (Sontag *et al.*, 1993). SV40 small t antigen acts as a surrogate inhibitory B-type regulatory subunit for PP2A. Such experiments indicate that PP2A is an important negative regulator, but do not reveal which components are the target for inactivation by PP2A. Indeed, as previously discussed, PP2A dephosphorylates and inactivates both MKK and MAPK (Dent *et al.*, 1992; Kyriakis *et al.*, 1992). The recently identified inducible protein phosphatases, with dual tyrosine/threonine specificity, and selectivity for MAPK are ideal candidates for inactivating MAPKs *in vivo*, as discussed above, and there is much evidence from studies in fibroblasts and Cos cells to suggest that this is indeed the case. For example, in NIH 3T3 fibroblasts, the inactivation of ERK2 is initiated 60 minutes after serum stimulation, the time at which the MKP-1 protein first appears (Sun *et al.*, 1993). However, in many cells the activation of MAPKs is a transient event, even in the continuing presence of the activating stimulus. For instance, some growth factors or hormones, such as insulin in adipocytes, or EGF in PC12 cells, produce a rapid and transient activation of MAPK, the inactivation of which is initiated after 10 minutes (Ray and Sturgill, 1987; Traverse *et al.*, 1992). In these situations the kinetics of inactivation are too rapid to be explained by *de novo* synthesis of dual specificity phosphatases. Recent experiments have now shown that the rapid

inactivation of ERK2 in endothelial, adipose and PC12 cells after stimulation with EGF is not catalysed by CL100. Protocols for treating cells with actinomycin D and cycloheximide were established to eliminate the detection of CL100 mRNA and protein, and under such circumstances ERK2 inactivation was not affected (Wu *et al.*, 1994; Alessi *et al.*, 1995). However, dephosphorylation of the Tyr residue was sensitive to sodium vanadate, implicating the involvement of a tyrosine phosphatase distinct from CL100. By contrast, dephosphorylation of the Thr residue was elicited by a vanadate-insensitive activity, and indeed, biochemical studies have identified PP2A activity as a rate-limiting step in the inactivation of ERK2 in PC12 cells (Alessi *et al.*, 1995). In a number of situations CL100 induction is not accompanied by the inactivation of MAPK, for example, the sustained activation of ERK2 in response to NGF in PC12 cells (Wu *et al.*, 1994; Alessi *et al.*, 1995). This raises the possibility that the MAPKs may be protected from CL100-like phosphatases, perhaps spatially or through a phosphatase-inhibitor. In *Xenopus* oocytes, there is a rapid inactivation of MAPK following fertilisation, and Sarcevic *et al.* (1993), have purified an as yet uncharacterised tyrosine phosphatase from the cytosolic fraction of *Xenopus* oocytes, which targets the regulatory Tyr residue of MAPK. This corroborates the findings of Alessi *et al.* (1995), who identify a tyrosine phosphatase activity in PC12 cells in addition to PP2A. However, more recently, Lewis *et al.* (1995), have cloned the *Xenopus* homolog (XCL100) of the human CL100 phosphatase. Interestingly, XCL100 is constitutively expressed in oocytes, whilst in a *Xenopus* epithelial cell line, this enzyme shows similar properties to previously identified dual specific phosphatases, such as induction by serum and cellular stress. Thus, further investigations are necessary to determine the precise role of XCL100 in the regulation of MAPK activity during early development.

Based on the studies outlined above, a model for the physiological inactivation of MAP kinase has been tentatively proposed (Keyse, 1995). Stimuli which lead to a very rapid and transient activation of MAP kinase do not result in significant translocation of the kinase to the nucleus. Therefore, the activity of MAP kinase is held in balance between the activities of MKK and PP2A in the cytoplasm. A separate

tyrosine phosphatase is also implicated in the regulation of cytosolic MAPK, however, it is the dephosphorylation of the phosphothreonine by PP2A that is the rate-limiting step. Hence, class-specific phosphatases that selectively dephosphorylate the Thr or Tyr phosphorylation sites of MAPK may control its inactivation under certain circumstances. In contrast, sustained activation of MAPKs is accompanied by translocation to the nucleus. This separates MAPK from both MKK and PP2A, which remain cytosolic. Once in the nucleus, MAPK is subject to regulation by dual specificity phosphatases, refer to figure 1.6, p58.

However, this model is only hypothetical. It is possible that the cellular targets of the CL100 phosphatase are not the classical p42 and p44 isoforms of MAP kinase. Indeed the specificity of the CL100-like phosphatases for the MAPK isoforms is yet to be determined. It is envisaged that each MAPK isoform will be regulated by both a specific MKK and a specific MAPK phosphatase. Will each of the MAPK phosphatases then be stimulated in a specific pathway, in a similar fashion to the upstream activators of MAPK? Multicyclic enzyme cascades involving protein kinases and phosphatases, such as the MAPK pathway, respond to a great number of regulators, with a high degree of amplification at each step. Cross-talk between parallel signal pathways regulated by protein phosphorylations is inevitable, and mechanisms of positive and negative feedback provide a further network of complexity. Furthermore, cell-type specific differences have been illustrated in the MAPK activation pathway, and subsequently, all these influences are also going to be fundamental in the regulation of the MAPK phosphatases. It is of course entirely possible that the MAPKs are not the only physiological substrates for the dual specific phosphatases.

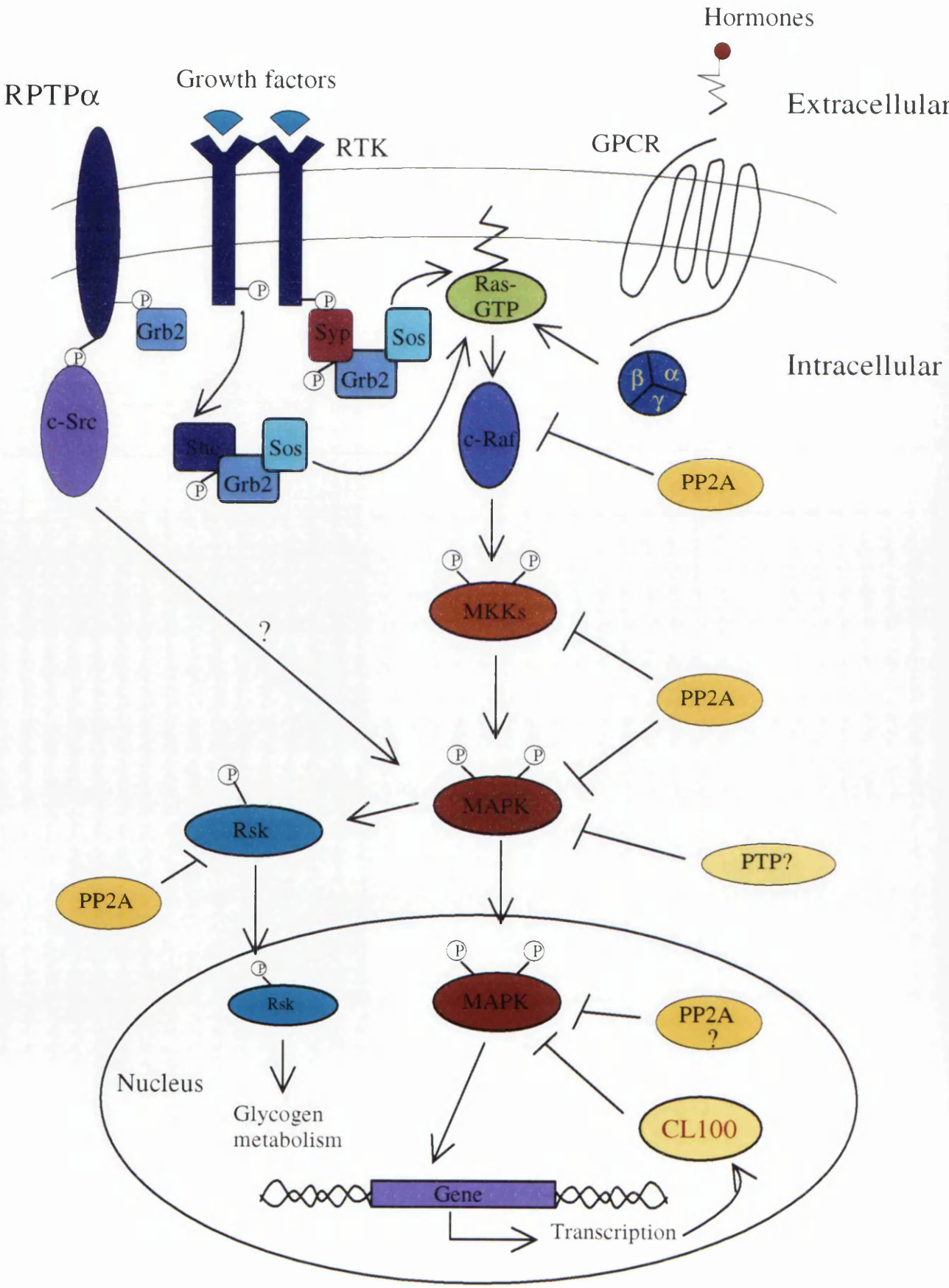
Figure 1.6.

Model for the participation of protein phosphatases in the regulation of the MAP kinase cascade.

The protein tyrosine phosphatase Syp, binds through its SH2 domains to autophosphorylated growth factor receptors, which then phosphorylate Syp such that it complexes with Grb2/Sos to activate p21Ras. The receptor tyrosine phosphatase RPTPα is also able to potentiate the activation of the MAP kinases through the dephosphorylation and activation of c-Src, although the components of this pathway are not known. Conversely, RPTPα can sequester Grb2 molecules preventing the activation of p21Ras, since RPTPα is constitutively phosphorylated on tyrosine residues (by an as yet unknown mechanism), and can therefore bind the SH2 domain of Grb2. Sos is not detected in such complexes. See text for details.

The physiological protein phosphatases for Raf-1, MKKs and RSK are not known, although the serine/threonine phosphatase PP2A, targets these enzymes *in vitro*. Transient activation of MAPK leads to a subsequent, rapid inactivation of the protein in the cytoplasm by PP2A, and an as yet uncharacterised PTP. Sustained activation of MAPK mediates translocation to the nucleus, where it remains active until dephosphorylated on both Thr and Tyr by the dual-specific phosphatases, such as CL100. It is not known if PP2A is nuclear. For full details see text.

Figure 1.6.


Hormones
RPTPα
Growth factors
Extracellular
RTK
GPCR
Ras-GTP
Grb2
Syp
Sos
Grb2
β
α
γ
Intracellular
c-Src
Shc
Sos
Grb2
c-Raf
PP2A
MKKs
?
PP2A
MAPK
Rsk
PTP?
PP2A
Rsk
MAPK
PP2A
?
Nucleus
Glycogen metabolism
CL100
Gene
Transcription

1.9. Summary and aims.

Cellular proliferation and differentiation is potently stimulated by a variety of cytokines and growth factors which specifically interact with receptor tyrosine kinases. Pathways have been defined involving cascades of protein kinases capable of inducing complex sets of immediate early genes, functionally significant in cell cycle regulation and oncogenic transformation. Thus, protein phosphorylation plays a central role in these events, and is regulated by the opposing activities of protein kinases and phosphatases. An essential kinase cascade is the mitogen-activated protein kinase (MAPK) pathway, implicated in the control of many cellular processes. Currently three MAPK pathways are known in mammalian cells, the best understood of which involves the extracellular signal-regulated kinases (ERKs), and in this regard they have been defined as the paradigm for all signalling cascades. Distinct from this classical pathway are the two recently identified MAPK subfamilies, the stress-activated protein kinases (SAPKs/JNKs) and p38/RK.

In contrast to the well characterised activation of MAP kinases, much less is understood regarding their inactivation. This can be accomplished by dephosphorylation of either the regulatory phosphothreonine or phosphotyrosine residue, or both. The recent identification of a family of phosphatase genes demonstrating dual specificity towards threonine/serine and tyrosine residues, harbouring a distinct substrate specificity for the MAP kinases, has led to the suggestion that these enzymes may be the physiologically functional phosphatases that target, and inactivate, the MAP kinases.

This study was designed to isolate further members of this dual specificity phosphatase family. At the start of this project only two such phosphatase genes had been identified, namely CL100 (Keyse and Emslie, 1992) and PAC-1 (Rohan *et al.*, 1993). The technique of polymerase chain reaction (PCR) was employed to amplify DNA sequences by the simultaneous extension of complementary strands of DNA (Mullis and Faloona, 1987). Highly degenerate primers, based on two conserved amino acid sequences between CL100 and PAC-1, were used in an attempt to isolate

further related dual specific phosphatases. The intent was to use the amplified DNA as a homologous hybridisation probe, allowing low stringency screening of gene libraries to isolate corresponding cDNA clones. The cDNA clones would then be fully sequenced, in the hope of deriving full length cDNAs. Five novel, partial cDNAs were detected from screening brain and liver cDNA libraries, which were predicted to encode protein phosphatases on the basis that the cDNAs showed sequence homology to the PTPase catalytic motif. Sequencing of one clone, TYP 1, revealed that it was a full length cDNA. Thus, the subsequent aims of this thesis were then to assess whether TYP 1 demonstrated phosphatase activity towards phosphoproteins, and to characterise the gene by investigating its regulation of expression, both at the RNA and protein level. The cell lines chosen for this study were derived from squamous cell carcinomas (SCC). The EGF receptor is often amplified, and frequently overexpressed in squamous cell carcinomas (Stanton *et al.*, 1994; Ozanne *et al.*, 1986; Cowley *et al.*, 1984; Ullrich *et al.*, 1984), being a consistent marker of squamous cell malignancies *in vivo* as well as in cell culture. Inhibition of ligand binding to the EGF receptor by specific antibodies prevents growth of SCC in culture (Masui *et al.*, 1984), and therefore it has been suggested that overexpression of the EGF receptor may enable the cells to respond to low levels of ligand giving them a selective growth advantage over their neighbours. Interestingly, SCC are growth inhibited by concentrations of EGF that are mitogenic for normal keratinocytes or fibroblasts. It is established that the EGF receptor is the initiating kinase in a well characterised protein kinase cascade, and as such, SCC cells therefore provide an ideal cellular context in which to study protein phosphatases, enzymes which presumably play a role in regulating this kinase cascade.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Tissue Culture

Cell Lines

Swiss 3T3 feeders	An immortal murine fibroblast cell line. A gift from Dr. Ken Parkinson, Beatson Institute.
A431	A vulval squamous cell carcinoma cell line, obtained from Dr. Brad Ozanne, Beatson Institute.
BICR6	A cell line derived from squamous cell carcinomas of the head and neck by Dr. Kirsten Edington, Beatson Institute.
MS-2	A cervical squamous cell derived by Dr. Margareta Nikolic, Beatson Institute.
SCC12F	A squamous cell carcinoma cell line. A gift from Dr. Ken Parkinson.
HKK	A human fibroblast cell strain. A gift from Dr. Ken Parkinson.
Cos1	A cell line derived from the transformation of Simian CV-1 cells with an origin-defective SV40 genome, (Gluzman, 1981). A gift from Dr. Sally Leevers, Chester Beatty Institute for Cancer Research, London.

Beatson Institute Central Services

Sterile phosphate buffered saline (PBS)
Sterile glassware and pipettes

Becton Dickinson U. K. Ltd.

Sterile plastic plates

Fisons Scientific Equipment U.K.

Dimethyl sulphoxide (DMSO)

Gibco Europe Life Technologies Ltd., U.K.

Dulbecco's modified Eagles medium (DMEM)
Foetal calf serum (FCS)
Gentamicin
Glutamine
Methionine-free DMEM

Sodium bicarbonate
Sodium pyruvate

Nunc, Denmark

Cryotubes
Sterile plastic flasks

Research and Diagnostic Sytems, Inc. U.S.A.

Transforming growth factor β1 (TGFβ1), Human platelet derived.

Sigma Chemical Company Ltd., U.K.

Chloroquine, diphosphate salt
Cycloheximide
Diethylaminoethyl-Dextran (DEAE-Dextran)
Epidermal growth factor (EGF), from mouse submaxillary glands.
Hydrocortisone

Worthington Biochemical Company, U.K.

Trypsin

2.1.2. Immunocytochemistry

Antibodies

anti-TYP 1 antibodies were obtained from Affiniti Research Products Ltd.

BDH Analar, U.K.

DPX mountant

Glass coverslips

Fisons Scientific Equipment, U.K.

Acetone, HPLC grade.

Methanol

Xylene

Nunc, Denmark

Chamber slides (glass)

Sigma Chemical Company Ltd., U.K.

Bovine serum albumin fraction V (BSA)

Diaminobenzoic acid

Hydrogen peroxide

Nickel chloride

Tween 20

Unipath Ltd., U.K.

Phosphate buffered saline (PBS)

Vector Laboratories, U.K.

Vectastain ABC peroxidase kit

2.1.3. Protein Biochemistry

Antibodies

anti-ERK2 polyclonal antibody, a kind gift of Anne Wyke, Beatson Institute, Glasgow.

anti-p54 antibody, a kind gift of Dr. Jim Woodgett, Ontario Cancer Institute, Canada.

anti-c-Myc antibody, 9E10, a kind gift of Dr. Sally Cowley, Chester Beatty Institute for Cancer Research, London.

Amersham International plc., U.K.

anti-rabbit Ig, horseradish peroxidase linked whole ab (from donkey)

anti-mouse Ig, horseradish peroxidase linked whole ab (from sheep)

[γ-^{32}P]ATP (5000Ci/mmol)

ECL western detection agent

[L-^{35}S]methionine (1000Ci/mmol), *in vitro* cell labelling mix

Rainbow colour markers (14,300-200,000 Da)

BDH, Analar, U.K.

Butan-2-ol

Ether

Glycerol

Magnesium acetate

Magnesium chloride

Sodium azide

Sodium dodecyl sulphate (SDS)

Sodium pyrophosphate

Trichloracetic acid (TCA)

Boehringer-Mannheim, Germany

EGTA (ethylene glycol tetra-acetic acid)

Eastman Kodak Company, U.S.A.

13255 Cellulose thin layer plate

X-OMAT AR X-ray film

Fisons Scientific Equipment, U.K.

Acetic acid

Ammonium persulphate (APS)

EDTA, sodium salt (ethylene diamine tetra-acetic acid)

Glycine
Hydrochloric acid
Sodium chloride

Gibco Europe Life Technologies Ltd., U.K.

Dithiothreitol (DTT)
Tris base, ultra pure

Merck, Germany

Ninhydrin

Millipore Corporation, U.S.A.

Immobilon P nitrocellulose

Premiere Beverages, U.K.

Marvel, non-fat dried milk

Promega Ltd., U.K.

TNT™ Coupled Reticulocyte Lysate System

Sigma chemical company Ltd., U.K.

Aprotinin
ATP
Bicinchononic acid
Bromophenol blue
Copper II Sulphate
Emetine, dihydrochloride
Leupeptin
Manganese II chloride
2-Mercaptoethanol
Myelin basic protein (MBP)
Phenymethylsulfonyl fluoride (PMSF)
Phosphoamino acids
Protein A sepharose
Pyridine
Sodium deoxycholic acid
Sodium fluoride
Sodium orthovanadate
TEMED (N,N,N',N'-tetramethylethylenediamine)
Triton-X-100

Severn Biotech Ltd., U.K.

30% Acrylamide: 0.8% bisacrylamide

Whatman International Ltd., U.K.

Whatman 3MM filter paper

Glass-microfibre filters (100mm)

2.1.3.1. Proteins

c-Jun protein a kind gift of Dr. Elizabeth Black, Beatson Institute, Glasgow.

GST-ERK2 protein a kind gift of Dr. Sally Cowley, Chester Beatty Institute, London.

GST-TYP 1 protein a kind gift of Dr. Alan Ashworth, Chester Beatty Institute.

MAP kinase kinase, (EE mutant), a kind gift of Dr. Sally Cowley.

^{32}P-labelled ERK2 a kind gift of Dr. Dario Alessi, Biochemistry department, University of Dundee.

2.1.4. Molecular Biology

Amersham International plc., U.K.

[α-^{32}P]dCTP (3000Ci/mmol)

[α-^{35}S]ATP (1000Ci/mmol)

Hybond N+ membrane

Beatson Institute Central services

L-broth

BDH, Analar, U.K.

Acrylamide

Butan-1-ol

Glucose

N N' Methylenebis-acrylamide

Potassium chloride

Xylene cyanol

Bibby-Sterilin Ltd., U.K.

Sterile bacteriological plates

Bio 101 Inc., U.K.

Geneclean II® kit

Boehringer-Mannheim, Germany

Caesium chloride

Calf intestinal alkaline phosphatase

DNAse-free RNAse A

Klenow fragment *E.coli* DNA polymerase

Mops, (4-Morpholinpropansulfonic acid)

Oligo $d(T)_{12}$

Protease K

Cinna/Biotecx Laboratories Inc., U.S.A.

RNAzol™ B

Cruachem Ltd., U.S.A.

Oligonucleotide purification cartridges

Triethylammonium acetate (TEAA)

Trifluoroacetic acid (TFA)

DuPont Instruments, U.K.

Glass corex tubes

Flowgen Instruments Ltd., U.K.

Agarose

Fisons Scientific Equipment, U.K.

Boric acid
Chloroform, HPLC grade.
Di-potassium hydrogen orthophosphate
Di-sodium hydrogen orthophosphate
Formaldehyde
Formamide
Isopropanol
Magnesium sulphate
Potassium acetate
Potassium dihydrogen orthophosphate
Sodium acetate
Sodium citrate
Sodium dihydrogen orthophosphate
Sodium hydroxide
Urea, ultra pure.

Gibco Europe Life Technologies Ltd., U.K.

Bactoagar
Bactotryptone
E.coli DH5α competent cells
ΦX 174 Hae-III digested DNA marker
Restriction enzymes
Yeast Extract

James Burrough Ltd., U.K.

Ethanol

Lab-Scan Ltd., Ireland, U.K.

Acetonitrile

Northumbria Biologicals Ltd., U.K.

T4 DNA ligase
T4 polynucleotide kinase

Nunc, Denmark

Sterile square bacteriological plates

Perkin-Elmer Cetus, U.S.A.

AmpliTaq DNA polymerase

Pharmacia AB, Sweden

Ficoll 400

dNTPs Ultrapure

Oligo-labelling kit

T7 DNA polymerase

Promega Ltd., U.K.

λ Hind-III digested DNA marker

PolyATract™ mRNA Isolation system I

Rathburn Chemicals Ltd., U.K.

Phenol

Sigma Chemical Company, U.K.

Ampicillin

Ethidium Bromide

Kanamycin

Lysozyme

Polyvinylpyrrolidone

Sephadex G50

Yeast RNA type IV

Stratagene, U.S.A.

Lambda Zap II® Library

Technical Photo Systems, U.K.

Fuji RX medical X-ray film

United States Biochemical Corporation, U.S.A.

Sequenase® version II kit

Vernon-Carus Ltd., U.K.

Gauze swabs

2.1.4.1. Vectors

pBluescript®II KS	A 2.96kb phagemid, Stratagene, U.S.A.
pCITE-TYP 1	A 3.8kb plasmid containing an NcoI-Xho1 fragment of TYP1 cDNA cloned into an Nco1-Sal1 fragment of the pCITE-1 vector. The Cap-Independent Translation Enhancer (CITE) is located immediately downstream from a T7 promoter, Novagen, U.S.A.
pMT-TYP 1	A 5.1kb plasmid containing a Sma1-Asp718 fragment of TYP 1 cDNA cloned into a Sma1-Kpn1 fragment of the pMT2 expression vector, in which transcription is directed by the adenovirus major late promoter, (Kaufman *et al.*, 1989).
pMT-Myc-TYP 1	An Nco1-Xho1 fragment of TYP 1 cDNA was Myc-tagged at the amino terminus by ligation to the c-Myc epitope containing a 5' half-Not1 site, and a 3' half-Nco1 site (see Table 2). The resulting construct was cloned into an Not1-Xho1 fragment of the pMT2 vector.
pMT-CL100	CL100 cloned into pTZ19R (a kind gift of Dr. Stephen Keyse, Ninewells Hospital, Dundee) was subcloned into the pMT2 vector as an Nae1-EcoR1 fragment.
pSPT19	A 4.2kb plasmid containing an EcoR1 fragment of avian c-Jun cDNA, cloned downstream of a T7 promoter site. A kind gift of Dr. Elizabeth Black, Beatson Institute.

2.1.4.2. DNA Probes

CL100	An EcoR1-Xho1 fragment excised from pTZ19R.
EGF receptor	An EcoR1 fragment of the 3' end of the EGFR.
TYP 1	A PCR product from pBluescript-TYP 1 using the primers 4340 and -20 (see Tables 2 and 3).
TYP 2	A Xho1-Stu1 fragment excised from pBluescript-TYP 2.

2.1.5. Water

Deionised water for solutions was obtained from a Millipore MilliRO 15 system. Water for tissue culture, nucleic acid and protein procedures was further purified on a Millipore MilliQ system to 18MΩcm.

Table 2.

General oligonucleotide sequences

Oligonucleotide	Sequence
pBluescript II KS	see Maniatis *et al.*, 1989.
Reverse	5' GGA AAC AGC TAT GAC CAT G 3'
M13 -20	5' GTA AAA CGA CGG CCA GT 3'
KS	5' TCG AGG TCG ACG GTA TC 3'
SK	5' CGCTCT AGA ACT AGT GGA TC 3'
Myc epitope	Not1 M E Q K L I S E E D Nco1
4341	5' GGCCGCC ATG GAG CAG AAA CTT ATC TCC GAG GAA GAT C 3'
4342	3' CGG TAC CTC GTC TTT GAA TAG AGG CTC CTT CTA GGTA 5'
TYP 1 Antibody	5' R S P R A E S L R E D S T V 3'
	CGC AGC CCG CGC GCC GAG AGC CTC CGC GAG GAC AGC ACC GTG

Table 3.

Oligonucleotides for dual specificity protein phosphatases

Oligonucleotide	Sequence
Degenerate #	Y D Q G G P V E
3350	5' TA(c/t) GA(c/t) CA(a/g) GGN GGN CCN GTN GA 3'
3351	5' AT (gct)CC (a/t)GC (c/t)TG (a/g)CA (a/g)TG NAC 3'
	-based on VHCQAGI
Forward # TYP 1	
3964	5' GCT CAA AAG GCT GAT GAA CC 3'
4202	5' CAC AGC GGG CTA CAT CCT AG 3'
4262	5' CAC CGT GTC GCT GGT GGT G 3'
4337	5' AGG AGG GTC CTG TGG AGA TC 3'
4340	5' CTT CGA GTT CGT TAA GCA GC 3'
4342	5' CTG CGG GAG CGG GCA GAC CC 3'
Reverse # TYP 1	
4261	5' CAT CTC GTC CCG TTC ATC AGC 3'
4263	5' ACA GCC ACG TTG ACC GAC CT 3'
4338	5' ATC TCC ACA GGA CCC TCC TG 3'
4339	5' CGA TGT ACT CTA TGG CTT CC 3'
4343	5' ACT CGA ACT GCA GCA GCT GC 3'

2.2. Methods

2.2.1. Tissue Culture

2.2.1.1. Culture of Swiss mouse 3T3 feeder cells

Swiss 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 0.3% (v/v) sodium bicarbonate, 2mM glutamine, 1mM pyruvate, 150μg/ml gentamicin. Cells were seeded at 10^4/9cm dish, and incubated at 37°C in an humidified atmosphere containing 7% (v/v) CO_2. Medium was changed every third day and cells used for feeders upon reaching confluency.

For use as feeders, cells were trypsinised with 0.25% (v/v) trypsin in phosphate buffered saline (PBS, 0.14M NaCl, 27mM KCl, 10mM Na_2HPO_4, 15mM K_2HPO_4) and resuspended in fresh growth medium. Cells were then lethally irradiated by exposure to 60Gy of γ-irradiation from a ^{60}Co source. This prevents further cell divisions. The cells were either used immediately or stored for up to 48 hours at 4°C without loss of feeding capacity. Feeder cells were plated at a density of 10^6/9cm plate.

2.2.1.2. Culture of mammalian squamous cell carcinoma cell lines

A431 cells were maintained in DMEM containing 10% (v/v) FCS and supplements, as above.

The BICR6 cell line was grown in serum-containing medium in the presence of a 3T3 feeder layer, as previously described. The 3T3 layer is necessary to initiate colony formation (Rheinwald and Becket, 1981). The medium consists of DMEM with 10% (v/v) FCS and supplements as above, including 0.4μg/ml hydrocortisone. Hydrocortisone is required to make colony morphology more orderly and maintains an increased proliferation rate (Rheinwald and Green, 1975). The same conditions were used for the culturing of cell lines MS-2 and SCC12F, except that they did not require the presence of a 3T3 feeder layer, and the DMEM contained 2% (v/v) FCS.

All cells were incubated at 37°C in an humidified atmosphere containing 7% (v/v) CO_2. Stock cultures were subcloned at a ratio of 1:10 every 3 days. The cells were passaged by aspiration of the growth medium from the monolayer, followed by two successive exposures to 2.5ml of 0.25% (v/v) trypsin solution in PBS. The majority of the trypsin solution was removed and the monolayers incubated at 37°C until the cells could be detached by gentle agitation. Trypsin was inactivated by adding ten volumes of serum-containing medium to the detached cells, which was removed by centrifugation in a Beckman GP centrifuge at 1000rpm for 5 minutes at room temperature. The supernatant was removed and cells resuspended in the appropriate media at the required dilution.

Quiescent cultures were prepared as follows. Cells were plated at approximately 5 x 10^5/175cm^2 flask in growth medium and grown at 37°C for approximately three days. Medium was aspirated, and the sub-confluent monolayers were washed with an equivalent volume of serum-free DMEM. Cells were refed DMEM containing 0.5% (v/v) FCS and appropriate supplements, as above, and incubated for a further 60 hours at 37°C. This rendered all cell lines used quiescent.

2.2.1.3. Culture of human fibroblasts

Human fibroblasts HKK were grown in DMEM as for the squamous cell line BICR6, but containing 15% (v/v) FCS. Stock cultures were maintained, rendered quiescent and passaged as for squamous cells.

2.2.1.4. Culture of monkey Cos1 kidney cells

Cos1 cells were grown in DMEM containing 10% (v/v) FCS and supplements as for Swiss 3T3 cells. Stock cultures were maintained and passaged as for squamous cells.

2.2.1.5. Frozen cell stocks

Stocks of all cells were kept frozen in liquid nitrogen. Cells trypsinized as above were resuspended at a concentration of 10^6 cells/ml in ice-cold medium containing

20% (v/v) FCS plus 10% (v/v) dimethyl sulphoxide (DMSO), (DMSO is toxic to cells at room temperature). Cells were aliquoted into 1.5ml Nunc cryotubes and frozen at -70°C overnight, where they were subsequently placed into liquid nitrogen for long term storage. Cells were thawed by transferring the ampoule directly from liquid nitrogen to water at 37°C. Once thawed cells were added to a large volume of pre-warmed growth medium.

2.2.1.6. Transient transfection of COS cells

For transient transfection, COS-1 cells were seeded at a density of 4 x 10^5 cells/10cm plate in DMEM containing 10% (v/v) FCS and grown for 20 hours prior to a 4 hour incubation at 37°C with 5ml/10cm plate of DMEM containing 10% (v/v) FCS, 0.04% (w/v) DEAE-dextran, 100μM chloroquine phosphate, and 0.5μg expression plasmid, either pMT-Myc-TYP 1, pMT-TYP 1 or parental pMT. After 4 hours, the medium was removed and the cells were incubated for precisely 2 minutes with 5ml of PBS containing 10% (v/v) DMSO. This was removed and replaced with DMEM, 10% (v/v) FCS for 24 hours. The cells were then treated and harvested as required.

For cycloheximide treatment, transfected COS cells were treated with 10μg/ml cycloheximide for the indicated times, in the presence or absence of 30ng/ml EGF which was added 15 minutes prior to cycloheximide addition.

2.2.1.7. Stimulation of cells with growth factors and heat shock

A431 cells maintained in DMEM supplemented with 10% (v/v) FCS were grown to subconfluency in 175cm^2 flasks and mitogen stimulated with 30ng/ml EGF for the indicated periods of time. For serum stimulation of all squamous cell lines and human fibroblasts, medium was aspirated from subconfluent quiescent cultures grown in flasks, and replaced with DMEM containing 10% (v/v) FCS for the indicated times. Note for manipulation of BICR6 cells, cells were grown in the absence of Swiss 3T3 feeders for two passages before any treatment. Heat shock was administered by immersing flasks containing subconfluent cells into a 44°C water bath for 30 minutes.

Following treatment, flasks were returned to 37°C and incubated for the indicated periods of time. For TGFβ treatment, growing subconfluent cells plated in flasks were stimulated with 10ng/ml TGFβ for the indicated times.

2.2.2. Immunocytochemistry

2.2.2.1. Staining for TYP 1

COS1 cells transfected with TYP 1 as described in section 2.2.1.6, were transferred to glass chamber slides 24 hours post-transfection, and grown for a further 24 hours prior to fixation. Non-transfected COS cells and COS cells transfected with parental pMT vector were used as negative controls. Slides were washed twice in ice-cold PBS, once in a 1:1 mix of methanol : acetone, and fixed for 10 minutes in 1:1 methanol : acetone. The slides were air-dried for 30 minutes and used immediately or stored at -20°C in airtight conditions. Cells were incubated with goat blocking serum from the rabbit peroxidase Vectastain kit at 1:10 dilution in PBS/BSA (0.5% (w/v) bovine serum albumin fraction V in PBS) overnight at 4°C in a humid box. Rabbit anti-TYP 1 antibodies (1:4000 dilution in PBS/BSA) or normal rabbit sera were added for 1 hour at room temperature, and the slides rinsed in a high salt solution (PBS containing 0.15M NaCl, 0.05% (v/v) Tween 20), three times for 10 minutes each. The secondary anti-rabbit biotinylated antibody from the kit (1:100 in PBS/BSA) was incubated for 1 hour at room temperature, cells washed as previously described and then exposed to a streptavidin-horseradish peroxidase amplification mix (solutions A and B from the kit) for 1 hour. The PBS/salt rinses were repeated again and the colour developed using a Ni-DAB solution (PBS containing 0.012% (v/v) hydrogen peroxide, 0.2% (w/v) $NiCl_2$, 0.6mg/ml diaminobenzoic acid) for 10 minutes. The cells were dehydrated by sequential immersion into 70% (v/v) ethanol, 90% (v/v) ethanol, 2X 100% ethanol and 100% xylene, overlaid with DPX mountant and a glass coverslip.

2.2.3. Protein Biochemistry

2.2.3.1. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to resolve proteins according to their apparent molecular weight. The acrylamide concentration in the gels varied depending on the technique being used. For most applications gels were 10% with respect to acrylamide, whereas for *in vitro* MAP kinase activity assays, the gels used were 15%.

A 10% or 15% SDS-PAGE gel (10% or 15% acrylamide:*bis*-acrylamide {stock is 30% (w/v) acrylamide, 0.8% (w/v) *bis*-acrylamide}, 375mM Tris pH 8.8, 0.1% (w/v) SDS, 0.1% (v/v) ammonium persulphate (APS), 0.04% (v/v) TEMED) was poured between glass plates, overlaid with H_2O-saturated butan-2-ol, and allowed to polymerise for 1 hour. The gel front was washed with H_2O to remove the butanol and blotted dry with Whatman 3MM paper. The stacking gel (4.8% acrylamide : *bis*-acrylamide, 125mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1% (v/v) APS, 0.04% (v/v) TEMED) was poured on top of the resolving gel, a comb inserted and allowed to polymerise. Due to the difference in pH between the stacking and resolving gel, the stacking gel should only be poured 1 hour before using the gel to minimise merging of the pH's. The gel was assembled in the tank and the wells were flushed extensively with running buffer (25mM Tris pH 8.3, 191mM glycine, 3.5mM SDS) and bubbles removed from the lower surface of the gel plates before the samples were loaded.

Protein samples (50μg) were denatured in sample buffer (62.5mM Tris pH 6.8, 10% (v/v) glycerol, 2.3% (w/v) SDS, 5mM EDTA, 100mM DTT, 0.002% (w/v) bromophenol blue) by heating at 95-100°C for 5 minutes. Proteins purified by immunoprecipitation were resuspended and denatured in an equivalent volume of sample buffer. Rainbow™ markers (Amersham) were included on all gels. Electrophoresis was carried out at 120V for 4-5 hours.

2.2.3.2. Preparation of TYP 1 antiserum

A synthetic oligonucleotide corresponding to amino acids 55 to 68 of TYP 1 (see Table 2, p72), was conjugated to keyhole limpet haemocyanin and used for immunisation of New Zealand White rabbits by conventional protocols (Affiniti Research Products Ltd., U.K.). The specificity of the antiserum was tested by both western blot analysis (see 2.2.3.7) and immunoprecipitation of transiently transfected COS cells with TYP 1 expression plasmids (see 2.2.3.5).

2.2.3.3. Protein extraction from mammalian cell lines

Cell cultures growing on 9cm plates were washed twice in ice-cold PBS, scraped off the plate in 200µl PBS and pelleted in pre-chilled eppendorf tubes by microcentrifugation at 4°C, 6000g for 2 minutes. The cell pellet was resuspended in 50µl P buffer (20mM Tris pH 8.0, 40mM Na_2P_2O, 50mM NaF, 5mM $MgCl_2$, 100µM Na_3VO_4, 10mM EGTA), 50µl S buffer was added (P buffer including 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 20µl/ml aprotinin, 20µg/ml leupeptin, 3mM phenylmethylsulfonyl fluoride (PMSF)), and the lysate incubated on ice for 15 minutes. The suspensions were centrifuged at 4°C, 12,000g for 10 minutes. The supernatant was stored at -70°C.

Protein concentration was determined by analysis of the sample and 1:10 dilutions using a bicinchoninic acid assay. 10µl of each sample was aliquoted into specific positions on a 96 well plate, 200µl of reaction mix (1 volume $CuSO_4$ and 50 volumes bicinchoninic acid) was added to each sample, and the colour allowed to develop at 37°C for 40 minutes. Spectrophotometric analysis was at A590nm using a Dynatech MR7000 spectrophotometer. Calibration was carried out using a range of BSA standards.

2.2.3.4. Immunoprecipitation and assay of MAP kinase activity

In an eppendorf tube, 200µl S buffer (see 2.2.3.3) was incubated with 50µl protein A-sepharose beads and 2µl anti-ERK2 antibody, or 1µl anti-p54 antibody (as indicated), and rotamixed for 1 hour at 4°C. For controls, pre-immune sera was used.

Following a brief centrifugation, the supernatant was removed and the cell lysates (one 9cm plate per immunoprecipitate) from transfected COS cells (see section 2.2.1.6) were incubated with the pre-bound antibody/protein A-sepharose beads overnight at 4°C with rotation. After brief centrifugation, the supernatant was removed and the beads washed three times in S buffer, not containing PMSF, once in 30mM Tris pH 8.0, and once in kinase buffer (30mM Tris pH 8.0, 20mM $MgCl_2$, 2mM $MnCl_2$).

Each immunoprecipitate was stored on ice and resuspended in a total of 30µl kinase buffer containing either 7.5µg myelin basic protein (MBP for ERK2 assay), or 2µg recombinant c-Jun protein (for p54 assay), 1µM ATP and 0.5µCi [γ-^{32}P]ATP. The kinase assay was initiated by incubation at 30°C for 30 minutes. The reactions were terminated by the addition of SDS loading buffer, and samples resolved on 15% SDS-PAGE gel. To reduce background radioactivity the proteins were transferred to nitrocellulose (see 2.2.3.7), the filters were wrapped in Saranwrap™ and exposed to Kodak X-Omat AR film with intensifying screens at -70°C.

2.2.3.5. Methionine labelling and immunoprecipitation of TYP 1

COS cells, 24 hours post-transfection (see section 2.2.1.6), were labelled in 90mm plates with [L-^{35}S] methionine as follows. Culture medium was aspirated and transfected cells washed once with serum-free DMEM lacking methionine. Cultures were then incubated for 1 hour in 3ml/plate of the same medium containing 300µCi of [^{35}S]-methionine. For some applications, the cells were stimulated for 15 minutes with 30ng/ml EGF in methionine free medium prior to [^{35}S]-methionine addition. After 1 hour of cell labelling, the medium was aspirated, and the cultures washed with DMEM containing 10% (v/v) FCS and supplements (see section 2.2.1.4). The cultures were then incubated in 5ml/plate of the same medium for the indicated periods of time prior to harvesting, as described in section 2.2.3.3.

Immunoprecipitates were prepared essentially as described in section 2.2.3.4, with the following changes. The lysates were first pre-cleared by incubation with 50µl protein A-sepharose beads and 20µl of rabbit pre-immune sera for 1 hour at 4°C with rotation. After a brief centrifugation, the supernatant was incubated with 20µl

anti-TYP 1 antibody overnight, at 4°C with rotation. Following this, 50µl protein A-sepharose beads was added and incubated for a further hour at 4°C with rotation. The immunoprecipitates were washed with S buffer as described in 2.2.3.4, and resuspended in SDS-loading buffer, denatured and loaded onto a 10% SDS-PAGE gel. After electrophoresis, the gel was fixed in 10% (v/v) acetic acid, 15% (v/v) methanol for 40 minutes, and dried under vacuum at 70°C for 1.5 hours. The gel was exposed to X-ray film overnight at room temperature.

2.2.3.6. TCA-precipitation of labelled proteins

To ensure that cycloheximide treatment of COS cells inhibited protein synthesis, growing COS cells were treated with 10µg/ml cycloheximide for 30 minutes (see section 2.2.1.6). Cultures were then washed with serum-free DMEM, lacking methionine, and labelled with ^{35}S-methionine for 1 hour as described in section 2.2.3.5. Control cultures were not treated with cycloheximide. Cell lysates were then prepared as described in 2.2.3.3. A 10µl sample of a 10-fold dilution of labelled lysate was mixed with 1ml of ice-cold 20% (w/v) trichloracetic acid, and incubated on ice for 30 minutes. The precipitate was collected on a Whatman GF-C filter and washed extensively with cold 5% (w/v) TCA. Filters were washed with 95% (v/v) ethanol, air-dried and counted in 10ml Ecoscint.

2.2.3.7. Western blotting

For western blot analysis, 50µg protein samples were resolved on 10% SDS-PAGE gels (see section 2.2.3.1). After electrophoresis, the gel was carefully removed from the glass plates, discarding the stacking gel, and soaked in transfer buffer (25mM Tris, 191mM glycine, 3.5mM SDS, 20% (v/v) methanol) for 30 minutes. The protein was transferred in the above buffer onto immobilon-P nitrocellulose using a Bio-rad wet trans-blot apparatus run at 30V, 4°C overnight.

The filter was blocked overnight at 4°C in TBST (20mM Tris pH 7.5, 150mM NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) non-fat dried milk and 0.025% (w/v) sodium azide, and incubated with the primary antibody for 3 hours at room

temperature, typically TYP 1 antisera diluted 1:1000 in blocking buffer, or monoclonal antibody 9E10 diluted 1:1000, which specifically recognises the c-Myc epitope. The filter was subsequently washed 3 times for 5 minutes each in TBST, and incubated with the secondary antibody for 1 hour at room temperature, anti-rabbit IgG conjugated to horseradish peroxidase (1:3000) for TYP 1 antisera, or anti-mouse IgG conjugated to horseradish peroxidase (1:15,000) for c-Myc antisera. The filter was washed extensively in TBST and immersed in ECL detection reagent for 1 minute. Following which the filter was lightly blotted, wrapped in Saranwrap™ and exposed to Kodak X-Omat AR for 30 seconds-3 minutes.

2.2.3.8. *In vitro* protein phosphatase assay

In vitro phosphatase activity of recombinant TYP 1 protein was assayed against activated, ERK2 kinase. Recombinant GST-ERK2 MAP kinase (2μg) was incubated on ice with 100ng activated MAP kinase kinase (EE mutant, Alessi *et al.*, 1993a), 100μM ATP, and 10mM magnesium acetate in a total volume 20μl buffer B (100mM Tris pH 7.5, 0.2mM EGTA, 0.1% (v/v) 2-mercaptoethanol). The reaction was initiated by incubation at 30°C for 30 minutes. After which, the reaction was diluted 5-fold with ice-cold BSA buffer (50mM Tris pH 7.5, 0.1mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1mg/ml BSA). The activated, labelled ERK2 kinase was stored at -70°C.

The phosphatase activity of TYP 1 protein was assayed indirectly by determining the activity of ERK2 against MBP. 10μl of the above activated ERK2 substrate was preincubated with 20ng recombinant purified TYP 1 protein for 10 minutes at 30°C, and placed on ice. The activity of the ERK2 protein was assayed for by the incorporation of phosphate into MBP, exactly as described in section 2.2.3.4. The reactions were resolved by gel-electrophoresis as described in section 2.2.3.1. Fixed, dried gels were exposed to X-ray film at -70°C overnight.

2.2.3.9. In vitro dephosphorylation of recombinant ERK2

Recombinant GST-ERK2 MAP kinase was ^{32}P-labelled and activated with MAP kinase kinase, purified from rabbit skeletal muscle, in the presence of [γ-^{32}P]ATP exactly as described in Nakielny *et al.* (1992). On ice, activated ERK2 (20U/ml, 125µg) was mixed with recombinant, purified TYP 1 protein (0-12.5µg/ml) in a final volume of 10µl B buffer (see 2.2.3.8), in the presence or absence of 2mM sodium vanadate. The reaction was initiated by incubation at 30°C for 10 minutes. After which, 2µl aliquots of each reaction were mixed with SDS-loading buffer and analysed by 10% SDS-PAGE gel (see section 2.2.3.1). The gel was fixed, dried and exposed to X-ray film with intensifying screens at -70°C overnight.

2.2.3.10. One dimensional phosphoamino acid analysis

The ^{32}P-labelled ERK2 from above (10µl samples) was precipitated with 160µl of 20% (w/v) TCA (see section 2.2.3.6). After incubation on ice for 30 minutes, the protein precipitate was collected by centrifugation for 5 minutes at 14,000g. The pellets were washed once with 5% (w/v) TCA, followed with ether, and dried under vacuum. The eppendorf tubes containing the dried pellets were transferred to Wheaton vials, and 600µl 6M HCL added to the vial (not the eppendorf tube). The vials were sealed under vacuum and hydrolysis carried out for 1.5 hours at 90°C, using a Waters PICOTAG system. Samples were allowed to cool to room temperature, and re-dried under vacuum. Finally the samples were redissolved in 3µl of PAA buffer (4.5% (v/v) acetic acid, 0.5% (w/v) pyridine in H_2O) containing 20mM of each unlabelled phosphoamino acid (phosphoserine, threonine, tyrosine) to serve as markers following electrophoresis.

Aliquots (0.5µl) were spotted at a point 2cm away from one edge of a 20cm x 20cm cellulose thin layer plate (Kodak, 13255 cellulose), keeping more than 1.5cm between each sample point. A further two 0.5µl aliquots were applied, allowing the plate to dry between applications. Wicks of Whatman paper were placed on opposite sides of the plate and wetted with PAA buffer, such that the buffer permeated across the plate, the two buffer fronts meeting at the area containing the samples.

Hydrolysates were resolved using a flat bed electrophoresis unit precoated with glycerol to allow thermal conduction, run at 1000V for 50 minutes. The plate was maintained below 10°C during electrophoresis using a block cooled with tap water. Following electrophoresis, the plate was dried using a hair dryer and immersed for a few seconds in 0.5% (w/v) ninhydrin in acetone to visualise the standards. Dried plates were exposed to X-ray film overnight at -70°C.

2.2.3.11. *In vitro* translation

In vitro translation was carried out using the TNT™ Coupled Reticulocyte Lysate System from Promega, essentially as described by Promega instructions. This system incorporates transcription from SP6, T3 or T7 RNA polymerase promoters directly in the translation mix, which uses standard rabbit reticulocyte lysates.

Reagents were removed from storage at -70°C and thawed on ice. To an eppendorf tube placed on ice, 25µl rabbit reticulocyte lysate was added together with 2µl reaction buffer, 1µl amino acid mix minus methionine (1mM), 1µl RNasin ribonuclease inhibitor (40 units), 4µl [L-^{35}S]methionine (1000Ci/mmol, Amersham), 1µg DNA template (pCITE-TYP 1 and/or pSPT19-c-Jun) and 1µl RNA polymerase (T7) in a total volume 50µl. The components were gently mixed and incubated at 30°C for 2 hours. For non-radioactive control reactions, a complete amino acid mix was used. After translation, the reactions were terminated by the addition of 0.1mM emetine. To determine TYP 1 phosphatase activity towards c-Jun, 10µl samples of translated TYP 1 and c-Jun, treated with 0.1mM emetine, were mixed and incubated at 30°C for 30 minutes. All samples (20µl) were mixed with SDS-loading buffer, denatured and loaded onto 10% SDS-PAGE gel, as described in 2.2.3.1. Following electrophoresis, the gel was fixed for 30 minutes in (v/v)10% acetic acid, 15% (v/v) methanol, and dried under vacuum at 70°C for 1.5 hours. The gel was exposed to X-ray film at room temperature.

2.2.4. Molecular Biology

2.2.4.1. Preparation of genomic DNA from cell lines.

DNA was prepared using the salting out method as described by Miller *et al.* (1988). Cells were lysed in 3ml lysis buffer (10mM Tris-HCL, 400mM NaCl, 2mM EDTA, pH 8.2) and digested overnight at 37°C with 0.2ml 10% (w/v) SDS and 0.5ml protease K solution (1mg protease K in 1% (w/v) SDS, 2mM EDTA). After digestion, 1ml saturated NaCl (approximately 6M) was added and the tube shaken vigorously for 15 seconds, followed by centrifugation (Beckman J2-21) at 3000g for 5 minutes to pellet the protein. Exactly two volumes of room temperature ethanol was added to precipitate the DNA strands, which were spooled out on a glass pipette, air-dried and resuspended in 100-200µl TE (10mM Tris-HCL, 0.2mM EDTA, pH 7.5). DNA concentrations were determined by measuring absorbance at 260nm and using the conversion of 1OD unit at 260nm is equivalent to a concentration of 50µg/ml. The ratios 260nm/280nm should be 1.8-2.0 showing good deproteinization of the DNA sample. Genomic DNA was stored at 4°C.

2.2.4.2. Total RNA extraction from mammalian cell lines.

Extraction of total RNA from mammalian cell lines was carried out following the manufacturer's protocol for RNAzol™ B (Cinna/Biotecx). Sub-confluent cells grown in 175cm^2 flasks were washed twice in ice-cold PBS which was completely removed by aspiration. Cells were subsequently lysed directly in the culture flask by the addition of 6ml RNAzol™ B (1ml/3.5cm dish). A disposable cell scraper was implemented to homogenise the cells, and the RNA was solubilised by passing the lysate a few times through the pipette. Chloroform was added (0.2ml per 2ml lysate) and the glass corex tubes vigorously shaken for 15 seconds, then incubated on ice for 5 minutes. The samples were centrifuged at 12,000g, 4°C for 15 minutes after which the upper, colourless aqueous phase was transferred to a fresh corex tube and an equal volume of isopropanol added. The samples were stored overnight at -20°C. The RNA precipitate was pelleted by centrifugation at 12,000g, 4°C for 25 minutes. The RNA pellet was washed once with 2ml ice-cold 75% (v/v) ethanol by vortexing and centrifugation at

7,500g, 4°C for 8 minutes. The RNA was air-dried and redissolved in 500µl diethylpyrocarbonate (DEPC) RNase-free water. Note, all solutions and tubes should be pre-treated with DEPC to prevent RNase contamination. The RNA concentration was determined by measuring the absorbance at 260nm and using the conversion of 1OD unit being equivalent to 40µg/ml. RNA was stored at -20°C.

2.2.4.3. Preparation of poly (A)+ RNA

Poly (A)+ RNA was prepared using the PolyATtract® mRNA Isolation System I from Promega. The system uses a biotinylated oligo (dT) primer to hybridise at high efficiency in solution to the 3' poly A region present in eukaryotic mRNA species. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand.

In a sterile, RNase-free eppendorf, 1mg of total RNA was combined with RNase-free H_2O to a final volume of 500µl, and placed in a 65°C heating block for 10 minutes. After which 13µl 20X SSC and 3µl biotinylated-oligo (dT) probe (both reagents supplied) was added, mixed gently and incubated at room temperature until completely cooled. Streptavidin-paramagnetic particles (supplied) were resuspended gently using a pipette and 0.6ml aliquoted per 1mg total RNA into a screw top eppendorf. The beads were captured by placing in a magnetic stand and washed three times with 0.5X SSC (0.3ml per wash). Washed beads were resuspended in 0.5ml of 0.5X SSC, and the entire contents of the annealing reaction added. This was incubated at room temperature for 10 minutes. The beads were then captured using the magnetic stand and washed four times with 0.1X SSC (0.3ml per wash). After the final wash, as much of the aqueous phase as possible was removed using an aspirator. The mRNA was eluted from the solid phase by two successive washes with RNase-free deionized H_2O (100µl and 150µl). The pooled eluate (0.25ml total) was frozen at -20°C and then freeze-dried to a volume of approximately 10µl for northern analysis. The mRNA was stored at -20°C, and typically yielded 10µg.

2.2.4.4. First strand cDNA synthesis

For cDNA preparation, poly (A)+ RNA (10μg) was converted into first strand cDNA using 500 U Mo-MLV reverse transcriptase in a buffered solution (50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$) containing 10mM DTT, 0.1mM Oligo $(dT)_{12}$ and 0.25mM each dNTP in a total volume of 50μl, incubated for 1 hour at 37°C. The cDNA synthesis reactions were terminated by heating at 65°C for 10 minutes and then stored at 4°C. For PCR applications, cDNA was purified using Geneclean (see 2.2.4.9) and eluted in 50μl TE.

2.2.4.5. Minipreparations of plasmid DNA

Small amounts of plasmid DNA were prepared using the alkaline lysis method as described by Maniatis *et al.* (1989). Single colonies of bacteria carrying the required plasmid were picked from agar plates using a sterile loop and grown in 5ml L-broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0) supplemented with the appropriate antibiotic (50μg/ml ampicillin) at 37°C in a shaking incubator. Bacteria were pelleted from 5ml of the overnight culture at low speed for 1 minute in a bench top centrifuge. The drained pellet was resuspended in 200μl of solution I (50mM Glucose, 25mM Tris-HCL pH 8.0, 10mM EDTA pH 8.0) and transferred to an eppendorf. After incubation for 5 minutes at room temperature, alkaline lysis was effected by the addition of 400μl of freshly made, ice-cold solution II (0.2M NaOH, 1% (w/v) SDS). The samples were mixed by inversion and placed on ice for 5 minutes. Lysis was terminated by the addition of 300μl ice-cold solution III (3M KAc pH 4.8), vortexed and incubated on ice for a further 5 minutes. The precipitated cell debris was pelleted in a bench top microcentrifuge for 2 minutes and the supernatant removed to a fresh eppendorf tube. At this point RNase treatment was employed, which involved incubation with 10mg/ml RNase for 10 minutes at 60°C. The nucleic acids were precipitated by adding two volumes of ice-cold ethanol to the supernatant, standing at room temperature for 5 minutes and microcentrifuged for 10 minutes. The pellet was washed in 1ml -20°C 70% (v/v) ethanol, microcentrifuged for 10 minutes and all ethanol removed using an aspirator. The pellet was air-dried and

dissolved in 25µl TE. Plasmid DNA was stored at -20°C. Typically, 2µl of this minipreparation was digested for mapping purposes.

2.2.4.6. Large scale preparation of plasmid DNA

A 10ml overnight culture was transferred to 500ml L broth containing antibiotic (50µg/ml ampicillin) and shaken at 37°C overnight. Cells were pelleted from the overnight culture in polypropylene bottles at 5000rpm, 4°C for 10 minutes, rinsed with 50mM Tris-HCL pH 8.0 and resuspended in 25ml lysozyme solution (25mM Tris-HCL pH 8.0, 10mM EDTA, 50mM glucose containing 5mg/ml lysozyme) for 30 minutes at room temperature. Following this, 40ml solution II (0.2M NaOH, 1% (w/v) SDS) was added, mixed well and placed on ice for 15 minutes. Protein and detergent was then precipitated by adding 20ml solution III (3M KAc pH 4.8), mixed by inversion and kept on ice for a further 15 minutes. The flocculates were centrifuged at 10,810g, for 5 minutes at 4°C. The supernatant was filtered through a gauze, and the DNA precipitated by adding 0.6 volumes of -20°C isopropanol and immediately centrifuged at 10,810g, room temperature for 5 minutes. After discarding the supernatant, the pellet was drained thoroughly and then resuspended in 5ml TE, pH 8.0.

Plasmid DNA was purified on a CsCl gradient. Precisely 7.5g CsCl was dissolved in the 5ml DNA solution in a plastic universal, and 5mg ethidium bromide added. The refractory index was adjusted to 1.3860-1.3900 with TE pH 8.0 or CsCl. The CsCl solution was balanced in Beckman polycarbonate tubes and centrifuged in a Beckman TL-100 ultracentrifuge (rotor TLA-100) at 40,000 rpm for 40 hours at 20°C.

The plasmid band was removed from the gradient using a syringe with a 19 gauge needle and extracted with an equal volume of water-saturated isobutanol until all the ethidium bromide had been removed. The DNA was precipitated by adding an equal volume of water and 2 volumes of room temperature ethanol, and left standing at room temperature for 15 minutes. The DNA was pelleted in glass corex tubes by centrifugation at 11,950g, 4°C for 15 minutes. The pellet was resuspended in

1ml deionised H_2O and re-precipitated with 0.1 volumes 3M NaOAc pH 5.0 and 2 volumes ethanol at -20°C for 1 hour. DNA used for transfection of cells in culture was phenol/chloroform extracted before precipitation. The DNA pelleted as above, was rinsed with 70% (v/v) -20°C ethanol, freeze-dried and dissolved in TE, pH 8.0. The DNA concentration was measured as described in section 2.2.4.1.

2.2.4.7. Restriction enzyme digest of DNA

Plasmid DNA (<2μg) was incubated with 5-10 units enzyme in a buffered solution as specified and supplied by the manufacturer for 1-2 hours at 37°C. Larger, preparative digests were carried out in proportionally larger volumes. For double digests, manufacturer's information was consulted and the appropriate buffer used.

Digestion of genomic DNA (30-50μg) was carried out essentially as described except digestion was continued overnight. Following this, a 5μl sample was analysed by gel electrophoresis (see below) to determine the extent of digestion. Where necessary a further aliquot of enzyme was added and incubation continued. Due to the inhibitory effects of high glycerol concentrations, the volume of enzyme used never exceeded 1/10th of the total volume.

All reactions were terminated by the addition of DNA loading buffer and digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.4.8. Agarose gel electrophoresis

Flat bed electrophoresis apparatus was used. Non-denaturing agarose gels (0.8%-1.5% (w/v) agarose) were cast in 1X TAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA, pH 8.0) containing 0.5μg/ml ethidium bromide. The gel was submerged in 1X Tae buffer. Samples for electrophoresis, and molecular weight standards (bacteriophage λ Hind-III digested or ΦX174 Hae-III digested) were mixed with one tenth volume of gel loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol in TE), loaded and resolved by electrophoresis towards the anode at 100V for 30-60 minutes. The separated DNA was visualised by illumination with

short wave (312nm) UV light and photographed through a red filter using polaroid type 57 high speed film or using an appligene imager.

2.2.4.9. Elution of DNA fragments from agarose gels

Geneclean II® (BIO 101) was used according to manufacturer's instructions. The required DNA fragment was excised from the gel and the agarose block containing the fragment was weighed and volume calculated (1g = 1ml). 2.5 volumes of 6M NaI was added and the agarose incubated at 55°C for 5 minutes to induce gel melting. Following this, 5µl glassmilk®, a suspension of silica matrix in water which binds single and double-stranded DNA without binding contaminants, was added for every 5µg or less DNA. This was mixed and left at room temperature for 5 minutes to allow binding. The glassmilk was pelleted by brief centrifugation and washed 3 times by mixing with 300µl NEW wash (a Tris and EDTA-buffered solution of NaCl, ethanol and H_2O), pelleting the glassmilk and removing the supernatant. Finally, half the desired volume of TE was added to the cleaned pellet and heated to 55°C for 5 minutes. The glassmilk was pelleted and the TE containing DNA removed to a fresh eppendorf. This was repeated to elute all the DNA. Typically for most applications DNA was eluted in 20µl final.

2.2.4.10. Ligation of DNA/PCR fragments

Vector DNA was digested as described in section 2.2.4.7. The DNA fragment to be inserted was also digested as above and then isolated by gel electrophoresis and purified as described in section 2.2.4.9.

To prevent the vector DNA from ligating back to itself without an insert, it was first dephosphorylated. The vector DNA was digested with the required enzyme as directed by the manufacturer and an aliquot removed to check that digestion was complete. The linearized plasmid DNA was dephosphorylated using calf intestinal alkaline phosphatase (5 units) essentially according to Maniatis *et al.* (1989), except that reactions were carried out in restriction buffer. The final volume (twice the digestion volume) was made up with H_2O and the reaction incubated at 37°C for

1 hour. The digested, dephosphorylated DNA was then isolated by electrophoresis and purified using Geneclean II® (see section 2.2.4.9).

PCR products were cloned by blunt-end ligation as described by Ashworth, (1993). PCR products electrophoresed in agarose and purified using Geneclean II® (eluted in 13µl water) were mixed with kinase buffer (70mM Tris-HCl pH 7.5, 10mM $MgCl_2$), 1mM ATP, 10mM DTT and T4 polynucleotide kinase (5 units) in a total of 20µl, and incubated at 37°C for 30 minutes. After which 0.5mM each dNTP and Klenow polymerase (5 units) were added, mixed and incubated 15 minutes at room temperature. The DNA was purified using Geneclean II® and eluted into 16µl H_2O.

Ligations were effected in a minimum volume (20µl). The insert was ligated to the dephosphorylated vector (10-20ng) at a ratio 3:1 respectively, (for PCR products the total 16µl was used). The vector and insert DNA were mixed on ice in a reaction containing ligase buffer (66mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1mM ATP, 1mM DTT) and T4 DNA ligase (5-10 units), and incubated at room temperature for a minimum of 2 hours.

2.2.4.11. Transformation of competent bacterial cells

Competent *E.coli* DH5α cells were thawed on ice and to each 200µl aliquot, the appropriate amount of plasmid DNA or ligation mix (10µl) was gently added. After incubation on ice for 30 minutes, the cells were heat shocked for 5 minutes at 37°C, and then returned to ice. 0.5ml of SOC medium (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to the cells, which were returned to 37°C for 40 minutes to allow expression of the antibiotic resistance marker. Cells were then pelleted by brief centrifugation and resuspended in 50µl of L-broth. All cells were spread onto an L-broth agar plate (15g Bacto-Agar/litre L-broth) containing antibiotic and incubated overnight at 42°C. Plasmid DNA was prepared from transformed colonies as described in section 2.2.4.5, or amplified by PCR as in section 2.2.4.16b.

2.2.4.12. Radioactive labelling and purification of DNA probes

DNA fragments were ^{32}P labelled using an oligolabelling kit (Pharmacia) as per manufacturer's instructions. DNA (50ng-100ng) in a volume of no more than 34µl was denatured by heating for 2 minutes at 100°C, and then placed on ice prior to the addition of 10µl reagent mix (buffered aqueous solution containing dATP, dGTP, dTTP and random hexadeoxy-ribonucleotides), 50µCi [α-^{32}P]dCTP and 10 units Klenow fragment. The reaction mix was incubated at 37°C for 1 hour.

The labelled probe was purified on a sephadex-G50 column. Eppendorf tubes pierced at the bottom were packed with sephadex-G50 equilibrated in TE buffer (pH 8.0), excess liquid was removed by centrifugation at 2000rpm for 3 minutes. Samples were subsequently spun at 2000rpm for 5 minutes, and the unincorporated nucleotides retained in the beads. Radioisotope incorporation in the probe was monitored using a scintillation counter.

2.2.4.13. Southern analysis

30µg of digested genomic DNA samples (see section 2.2.4.7) were electrophoresed on a 0.8% (w/v) agarose gel together with Hind-III digested λ DNA to serve as markers. Electrophoresis was overnight at 30V in 1X TAE buffer. The DNA was photographed and the gel depurinated by soaking in 0.25M HCl until the dye front changed to a yellow colour. The gel was rinsed in deionised H_2O and the DNA transferred overnight onto Hybond N+ membrane by upward capillary blotting using 0.4M NaOH, essentially as described in Maniatis *et al.* (1989). The blot may be stored wrapped in Saranwrap™ at 4°C.

The detection of specific DNA species was carried out using the method of Church and Gilbert, (1984). Membranes were pre-hybridised at 65°C for 1 hour in sealed plastic bags containing approximately 25ml aqueous hybridisation solution (0.5M Na_2HPO_4 pH 7.2, 1mM EDTA, 7% (w/v) SDS). Probes, labelled by the random priming method above, were denatured at 100°C for 2 minutes and incubated with the membranes in a minimal volume of fresh hybridisation solution (5 x 10^6cpm/ml). Membranes were hybridised overnight at 65°C in a shaking water bath.

To remove non-specific binding the membranes were washed at high stringency (40mM Na_2HPO_4, 1% (w/v) SDS), with multiple quick washes at room temperature followed by a final wash at 65°C for 20 minutes. Excess liquid was removed and the membrane wrapped in Saranwrap™ and exposed to photographic film (either Kodak X-OMAT AR or Fuji RX) with intensifying screens at -70°C.

2.2.4.14. Northern Analysis

Northern analysis was carried out essentially as described by Maniatis *et al.* (1989). Poly (A)+ RNA samples (10µg) were mixed with 3 volumes RNA loading buffer (2ml contains 350µl formaldehyde, 1ml formamide, 150µl RNA loading dye {50% (v/v) glycerol, 0.1mM EDTA, 0.6% (w/v) bromophenol blue, 0.6% (w/v) xylene cyanol) 30µl ethidium bromide - 10mg/ml stock, 200µl 5X Mops buffer containing 0.1M Mops, 40mM sodium acetate, 5mM EDTA, pH 7.0) and heated for 10 minutes at 65°C. The samples were loaded onto a 1% (w/v) agarose gel containing 20% (v/v) 5X Mops, 17.5% (v/v) formaldehyde. Electrophoresis was overnight at 30V in 1X Mops buffer which was constantly recirculated. The RNA was photographed and the gel soaked in deionised H_2O for 10 minutes to leach out the formaldehyde. Transfer of the RNA to Hybond N+ membrane was the same as for southerns, except that the buffer used was 20X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0). The membrane was UV-crosslinked using a UV Stratalinker 1800 (Stratagene).

Membranes were prehybridised in 10ml of hybridisation solution (50% (v/v) deionised formamide, 10X Denhardt's solution {100X stock contains 10g ficoll, 10g polyvinylpyrrolidone, 10g BSA in 500ml H_2O}, 5X SSPE {20X stock contains 3M NaCl, 0.2M $NaH_2PO_4.H_2O$, 20mM EDTA, pH 7.4} 2% (w/v) SDS, 1mg/ml yeast RNA} at 42°C for a minimum 5 hours, using Hybaid™ bottles in a Mini oven MK II (as per manufacturer's instructions, rotation speed 7). The hybridisation solution was replaced with fresh solution containing the radiolabelled probe (prepared as in section 2.2.4.12) at a concentration of 5 x 10^6cpm/ml, and hybridised at 42°C overnight. The membrane was washed at low stringency (2X SSC, 0.05% (w/v) SDS)

several times at room temperature, and then at high stringency (0.1X SSC, 0.1% (w/v) SDS) at 50°C for 40 minutes with one change of fresh wash solution. Excess solution was shaken from the membrane, and the blot covered in Saranwrap™ and exposed to X-ray film (as above) at -70°C with intensifying screens.

Radioactive probe was stripped from the membrane by gentle agitation of the blot for 10 minutes in sterile H_2O containing 0.5% (w/v) SDS which had been heated to 100°C. The solution was allowed to cool before the membrane was removed. The membrane can be stored wrapped at -20°C.

2.2.4.15. Oligonucleotide synthesis and purification

Oligonucleotides were synthesised on an Applied Biosystems 381A DNA synthesiser or 392 DNA/RNA Synthesiser using the manufacturer's protocols and Cruachem reagents. Primers were synthesised with or without trityl group protection. All these primers were firstly deprotected by incubation overnight at 55°C.

"Trityl on" primers were detritylated using an Applied Biosystems oligonucleotide purification cartridge. 5ml acetonitrile was passed through the column to waste at a rate of 1 drop/sec using a syringe. This was followed by 5ml 2M triethylammonium acetate. The deprotected oligonucleotide ammonium solution was diluted with an equal volume of distilled H_2O and passed through the column the same way. The eluate was collected and passed through a second time. The cartridge was then flushed through with 5ml 10% (v/v) ammonia and 10ml distilled H_2O. The oligonucleotide was detritylated while bound to the support by passing 2.5ml 2% (v/v) trifluoroacetic acid through the column, allowing it to stand for 5 minutes and repeating. The cartridge was then flushed with 10ml distilled H_2O. The oligonucleotide was eluted drop-by-drop with 3 ml 20% (v/v) acetonitrile, freeze-dried overnight and dissolved in 250ml TE pH 8.0. Primer concentration was determined by measuring absorbance at 260nm and using the conversion that $33OD_{260}$ is equivalent to 1mg/ml. Primers were stored at -20°C.

"Trityl off" primers were purified by desalting by precipitation with butan-1-ol. 1ml butan-1-ol was added per 150µl primer solution and microcentrifuged at 13,000g

for 20 minutes at room temperature. Excess butanol was removed by centrifugation under vacuum and the primer dissolved in 150µl TE pH 8.0. Primer concentrations were calculated as above.

2.2.4.16. Polymerase chain reaction (PCR)

a) Generation of novel phosphatase probes

Approximately 10ng A431 cDNA (see section 2.2.4.4) was used as a template in conjunction with 50pmol of each degenerate oligonucleotide primer (see Table 3, p72) in a PCR reaction, essentially as described in Ashworth, (1993). A master mix comprising of 200µM of each dATP, dCTP, dTTP, and dGTP, 1X PCR buffer (15mM Tris-HCl pH 8.8, 60mM KCl, 2.25mM $MgCl_2$), 50pmol of each oligonucleotide and 2.5 units *Taq* polymerase (a themolabile DNA polymerase from *Thermus aquaticus*), was aliquoted into 0.5ml polypropylene microcentrifuge tubes to a volume of 49µl. The template (1µl) was added last to reduce the chances of cross contamination, thorough mixing is unnecessary, and the reactions were overlaid with 100µl of light mineral oil. DNA amplification was performed using a Techne PHC-1 thermal cycler for 30 cycles of denaturation (94°C, 20 seconds), annealing (52°C, 20 seconds), and extension (72°C, 45 seconds), with a final extension (72°C, 10 minutes). Samples were stored at 4°C. The reaction products (5µl of each sample), were resolved by agarose gel electrophoresis (see section 2.2.4.8) and fragments of the expected size, 260bp, were excised and purified using the Geneclean kit (eluted in 15µl as described in section 2.2.4.9). The PCR products were cloned into pBluescript® II KS (Stratagene) by blunt-end ligation (see section 2.2.4.10) and sequenced directly from bacterial colonies (see below).

b) Amplification of DNA from bacterial colonies

It is most convenient to sequence directly from bacterial colonies harbouring plasmids containing the subcloned PCR product. This is done by PCR on the bacterial colonies, whereby the PCR product can then be sequenced. PCR was carried out essentially as described in the previous section. Primers were complementary to

regions flanking the restriction site used for subcloning, namely Reverse and M13-20 (see Table 2, p72). Using a disposable microbiological loop, bacterial colonies were picked and the loop twirled in the PCR master mix for 2-3 seconds. The loop was then used to innoculate 5ml L-broth cultures such that a stock of the bacterial colony was made, as described in section 2.2.4.5. Mineral oil was then added to the PCR tubes and DNA amplification continued as described above in section a). PCR products were analysed by agarose gel electrophoresis, bands were excised, the DNA purified by the Geneclean procedure and sequenced directly by the method described below.

If further sequencing of the bacterial clone was required, PCR analysis was performed on the bacterial culture after overnight growth as follows. 100µl bacterial culture was centrifuged for 1 minute in a microfuge, and the supernatant discarded. The pellet was resuspended in 50µl H_2O and the tube boiled for 5 minutes, centrifuged for 1 minute, and 1µl of the supernatant used in the PCR reaction, as described above in section a).

2.2.4.17. Sequencing

a) Direct sequencing of PCR products

Sequencing was carried out according to the chain termination reaction of Sanger *et al.* (1977), essentially as described in Ashworth, (1993). The PCR product was purified using Geneclean and eluted in 15µl H_2O. In a flip-top eppendorf, 7µl (0.25-1.0µg) PCR product was mixed with 1µl (10pmol) of the appropriate sequencing primer, (see Tables 2 and 3, p72) and placed in a heating block at 95-100°C for 5 minutes. Typically the primers used were internal to the primers used in the PCR reaction. The tube was placed on dry ice for 1 minute to freeze, and then centrifuged briefly (2-3 seconds) in a microcentrifuge. The annealed DNA was labelled by adding 7.5µl of a previously prepared reaction mix composed of 2µl 5X sequencing buffer (5X is 200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl), 1µl DTT (100mM), 2µl labelling mix (7.5µM dCTP, dGTP, and dTTP diluted 1:4 in H_2O), 0.5µl [α-^{35}S]ATP, and 2µl (2 units) T7 DNA polymerase (Pharmacia; diluted 8 fold in 10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA). This was centrifuged briefly

and incubated at room temperature for 2 minutes. During this time, 3.5µl of this labelling mix was added to the lip of each of the four eppendorf tubes containing T, C, G, A termination mixes. The termination reaction was initiated by brief centrifugation, and the tubes then placed in a 37°C water bath and incubated for 5 minutes. The reactions were stopped by addition 4µl formamide/dye mix (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue), and the samples heated to 95°C for 2 minutes prior to loading onto a polyacrylamide gel as described below in section c).

b) Preparation and sequencing of double-stranded plasmid DNA

Minipreparation plasmid DNA was prepared as in section 2.2.4.5, and 12µl DNA used for sequencing for each primer. The DNA was denatured by adding 0.1 volumes of 2M NaOH, 2mM EDTA and incubating at 37°C for 30 minutes. The mix was neutralised by the addition 0.1 volume 3M sodium acetate pH 4.5-5.5, and the DNA precipitated using 2X volume ethanol at -70°C for 15 minutes. The DNA precipitate was microcentrifuged for 10 minutes, washed with 70% (v/v) ethanol, air-dried and redissolved in 7µl H_2O.

Sequencing was carried out essentially as described in the sequenase® kit (United State Biochemicals). 7µl denatured DNA was added to 2µl 5X sequencing buffer (as above) and 1µl (10pmol) appropriate primer (see Table 3). The primer was annealed by incubation at 65°C for 2 minutes followed by a slow cool to room temperature. The annealed DNA was labelled using the same method as described in section a).

c) Analysis of sequencing reactions

Sequencing reactions were analysed by denaturing gel electrophoresis. A 6% polyacrylamide gel (acrylamide to bisacrylamide 19:1) in TBE (1x TBE is 90mM Tris, 90mM boric acid, 2mM EDTA) containing 42% (w/v) urea was polymerised with 0.08% (v/v) APS and 0.8% (v/v) TEMED and immediately poured between gel plates separated by wedged (0.4-1.0mm) spacers. The gel was then placed in a vertical apparatus (BRL Life Technologies, model S2) with both ends submerged in 1X TBE

buffer. Samples were heated to 95°C for 2 minutes and 4µl loaded onto the gel using a sharks-tooth comb. The gel was run at 85W for approximately 3.5 hours. The gel was fixed in a solution of 10% (v/v) acetic acid for 45 minutes and transferred to 3MM Whatman paper, covered with Saranwrap™ and dried under vacuum at 80°C for 2 hours. The gel was exposed directly onto Kodak X-OMAT AR film at room temperature. Autoradiographs were read from the bottom (5' end of DNA) upwards.

2.2.4.18. cDNA library screening

A custom human brain, and rodent liver library constructed in the ZAP II vector was acquired from Stratagene, and plating and screening was carried out essentially as described in the Stratagene instruction manual, outlined briefly below.

a) Preparation of plating cultures

E.coli XL1-Blue carry the F' episome, which is required for both colour selection (not utilised in this case), and the *in vivo* excision process (see section 2.2.4.19). The Tn10 tetracycline gene is also located on the F' episome, therefore, in the presence tetracycline, the episome is selectively maintained.

XL1-blue cells were streaked onto an L-broth agar plate containing 12.5µg/ml tetracycline, and grown at 37°C until colonies appeared. A colony was used to innoculate 50ml L-broth supplemented with 0.2% (w/v) maltose and 10mM $MgSO_4$ and grown overnight with shaking at 30°C. This temperature ensures that cells will not overgrow since phage can adhere to dead cells. The culture was centrifuged at 2000rpm for 10 minutes, and the pelleted cells gently resuspended in 15ml of 10mM $MgSO_4$. The cells were diluted to OD_{600} = 0.5 with 10mM $MgSO_4$ and stored at 4°C for 3 days maximum. Approximately 1ml of OD_{600} = 0.5 was needed for each 245mm square plate and 200µl of OD_{600} = 0.5 cells for each 100mm plate.

b) Titering phage

The Lambda ZAP II phage was diluted in 4 serial, 10-fold dilutions in TM buffer (10mM $MgSO_4$, 50mM Tris-HCl pH 7.5). In a 15ml sterile tube, 200µl of XL1-blue

cells diluted to OD_{600} = 0.5 were mixed with 1µl of each Lambda phage dilution and incubated at 37°C for 15 minutes to allow the phage to attach to the cells. 3ml molten top agar (0.7% (w/v) agarose in L-broth cooled to 48°C) was added to the culture/phage mix and poured immediately onto warmed (42°C) 100mm L-broth plates. The plates were left to harden at room temperature and then incubated at 42°C overnight. The number of plaques per plate were counted and the titre calculated using the formula:

titer = (no. plaques x 1000 x dilution factor) pfu/ml.

c) Screening cDNA library

For both the human brain and rodent liver library, approximately 7.5 x 10^5 pfu/plate were plated on four large 245mm square L-broth plates with 1ml of OD600 = 0.5 XL1-Blue cells/plate and 30ml top agar/plate. These were incubated overnight at 42°C. The plates were refrigerated for 2 hours at 4°C prior to taking lifts, as this prevents the top agar from sticking to the nitrocellulose filter.

For each plate, Hybond N+ membrane (Amersham) was layered onto the plate and left for 1 minute. Five orientation marks were made with a needle through the membrane into the agar. After transfer, the filter was carefully lifted ensuring not to drag the membrane across the plate, and inverted over 3-4ml denaturation solution (1.5M NaCl, 0.5M NaOH) placed on a piece of Saranwrap™, and left for 2 minutes. After which the filter was transferred to 3-4ml neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH 8.0) for 5 minutes. The filter was rinsed in 50mM Na_2HPO_4, briefly blotted with 3MM Whatman paper, and UV-crosslinked using a UV Stratalinker 1800 (Stratagene). The agar plates were stored at 4°C.

The filters were prehybridised, hybridised and washed using the method of Church and Gilbert, (1984), as described in section 2.2.4.13. The probe was a mixed product of two PCR reactions, each using the degenerate oligonucleotides with 1µl culture supernatant from the two most frequently isolated novel phosphatase clones, which had been subcloned into pBluescript (see section 2.2.4.16b). The degenerate primers were used in the PCR instead of internal pBluescript primers,

because the library cDNA was also cloned into a pBluescript vector. Each PCR product was purified using Geneclean, and approximately 10ng DNA from each was mixed and radiolabelled as described in section 2.2.4.12. The filters were wrapped in Saranwrap™ and exposed overnight onto Kodak X-OMAT AR film with intensifying screens at -70°C.

The films were orientated against the filters using the needle marks, and positive clones determined using the strongest signals on the film. The end of an inverted pasteur pipet was used to "core" the putative clones from the stock agar plates, and the agar plug placed into an eppendorf tube with 500µl TM buffer and 20µl chloroform. The tube was briefly vortexed and incubated overnight at 4°C to elute the phage. The phage stock is stable for 1 year at 4°C.

For each positive clone, 100µl of a 200-fold dilution of eluted phage in TM buffer was added to 200µl XL1-Blue cells at OD_{600} = 0.5, incubated at 37°C for 15 minutes, mixed with 3.5ml molten top agar, and poured onto 100mm L-broth plates. Transfer to nitrocellulose filters and screening was carried out as previously described above. This procedure was repeated for a total of four platings, such that single, positive plaques were isolated.

d) In vivo excision of the pBluescript phagemid

In vivo excision of the cloned insert is dependent upon the simultaneous infection of XL1-blue cells with both the lambda vector (containing cloned insert) and the M13 helper phage (ExAssist™). The F' episome is required for pili formation, necessary for superinfection with the ExAssist helper phage. The M13 phage proteins recognise the sites of initiation and termination for DNA synthesis, which have been subcloned separately into the Lambda ZAP II vector. The newly synthesised phagemid is secreted from the *E.coli*, and rescued by transformation into SOLR cells. Subsequent bacterial colonies contain the pBluescript double-stranded phagemid with the cloned DNA insert. Helper phage will not grow, since they are unable to replicate in Su^- (non-suppressing) SOLR strains and do not contain ampicillin-resistance genes.

SOLR cells are also resistant to lambda phage infection, preventing lambda DNA contamination.

Single plaques, isolated as described above, were placed in 500µl TM buffer with 20µl chloroform and incubated overnight at 4°C. In a 50ml conical tube 200µl of OD_{600} = 1.0 XL1-Blue cells, prepared as in section 2.2.4.18a), were combined with 100µl eluted phage stock and 1µl ExAssist helper phage, and incubated at 37°C for 15 minutes. After which, 3ml of terrific broth (for 1 litre: 12g bacto tryptone, 24g bacto yeast extract, 4ml glycerol. After autoclaving add 100ml solution B {0.17M KH_2PO_4, 0.72M K_2HPO_4}) was added and incubated at 37°C with shaking for 2.5 hours. The tube was heated at 70°C for 15 minutes, and centrifuged for 5 minutes at 4000g. The supernatant containing the pBluescript phagemid, packaged as phage particles, was stored at 4°C.

To rescue the phagemid, 1µl supernatant from above was added to 200µl OD_{600} = 1.0 SOLR host cells, and incubated at 37°C for 15 minutes. The SOLR cells were prepared as described for XL1-Blue cells, see section 2.2.4.18a, with the following changes: SOLR cells were streaked on L-broth agar plates containing 50µg/ml kanamycin. Overnight cultures were grown in L-broth with no supplements. Following incubation, 10µl and 100µl of the phage/SOLR mix was spread onto L-broth plates containing 50µg/ml ampicillin, and incubated overnight at 42°C. Minipreparations of plasmid DNA were prepared as described in section 2.2.4.5.

CHAPTER 3

RESULTS

Isolation of Novel Protein Phosphatases

3. Isolation of novel protein phosphatases

3.1. Degenerate PCR and analysis of resulting clones

The human gene CL100, its murine homolog 3CH134, and PAC-1 show significant sequence identity to the vaccinia virus protein VH1, which has been demonstrated to dephosphorylate both phospho-serine and -tyrosine residues *in vitro*, as discussed in section 1.7.6. In order to isolate related cDNAs to this potentially novel, dual specificity phosphatase subfamily, degenerate PCR primers were designed based on the amino acid sequences conserved between CL100, 3CH134 and PAC-1, see table 3, p72 (Keyse and Emslie, 1992; Rohan *et al.*, 1993). The 3' primer spans the PTPase catalytic motif, a region highly conserved in all protein tyrosine phosphatases, inclusive of the VH1-like enzymes, whilst the 5' primer corresponds to a region only identified in the CL100 and PAC-1 genes. These primers were used to amplify related sequences from cDNA made from poly (A)+ RNA isolated from the human squamous cell line, A431. PCR fragments of the predicted size, 270 base pairs, were purified by gel electrophoresis and subcloned by blunt-end ligation into the EcoRV site of pBluescript KS II , as described in the Methods.

The cloned inserts were amplified by PCR using the universal Reverse and M13-20 primers, and subsequently sequenced, in both 5' and 3' directions, using the more internal, universal primers for pBluescript, KS and SK. Fifty individual bacterial clones were analysed in this way. Sequence analysis identified six of these to be identical to the CL100 gene, and have been designated PCR group A. A further twelve clones were found to be homologous to, but distinguishable from CL100, and within this twelve were divided into groups B-E. The predicted amino acid sequences of these five groups of PCR clones are illustrated in figure 3.1, p103. The nucleotide sequences are presented in the appendix, figure A. Of the residual clones that were analysed, eight showed sequence identity to the Na/K ATPase beta subunit, whilst the remainder proved to be PCR artifacts, or PCR products that were not sequenced, due to their being much smaller, or larger than the predicted 270 base pair product.

Figure 3.1.

Alignment of the predicted amino acid sequence of the five groups of clones isolated by degenerative PCR.

The residues underlined and in bold represent the degenerate PCR primers, amino acid sequences that are completely conserved are overlined, and "..." indicates sequence data not obtained. * denotes a stop codon.

```
                                                  ¯
Group A (CL100): Y G Q G G P V E I L P F L Y L G S A Y H A S
Group B        :               E I L P F L Y L G S A Y H A S
Group C        :               E I L P F L Y H A S A Y H A A
Group D        :               E P I R F L Y L L K L Y S P R
Group E        :               E I L H S L F L G S C S H G S

Grp A: R K D M L D A L G I T A L I N V S A N C P N H F E G
Grp B: K C E F L A N L H I T A L L N V S R R T S E A C M T
Grp C: R R D M L D A L G I T A L L N V S S D C P N H F E G
Grp D: K V L Y R S S A R L L G L K T P R L D Q T R Q L L K
Grp E: D L R G C R P V R S Q P V L N V S A S C P N H F . .

                ¯¯¯¯    ¯¯¯¯¯¯¯¯    ¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯¯    ¯¯¯¯
Grp A: H Y Q Y K S I P V E D N H K A D I S S W F N E A I D
Grp B: H L H Y K W I P V E D S H T A D I S S H F Q E A I D
Grp C: H Y Q Y K C I P V E D N H K A D I S S W F M E A I E
Grp D: D * . . . . . .
Grp E: . . . . . .

                        ¯       ¯
Grp A: S I K N A G G R V F V H C Q A G
Grp B: C V R E K G G K V L V
Grp C: C V K D C R G R V L V
Grp D: . . . . . .
Grp E: . . . . . .
```

Clones from groups D and E were only isolated once, and sequence data for these two clones could not be obtained using the 3' primer. However, these clones do show limited sequence homology to the CL100 gene, suggesting that they may be partial sequences of a miss-cloned cDNA, or perhaps more distantly related phosphatase genes, rather than PCR artifacts. In view of this, a mixed probe from groups B and C only, was used to screen the cDNA libraries.

3.2. Library screening and sequence analysis of partial cDNA clones

From the five groups of clones isolated by degenerative PCR, probes from groups B and C were chosen to screen the cDNA libraries, since these were the most complete and frequently isolated novel sequences, with six individual clones making up group B, and four clones in group C. Thus, a mixed hybridisation probe was used to screen 30 x 10^5 plaques from both a human brain, and a rodent liver cDNA library, plated at approximately 7.5 x 10^5 pfu/plate as described in the Methods. Low stringency screening of the libraries was performed for four separate platings of positive plaques, such that single plaques could then be identified. Six final hybridising clones were isolated in this way. These were analysed in more detail by using restriction mapping and partial DNA sequencing, using both pBluescript primers and the degenerate primers. The four clones from the liver cDNA library ranged between the sizes of 1 kb to 2.4 kb, and sequence data revealed that two of these were identical, overlapping clones. The clones isolated from the brain cDNA library yielded much larger cDNA products. All of the clones were found to encompass the PTPase catalytic motif, which typifies Tyr, and Ser/Thr phosphatase activity. The predicted amino acid sequences of these partial clones are shown in figure 3.2, p105, and are compared to CL100. The sequences designated TYP 1 and 2 refer to the clones isolated from the brain cDNA library, whilst TYP 3-5 represent the liver cDNA clones. The nucleotide sequences are presented in the appendix, figure B. (TYP represents the amino acid single letter code for threonine, tyrosine phosphatase).

Figure 3.2.

Sequence alignment of CL100 and the predicted amino acid sequences corresponding to the TYP partial cDNA's.

The residues underlined and in bold represent the regions corresponding to the degenerate oligonucleotides, the second region corresponding to the PTPase catalytic motif. Amino acid sequences that are completely conserved between CL100 and the various TYP cDNA's are overlined, and "...." indicates sequence data not obtained.

```
CL100:  Y G Q G G P V E I L P F L Y L G A S Y H A S R K D M
TYP1:   H D Q E G P V E I L P F L Y L G S A Y H A A R R D M
TYP2:   . . . . . V E I L S S L Y L G G A Y H A S K C K F
TYP3:   . . . . . V E I L P Y L F L G S C S H S S D L Q G
TYP4:   . . . . . .
TYP5:   . . . . . V E I L H S L Y L G S A Y H A S K C E F

CL100:  L D A L G I T A L I N V S A N C P N H F E G H Y Q Y
TYP1:   L D A L G I T A L L N V S S D C P N H F E G H Y Q Y
TYP2:   L D K L Y I T A Q L N V P . . . . . .
TYP3:   L Q A C G I T A V L N V S A S C P N H F E G L F R Y
TYP4:   . . . . . .
TYP5:   L A N L H I T A L L N V S Q R T S E A C M T H L H Y

CL100:  K S I P V E D N H K A D I S S W F N E A I D F I D S
TYP1:   K C I P V E D N H K A D I S S W F M E A I E Y I D A
TYP2:   . . . L V E E G H M A D I S S H F Q E A I D F I D C
TYP3:   K S I P V E D N Q M V E I S A W F Q E A I G F I D W
TYP4:   . . . . . A D I S S H F Q E A I D F I D C
TYP5:   K W I P V E D S H T A D I S S H F Q E A I D F I D C

CL100:  S K N A G G R V F V H C Q A G I S R S A T I C L A Y
TYP1:   V K D C R G R V L V H C Q A G I S R E A T I C L A Y
TYP2:   V R E K K G K V L V H C E A G F S C S P T . . . . .
TYP3:   V K N S G G R V L V H C . . . . . .
TYP4:   V R E G G G K V L V H C E A G V S R S P T I C M A Y
TYP5:   V R E K G G K V L V H C . . . . . .

CL100:  I M R T N R V K L D E A F E F V K Q R R S I I S P N
TYP1:   L M M K K R V R L E E A F E F V K Q R R S I I S P N
TYP4:   L M K T K Q F R L K E A F D I V K Q R R S V I S P N

CL100:  F S F M ---
TYP1:   F S F M ---
TYP4:   F G F M .
```

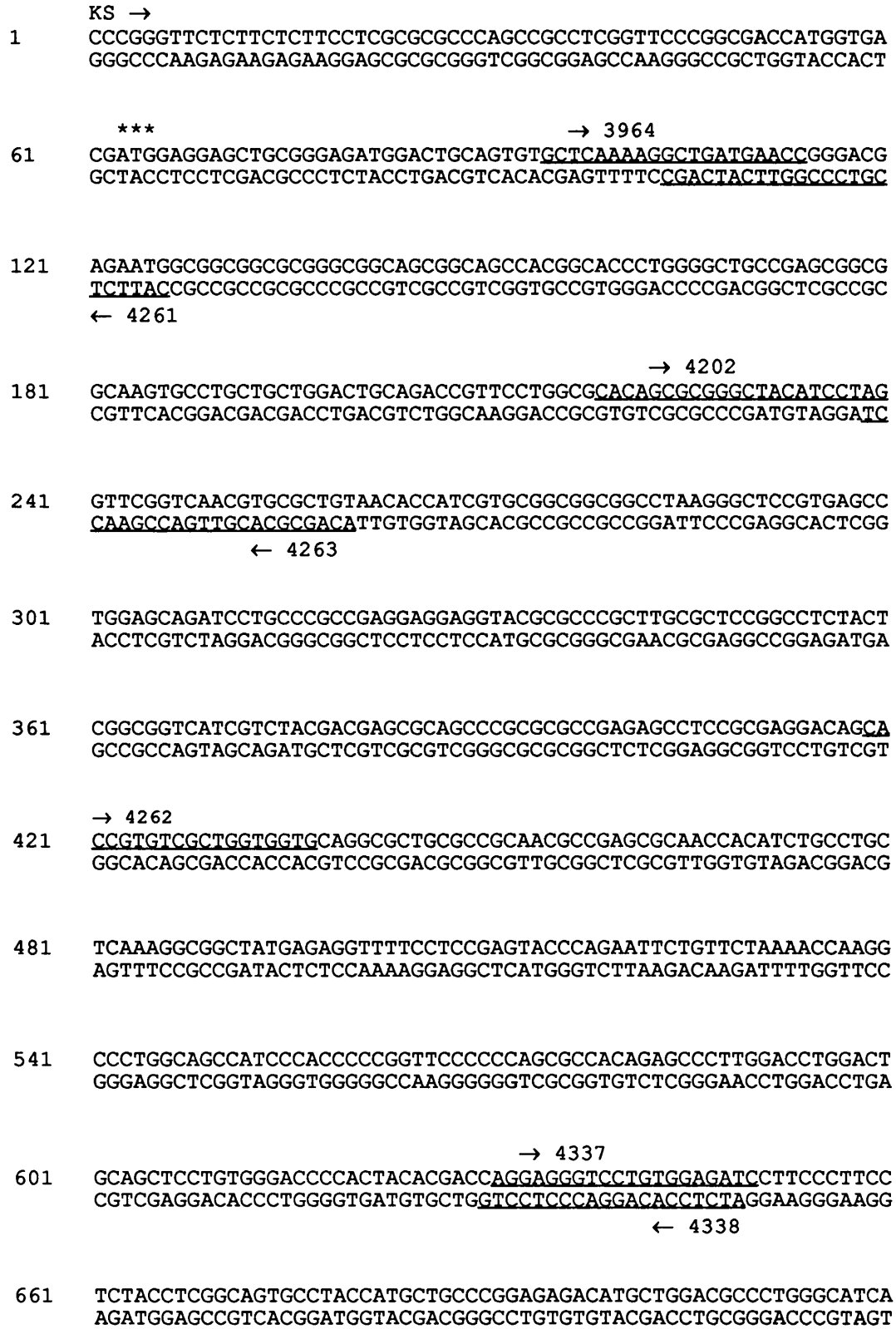
Since the initiation of this work, many research groups have also identified further CL100-like protein phosphatases, as previously discussed in the introduction, section 1.7.6.1. One of these genes VH3, was isolated by Kwak and Dixon (1995) through the screening of a human placental library. VH3 shows the highest level of expression in both the liver and the placenta, and encodes a cDNA of 2.1 kb. Interestingly, TYP 5, a 2.1 kb clone isolated from the liver demonstrates sequence identity to VH3, albeit one amino acid change from a phenylalanine (TTt/c) in VH3, to a serine (TCC) in TYP 5. This C for T substitution may be due to a PCR error, or simply that TYP 5 was isolated from a rat liver cDNA library, whilst VH3 is a human placental clone. VH3 also shows high sequence similarity to a further gene, B23, isolated from a mammalian epithelial cDNA library by Ishibashi *et al.* (1994). Both B23 and VH3 exhibit a greater degree of homology to each other, than either gene shows with CL100. TYP 5 is also one of the initial clones, group B, identified by PCR using the degenerative primers, whilst TYP 1 shows sequence identity with PCR group C.

3.3. Complete sequence analysis of TYP 1

To attempt to gain complete sequence for all the cDNAs above would have been very time consuming, particularly considering that some of the cDNAs indicated to be only partial clones at 1.0 kb, hence necessitating further screening in order to obtain full length clones. The sequencing of these clones was also technically difficult, as it became clear that they were extremely GC rich in places, causing a great number of compressions between these residues, making it difficult to decipher the true sequence. In this regard, efforts were concentrated on sequencing TYP 1 (2.7 kb), and TYP 2 (3.0 kb), the two largest clones isolated from the human brain cDNA library. Disappointingly, although much sequence data was obtained for TYP 2, it was problematic in interpreting an open reading frame running through the entire sequence. The complete nucleotide sequence of TYP 1 is shown in figure 3.3, p107. The positions of the oligonucleotides used in the sequence analysis are also illustrated in figure 3.3, and shown in table 3, p72.

Figure 3.3.

Positioning of the oligonucleotides used for sequencing TYP 1.



```
721   CGGCTCTGTTGAATGTCTCCTCGGACTGCCCAAACCACTTTGAAGGACACTATCAGTACA
      GCCGAGACAACTTACAGAGGAGCCTGACGGGTTTGGTGAAACTTCCTGTGATAGTCATGT

781   AGTGCATCCCAGTGGAAGATAACCACAAGGCCGACATCAGCTCCTGGTTCATGGAAGCCA
      TCACGTAGGGTCACCTTCTATTGGTGTTCCGGCTGTAGTCGAGGACCAAGTACCTTCGGT

                                                        >
841   TAGAGTACATCGATGCCGTGAAGGACTGCCGTGGGCGCGTGCTGGTGCACTGCCAGGCGG
      ATCTCATGTAGCTACGGCACTTCCTGACGGCACCCGCGCACGACCACGTGACGGTCCGCC
           ← 4339
      <
901   GCATCTCGCGGTCGGCCACCATCTGCCTGGCCTACCTGATGATGAAGAAACGGGTGAGGC
      CGTAGAGCGCCAGCCGGTGGTAGACGGACCGGATGGACTACTACTTCTTTGCCCACTCCG

                  → 4340
961   TGGAGGAGGCCTTCGAGTTCGTTAAGCAGCGCCGCAGCATCATCTCGCCCAACTTCAGCT
      ACCTCCTCCGGAAGCTCAAGCAATTCGTCGCGGCGTCGTAGTAGAGCGGGTTGAAGTCGA

1021  TCATGGGGCAGCTGCTGCAGTTCGAGTCCCAGGTGCTGGCCACGTCCTGTGCTGCGGAGG
      AGTACCCCGTCGACGACGTCAAGCTCAGGGTCCACGACCGGTGCAGGACACGACGCCTCC
                           ← 4345

                                    → 4342
1081  CTGCTAGCCCCTCGGGACCCCTGCGGGAGCGGGGCAAGACCCCCGCCACCCCCACCTCGC
      GACGATCGGGGAGCCCTGGGGACGCCCTCGCCCCGTTCTGGGGGCGGTGGGGGTGGAGCG

1141  AGTTCGTCTTCAGCTTTCCGGTCTCCGTGGGCGTGCACTCGGCCCCCAGCAGCCTGCCCT
      TCAAGCAGAAGTCGAAAGGCCAGAGGCACCCGCACGTGAGCCGGGGGTCGTCGGACGGGA

1201  ACCTGCACAGCCCCATCACCACCTCTCCCAGCTGTTAG*
      TGGACGTGTCGGGGTAGTGGTGGAGAGGGTCGACAATC* .... ← SK
```

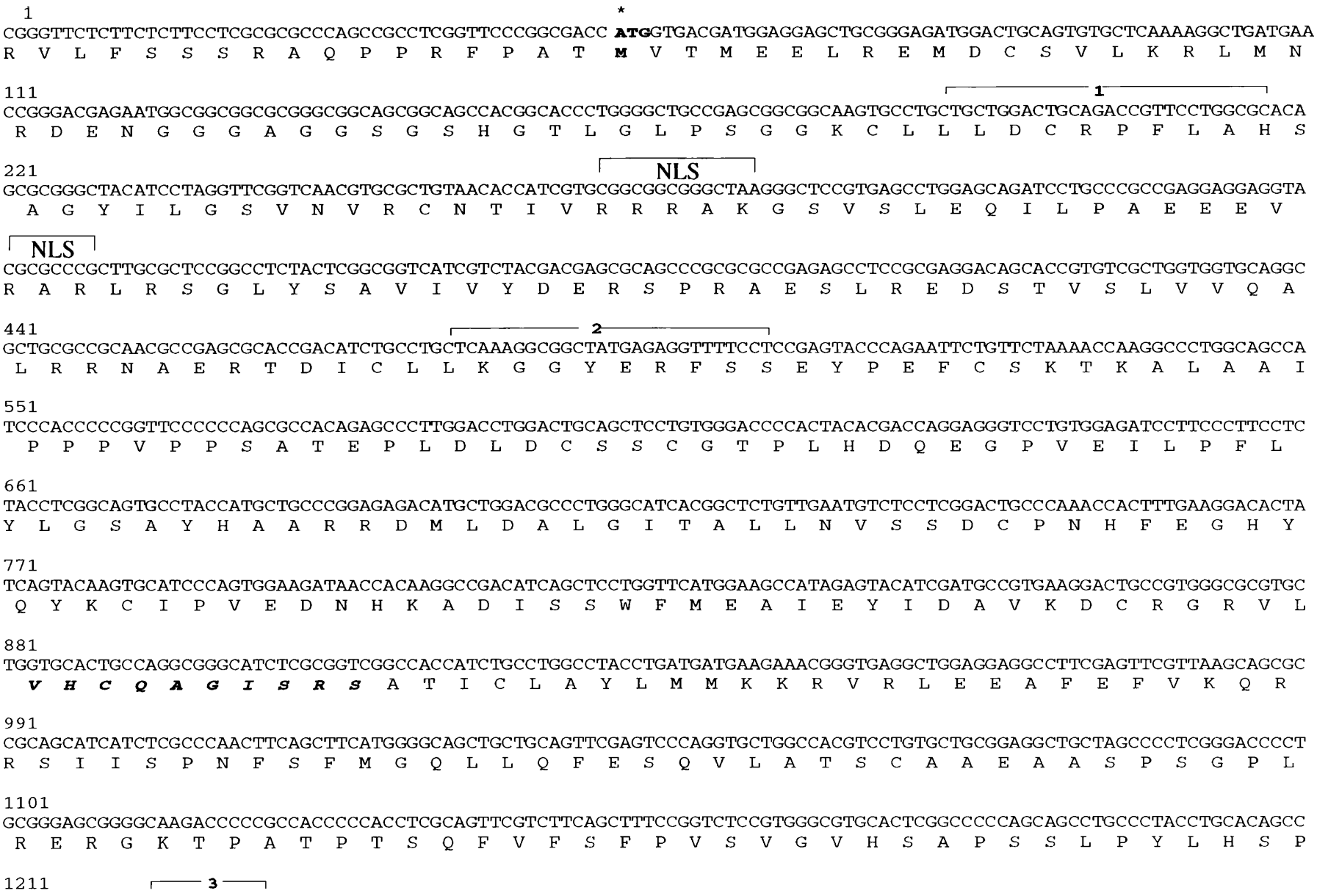
*** denotes first methionine
→ indicates forward primer
← indicates reverse primer
>__< PTPase catalytic motif
* denotes termination codon

Initially the technique of "shot-gun" cloning was used with frequent cutting restriction enzymes, such as Asp 718. Sequence data was pieced together using the Contig algorithm, GCG version 7.0. Oligonucleotides were then designed based on the sequence obtained, such that TYP 1 was sequenced in both 5' and 3' directions across the whole length of the cDNA. Nucleotide sequence analysis, see figure 3.4, p110, revealed an ATG at position 52, located within a consensus translation initiation site (Kozak, 1987), and therefore this is predicted to be the first methionine. The coding region (1182 bp) is flanked by a GC rich 5' untranslated region and a 3' untranslated region containing a polyadenylation signal, not shown. The TYP 1 cDNA encodes a predicted polypeptide of 394 amino acids with an estimated molecular mass of approximately 43 kDa. Consistent with this, the translation initiation site was verified by performing *in vitro* transcription and translation, and a single band of 43 kDa was observed, see section 5.3. The amino acid sequence is also illustrated in figure 3.4. This sequence does not contain the model seven-amino acid nuclear targeting sequence, as exemplified by the SV40 large T antigen. However, there are presently several examples of a newly emerging bipartite nuclear targeting sequence, comprising two basic amino acid elements, close together but not actually contiguous, in the primary sequence (Dingwall and Laskey, 1991). It would seem that there is no consensus sequence for the intervening spacer region. Although the bipartite sequence is defined as two basic amino acids, followed by a second cluster in which three out of the next five are basic, it is possible that the two regions marked "NLS" in figure 3.4, may impart nuclear localisation properties on the TYP 1 protein, which has been demonstrated to be nuclear, discussed later in section 5.6. Interestingly, these sequences, or variations of, are also present in the CL100-like phosphatase family, and indeed CL100, PAC-1, HVH2 and HVH3 have been demonstrated to localise to the nucleus, see section 1.7.6.1. The TYP 1 protein does not contain characteristic transmembrane sequences. However, there are MAP kinase phosphorylation sites at the extreme carboxy-terminus of the protein (Figure 3.4, region 3), which may be involved in the regulation of TYP 1.

Figure 3.4.

Nucleotide- and predicted amino acid sequence of TYP 1 cDNA.

Nucleotides are numbered on the left beginning at the first nucleotide of the cDNA. Amino acids are indicated in single letter code with the first predicted methionine in bold, marked by an asterisk. This is located within a consensus translation initiation site CCA/GCCATGG (Kozak, 1987). The termination codon is also denoted by an asterisk. The PTPase domain is indicated in bold italics, and the bracketed regions 1 and 2 show homology to the CH2 domains in Cdc25. The bracketed region 3 indicates potential MAP kinase phosphorylation sites, and NLS marks the two putative nuclear localisation signals. See text for details.

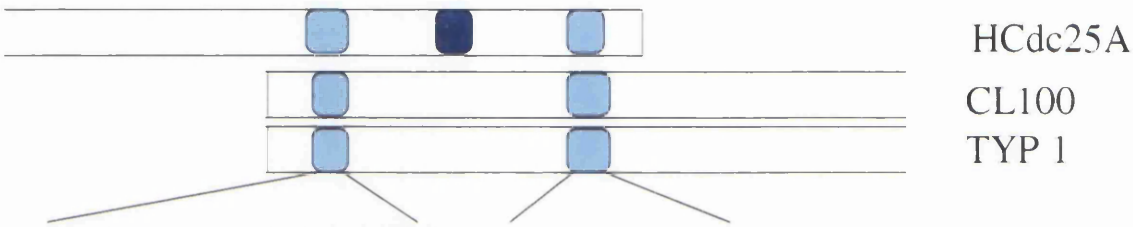

1
*
CGGGTTCTCTTCTCTTCCTCGCGCGCCCAGCCGCCTCGGTTCCCGGCGACC ATGGTGACGATGGAGGAGCTGCGGGAGATGGACTGCAGTGTGCTCAAAAGGCTGATGAA
R V L F S S S R A Q P P R F P A T M V T M E E L R E M D C S V L K R L M N
111
1
CCGGGACGAGAATGGCGGCGGCGCGGGCGGCAGCGGCAGCCACGGCACCCTGGGGCTGCCGAGCGGCGGCAAGTGCCTGCTGCTGGACTGCAGACCGTTCCTGGCGCACA
R D E N G G G A G G S G S H G T L G L P S G G K C L L L D C R P F L A H S
221
NLS
GCGCGGGCTACATCCTAGGTTCGGTCAACGTGCGCTGTAACACCATCGTGCGGCGGCGGGCTAAGGGCTCCGTGAGCCTGGAGCAGATCCTGCCCGCCGAGGAGGAGGTA
A G Y I L G S V N V R C N T I V R R R A K G S V S L E Q I L P A E E E V
NLS
CGCGCCCGCTTGCGCTCCGGCCTCTACTCGGCGGTCATCGTCTACGACGAGCGCAGCCCGCGCGCCGAGAGCCTCCGCGAGGACAGCACCGTGTCGCTGGTGGTGCAGGC
R A R L R S G L Y S A V I V Y D E R S P R A E S L R E D S T V S L V V Q A
441
2
GCTGCGCCGCAACGCCGAGCGCACCGACATCTGCCTGCTCAAAGGCGGCTATGAGAGGTTTTCCTCCGAGTACCCAGAATTCTGTTCTAAAACCAAGGCCCTGGCAGCCA
L R R N A E R T D I C L L K G G Y E R F S S E Y P E F C S K T K A L A A I
551
TCCCACCCCCGGTTCCCCCCAGCGCCACAGAGCCCTTGGACCTGGACTGCAGCTCCTGTGGGACCCCACTACACGACCAGGAGGGTCCTGTGGAGATCCTTCCCTTCCTC
P P P V P P S A T E P L D L D C S S C G T P L H D Q E G P V E I L P F L
661
TACCTCGGCAGTGCCTACCATGCTGCCCGGAGAGACATGCTGGACGCCCTGGGCATCACGGCTCTGTTGAATGTCTCCTCGGACTGCCCAAACCACTTTGAAGGACACTA
Y L G S A Y H A A R R D M L D A L G I T A L L N V S S D C P N H F E G H Y
771
TCAGTACAAGTGCATCCCAGTGGAAGATAACCACAAGGCCGACATCAGCTCCTGGTTCATGGAAGCCATAGAGTACATCGATGCCGTGAAGGACTGCCGTGGGCGCGTGC
Q Y K C I P V E D N H K A D I S S W F M E A I E Y I D A V K D C R G R V L
881
TGGTGCACTGCCAGGCGGGCATCTCGCGGTCGGCCACCATCTGCCTGGCCTACCTGATGATGAAGAAACGGGTGAGGCTGGAGGAGGCCTTCGAGTTCGTTAAGCAGCGC
V H C Q A G I S R S A T I C L A Y L M M K K R V R L E E A F E F V K Q R
991
CGCAGCATCATCTCGCCCAACTTCAGCTTCATGGGGCAGCTGCTGCAGTTCGAGTCCCAGGTGCTGGCCACGTCCTGTGCTGCGGAGGCTGCTAGCCCCTCGGGACCCCT
R S I I S P N F S F M G Q L L Q F E S Q V L A T S C A A E A A S P S G P L
1101
GCGGGAGCGGGGCAAGACCCCCGCCACCCCCACCTCGCAGTTCGTCTTCAGCTTTCCGGTCTCCGTGGGCGTGCACTCGGCCCCCAGCAGCCTGCCCTACCTGCACAGCC
R E R G K T P A T P T S Q F V F S F P V S V G V H S A P S S L P Y L H S P
1211
3
CCATCACCACCTCTCCCAGCTGTTAG
I T T S P S C *

The deduced TYP 1 amino acid sequence was used to search the Genbank/EMBL data base using the FASTA algorithm of the GCG programme, and throughout its entire length exhibits the highest degree of similarity to CL100, 62.8% identity, and its murine counterpart 3CH134, 62.5% identity, (Keyse and Emslie, 1992; Charles *et al.*, 1992). The TYP 1 sequence displays 50.8% identity with human PAC-1, whilst demonstrating 27.5% identity with VH1. Across the putative catalytic region, which includes the signature motif (I/V)HCXAGXGR(S/T)G, TYP 1 shares significant homology with VH1 and other VH1-like family members. In addition, two regions conserved between CL100 and Cdc25, a phosphatase whose activity is necessary for cell cycle regulation, are also present in TYP 1, and other CL100-like phosphatases. The functional significance of these regions is as yet unknown, but they may be involved in substrate recognition (Keyse and Ginsburg, 1993), or localising proteins to nuclear or cytoskeletal locations (Kwak and Dixon, 1994). These regions of homology are illustrated in figure 3.5, p112.

The pharmaceutical use of the nucleotide and amino acid sequences of the PCR groups, the partial TYP cDNA clones, and the full length sequence of TYP 1 as MAP kinase phosphatases are protected by an international patent application:
no. PCT/GB94/00694

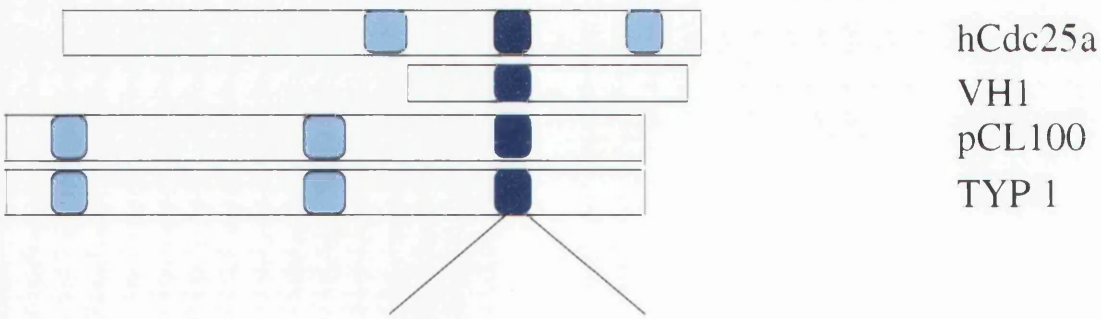
Figure 3.5.

A. Alignment of the CH2 domains of CL100, TYP 1 and human Cdc25A.




```
FVIIDCRYPYEYEGGHIKGAVNLHM...PELYVLKGGYKEFF   hCdc25A
CLLLDCRSFFAFNAGHIAGSVNVRF...AQVFFLKGGYEAFS   CL100
CLLLDCRPFLAHSAGYILGSVNVRC...TDICLLKGGYERFS   TYP 1
```

B. Alignment of the PTPase domains of human Cdc25A and the CL100-like phosphatases.




```
VVFHCEFSSERGPRMC   hCdc25A
VLVHCAAGVNRSGAMI   VH1
VFVHCQAGISRSATIC   CL100
VLVHCQAGISRSATIC   TYP 1
VLVHCQAGISRSATIC   PAC-1
VLVHCEAGISRSPTIC   VH3
```

In each figure, the catalytic phosphatase domain is shown in dark blue, and the CH2 domains in light blue. Identical residue motiffs are shown in bold.

CHAPTER 4

RESULTS

Regulation of TYP 1 and TYP 2 expression

4. Regulation of TYP 1 and TYP 2 expression

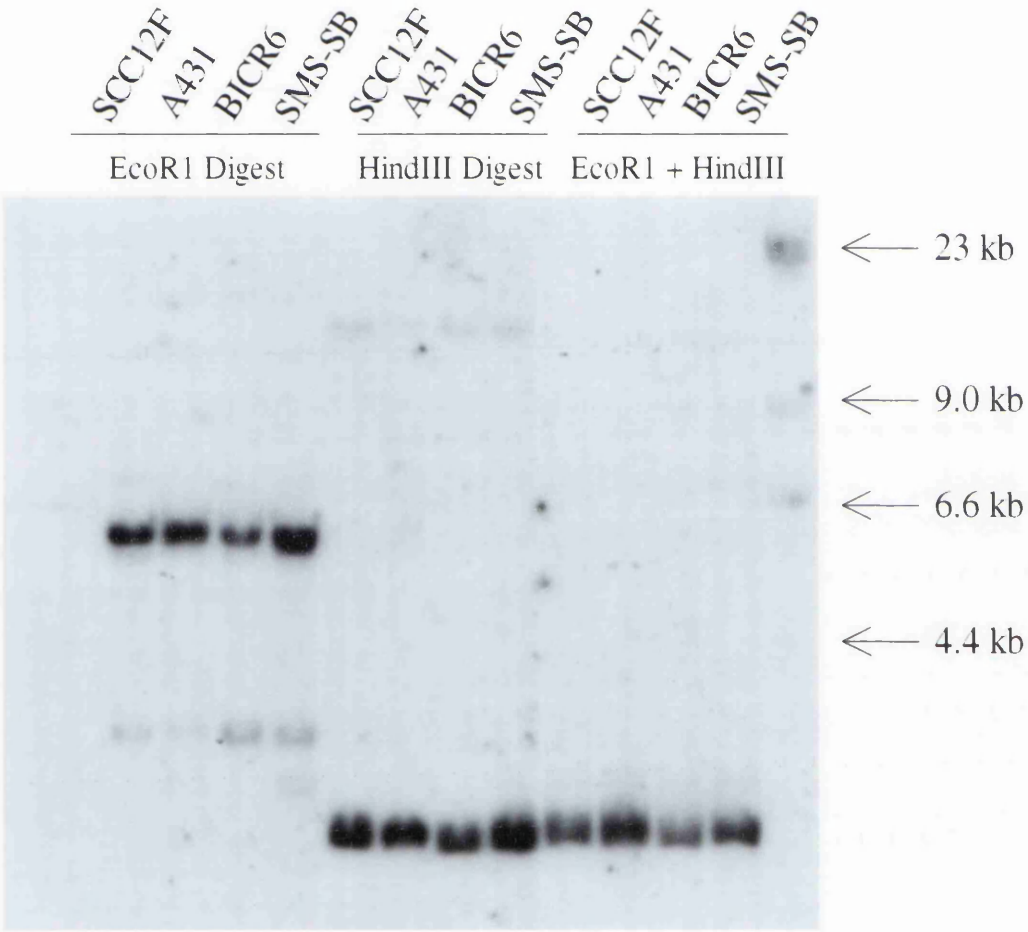
4.1. Genomic DNA analysis

The activity of protein kinases is significantly associated with an increase in cellular proliferation, which can frequently lead to neoplastic transformation under certain circumstances. Conversely, in this regard the protein phosphatases, which antagonise the kinases, have been viewed as potential tumour suppressor genes. Hence, the expression levels of TYP 1 and 2 were investigated across a panel of various squamous carcinoma cell lines, to establish if these genes are lost, or perhaps amplified in such tumours.

Southern analysis was performed on restriction enzyme-treated genomic DNA samples from various cancer cell lines, with the aim to investigate the gene dosage and amplification levels of TYP 1 and 2. The panel of cell lines include the squamous cell carcinomas A431, SCC12F, and BICR6, and a cell line isolated from a young girl with acute lymphoblastic leukemia, SMS-SB cells (genomic DNA was kindly supplied by Joe Winnie). SMS-SB cells were chosen as the control cell line, since both TYP 1 and TYP 2 are not expressed in these cells, as determined by northern analysis (data not shown), indicating that there is, presumably, no need for any genetic loss or amplification of the TYP phosphatase genes. As demonstrated in figure 4.1, p115 and figure 4.2, p116, there appears to be no significant loss, amplification or rearrangement of the TYP 1 and TYP 2 genes in the squamous cell lines studied, as judged by the expression levels in the SMS-SB cells.

Figure 4.1.

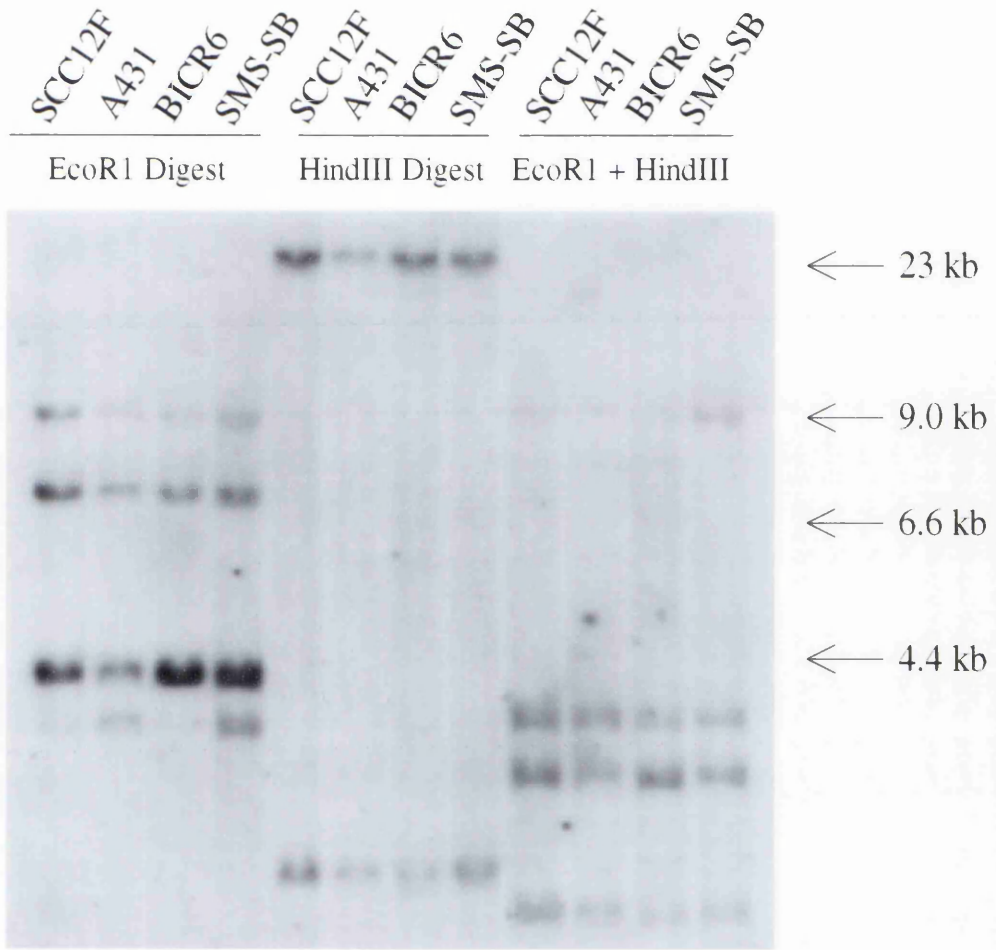
Detection of TYP 1 DNA sequences in various cell lines



Southern Analysis: 30µg samples of genomic DNA, isolated from various cell lines (as labelled), were digested overnight with the indicated restriction enzymes. Electrophoresis was carried out overnight through a 0.8% agarose gel, DNA transferred onto Hybond N+, and the membrane hybridised using a probe specific for TYP 1 (as detailed in Methods). Arrows indicate the Hind-III digested λ DNA markers.

Figure 4.2.

Detection of TYP 2 DNA sequences in various cell lines



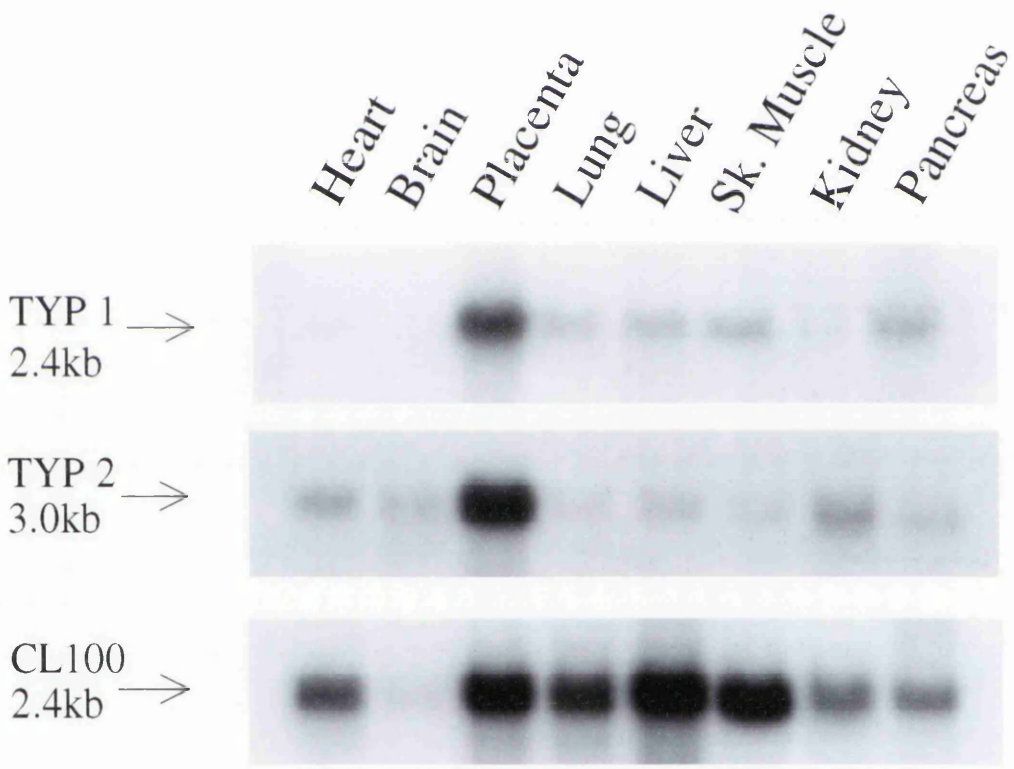
Southern Analysis: The membrane that was used in figure 4.1 for TYP 1 DNA detection was stripped from radioactivity, and re-hybridised with a TYP 2 DNA probe, as described in Methods. The arrows indicate the Hind-III digested γ DNA markers.

4.2. Tissue expression of TYP 1 and TYP 2

Initially, the expression of TYP 1 and 2 was investigated by northern analysis across a panel of multiple human tissues, and compared to the expression of CL100. Whilst TYP 1 and 2 are expressed at low, but detectable levels in all the tissues tested, their expression is most abundant in the placenta. TYP 1 additionally shows a slightly higher expression level in pancreatic tissue, whilst TYP 2 demonstrates a small increase in expression in the heart and kidney, as illustrated in figure 4.3, p118. The expression of CL100 is also ubiquitous across the tissues tested, but would appear to be less specific than TYP 1 and 2, with high mRNA levels seen in placenta, lung, liver and skeletal muscle. This data provides evidence that TYP 1 and 2, and CL100 may be regulated in a tissue-specific manner, and this is supported by the findings that other family members also show a differential pattern of tissue expression. For example, PAC-1 is strictly expressed in cells of hematopoetic origin (Rohan *et al.*, 1993), VH3 (Kwak and Dixon, 1995), and B23 (Ishibashi *et al.*, 1994) demonstrate a high expression in liver cell lines, whilst MKP-2 shows an increased expression in the central nervous system (Misra-Press *et al.*, 1995). Two mRNA species (2.4 and 7.0 kb) were detected by the TYP 1 probe; the 2.4 kb mRNA is consistent with the cloned cDNA, the larger 7.0 kb mRNA (not shown) may represent an alternative splice variant of TYP 1, or a closely related transcript. However, at present there is no evidence to distinguish between these two possibilities.

Figure 4.3.

Northern blot analysis of TYP 1, TYP 2, and CL100 mRNA distribution against poly (A) mRNA from human multiple tissues , (Clontech MTN blot).



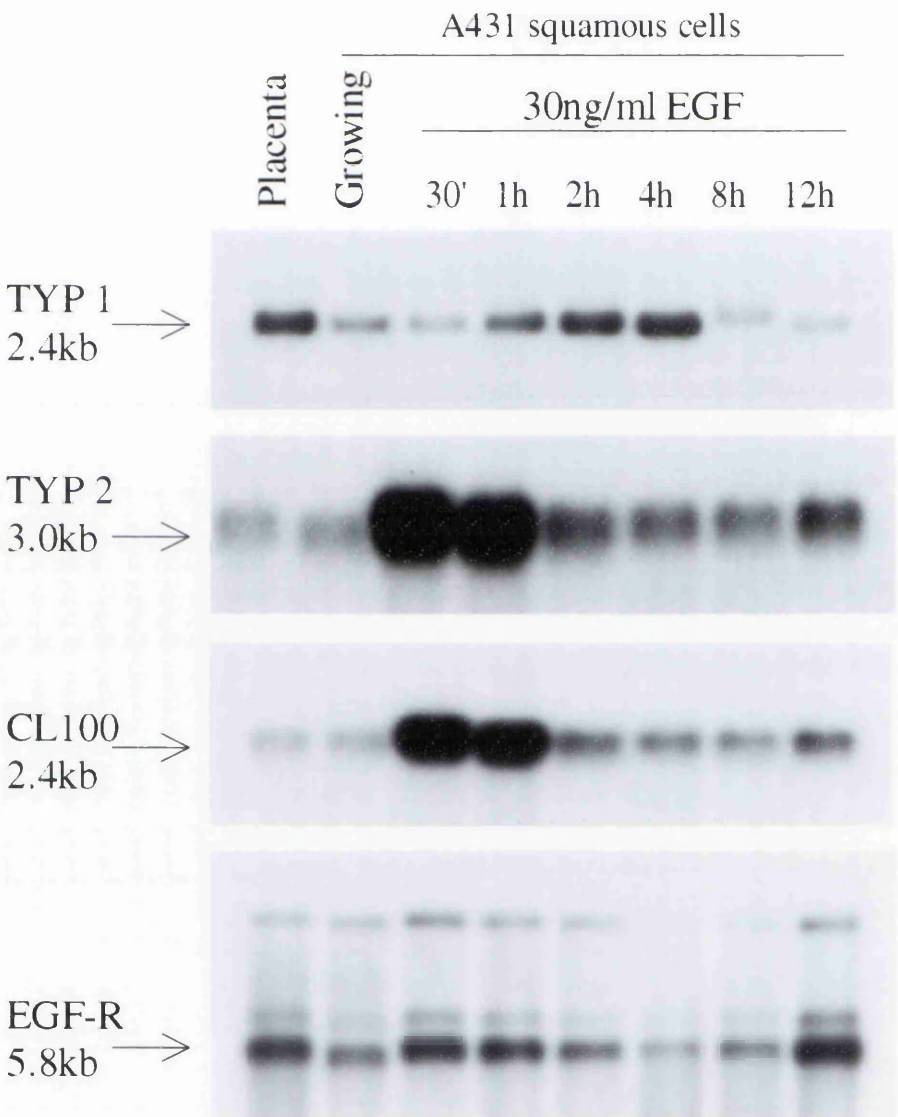
Each lane contains approximately 2 µg of highly pure poly (A) RNA isolated from human tissues as indicated. The membrane was initially hybridised with the TYP 1 probe, and then with TYP 2 and CL100 respectively, being stripped from radioactivity between each hybridisation, as detailed in the Methods.

4.3. Induction of TYP phosphatase mRNA by mitogens

One striking feature of the CL100-like dual specific phosphatases is that they are rapidly induced upon mitogenic stimulation. Consequently, the expression of TYP 1 and TYP 2 was investigated in two squamous carcinoma cell lines after stimulation with either the growth factor EGF, or serum, and compared to the mitogenic induction of CL100. As shown in figure 4.4, p120, TYP 1 expression in growing A431 cells is low, but shows an increased stimulation by 1 hour after EGF treatment, with a peak at 4 hours representing a 12-fold induction over untreated cells (as determined by laser densitometry using PDI image software analysis). Thereafter, the mRNA transcript declines, returning to base line by 8 hours. This is in contrast to CL100 and TYP 2, whose expression levels are rapidly induced, at least 50-fold over control levels by 30 minutes following EGF stimulation, with a slow return to base line by 12 hours. Placenta poly (A)+ RNA is included as an internal control for the detection of the TYP phosphatases, since the expression of both TYP 1 and 2 is particularly high in this tissue, as determined by northern analysis using the MTN tissue blot, figure 4.3. The EGF receptor is used as a loading control, and is encoded by a 5.8 kb mRNA. A transcriptional variant, possibly carrying a longer 3' untranslated region, is also detected at 10.5 kb. A431 cells also synthesise a 2.8 kb mRNA, a secreted polypeptide representing almost the entire extracellular EGF-binding domain of the receptor. However, this transcript is not recognised by the EGF receptor probe, which consists of a restriction fragment of the 3' end of the gene, inclusive of the kinase domain.

Figure 4.4.

Induction of TYPs and CL100 by EGF in A431 squamous cell carcinomas.



Northern analysis: Growing A431 cells were stimulated for 15 min with 30ng/ml EGF and incubated for the indicated periods of time before isolation of total RNA. 1mg/ml total RNA was used to prepare poly (A) mRNA, and 10 µg samples were electrophoresed overnight through a 1% agarose gel. RNA was transferred to Hybond N+, and the membranes sequentially probed with TYP1, TYP 2, CL100 and the EGF receptor as a loading control; the membranes were stripped from radioactivity between each hybridisation. See Methods for details.

The squamous carcinoma cell line, A431, cannot be rendered quiescent, thus cells harvested at each time point are an asynchronous population within the cell cycle. To ensure that the mRNA induction observed was not due to cell cycle variations, but a true response to mitogen exposure, expression levels of TYP 1 and 2 were also investigated in the cell line SCC12F, rendered quiescent after plating in 0.5% serum for 60 hours. Equivalent results were also obtained with quiescent SCC12F cells, as demonstrated in figure 4.5, p122. Stimulation with serum for 1 hour induced TYP 1 mRNA levels, which peak approximately 10-fold compared to starved cells by 2 hours. The expression of CL100 was induced in response to serum by 30 minutes, with a 20-fold greater stimulation than detected in quiescent cells within 1 hour. The time course of TYP 2 response to serum in quiescent SCC12F is not as immediate as CL100, peaking at 2 hours. However, both TYP 2 and CL100 mRNA levels decline by 4 hours, and have returned to basal levels by 8 hours. Whilst TYP 1 mRNA transcript, although also declining by 4 hours, is still significantly expressed at 8 hours.

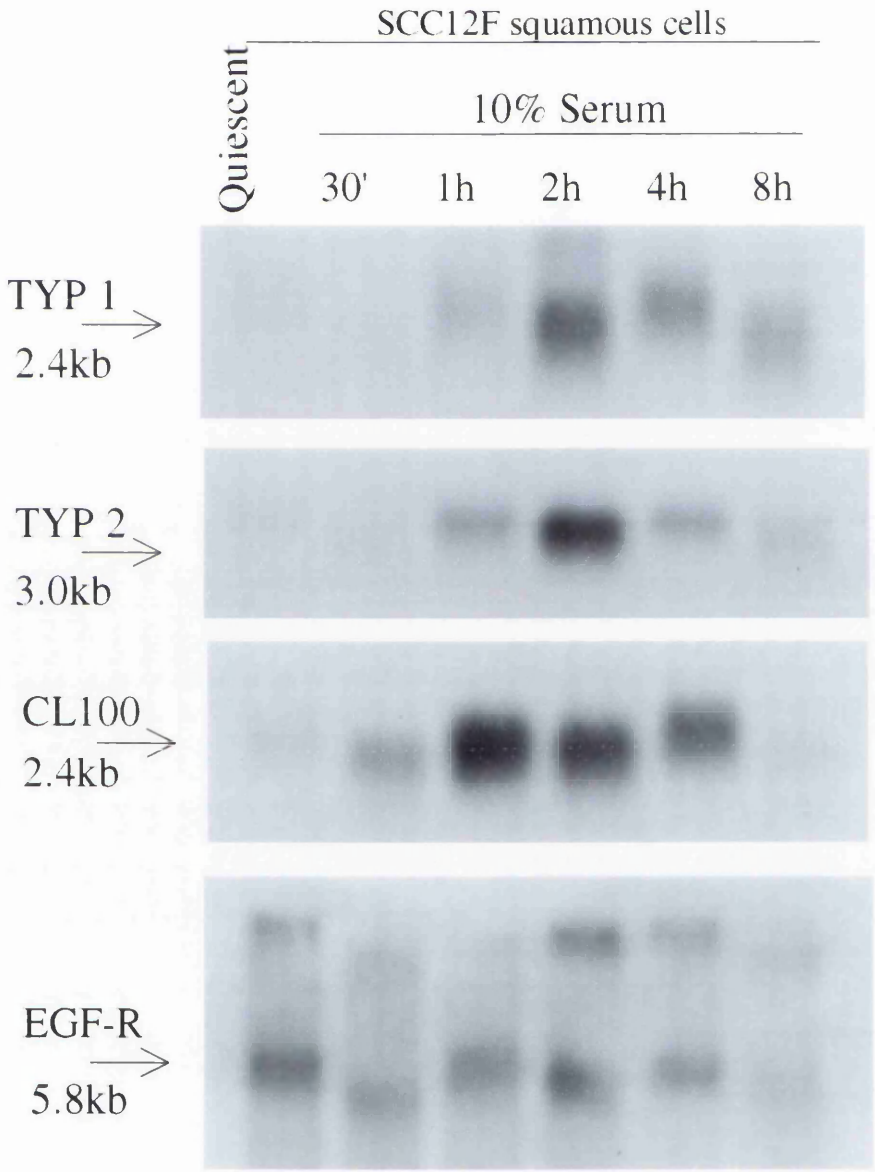
Thus, CL100 and TYP 2 are similarly activated in an immediate-early manner upon mitogenic stimulation. However, there are clear differences in the kinetics of induction of TYP 1, which appears to be induced slightly later in SCC12F squamous carcinoma cells, and remains up-regulated for a longer period of time than both TYP 2 and CL100.

4.4. Regulation of TYP phosphatase mRNA by cellular stress

The differential response between TYP 1 and CL100 mRNA expression upon mitogenic stimulation prompted the investigation into the effects of heat shock on the expression of TYP 1 and TYP 2, since Keyse and Emslie (1992) first isolated CL100 as a stress-induced gene. These studies confirm that CL100 expression is markedly induced by 30 minutes in SCC12F cells following heat shock, returning to basal levels after 2 hours treatment, consistent with previous reports. This is shown in figure 4.6, p123. TYP 2 expression follows the same profile as CL100, although induction is slower peaking at 1 hour rather than 30 minutes.

Figure 4.5.

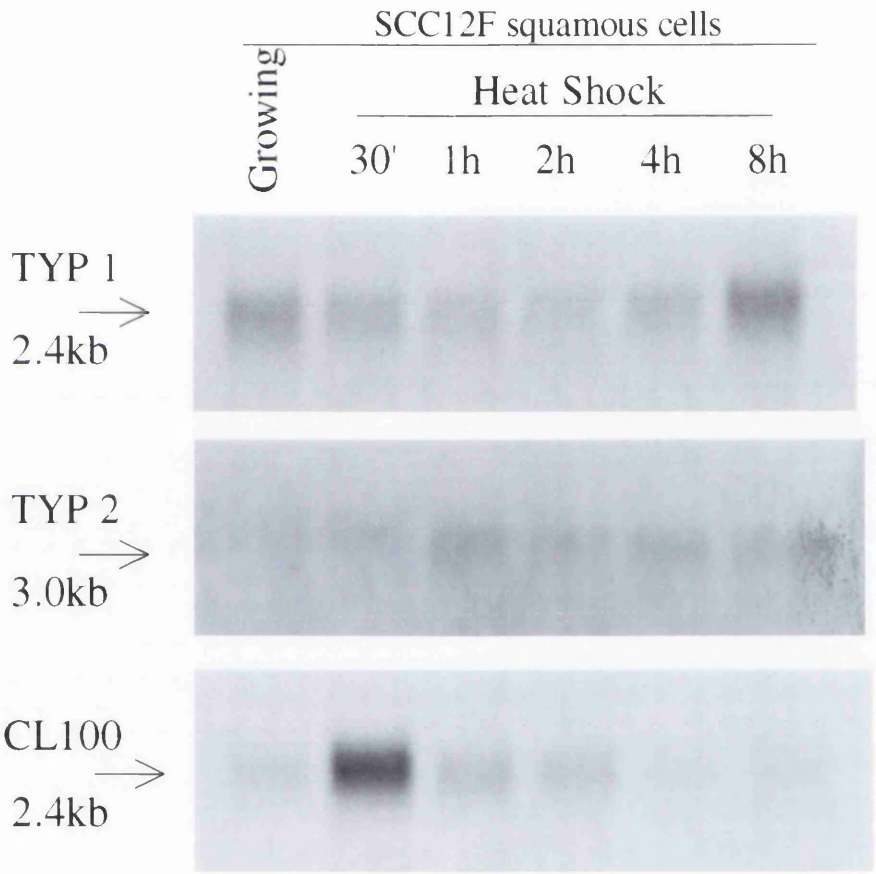
Induction of TYPs and CL100 mRNA by serum in SCC12F squamous cell carcinomas.



Northern analysis: SCC12F cells were grown in 0.5% serum for 60 hours to induced quiescence, and then were stimulated with 10% serum. Total RNA was isolated at indicated times after stimulation. 1mg/ml total RNA was used to prepare poly (A) mRNA, and 10μg samples were electrophoresed overnight through a 1% agarose gel. The RNA was transferred onto Hybond N+, and the membranes sequentially probed with TYP 1, TYP 2, CL100 and the EGF receptor as a loading control. Membranes were stripped from radioactivity between each hybridisation. See Methods for details.

Figure 4.6.

Induction of TYPs and CL100 by heat shock in SCC12F squamous cell carcinomas.



Northern analysis: Growing SCC12F cells were heat shocked by placing the flasks for 30 min in a 44°C water bath. The flasks were then returned to 37°C for the indicated times prior to RNA isolation. 1mg/ml total RNA was used to prepare poly (A) mRNA, and 10µg samples were loaded onto a 1% agarose gel and electrophoresed overnight. RNA was transferred to Hybond N+, and membranes were sequentially probed with TYP 1, TYP 2 and CL100. The membranes were stripped of radioactivity between each hybridisation. For details refer to Methods.

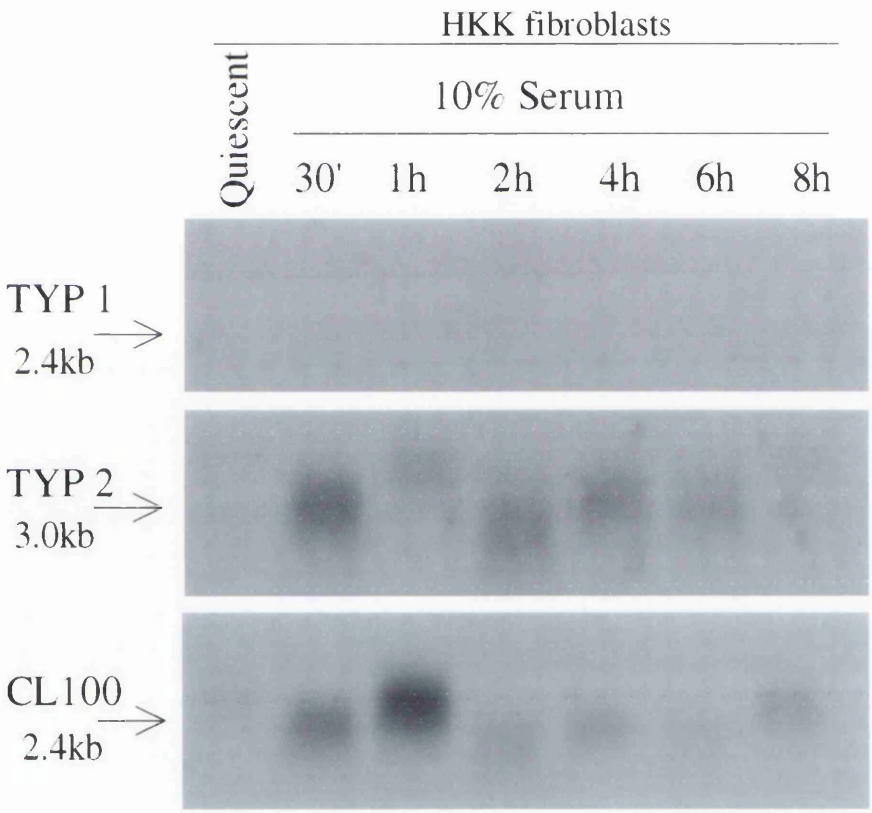
The expression level of TYP 2 in SCC12F cells after heat shock is also significantly lower compared to CL100 mRNA induction. However, TYP 1 mRNA is not induced upon heat shock in SCC12F cells, illustrated in figure 4.6. Indeed, the expression level of TYP 1 would appear to be slightly down-regulated by 2 hours compared to untreated growing cells, returning to near basal levels after 8 hours. Under these conditions, expression of TYP 1 mRNA in growing cells is much higher than that observed for TYP 2 and CL100, and interestingly, whilst the basal expression of TYP 1 is increased in growing, in comparison to quiescent SCC12F cells, as expected, TYP 2 and CL100 mRNA levels are equally low in both quiescent and growing cells, refer to figures 4.5 and 4.6.

4.5. TYP phosphatase expression in fibroblast cells

To further examine the expression of these genes in different cell types, the induction of TYP 1 and 2 in response to mitogens was subsequently investigated in human fibroblasts, on the basis that CL100 was initially cloned from a fibroblastic cell line (Keyse and Emslie, 1992). However, this data demonstrates that TYP 1 is not expressed in HKK fibroblasts, even following mitogenic stimulation, shown if figure 4.7, p125. Expression of CL100 is detected 30 minutes following serum stimulation, which increases approximately 15-fold compared to quiescent cells by 1 hour. Elevated levels of the transcript persist for at least 8 hours, although there is a significant decline after 1 hour. TYP 2 expression is also induced within 30 minutes, approximately 10-fold in comparison to quiescent cells, and is reminiscent of CL100 showing a slight decrease in mRNA levels after 1 hour, remaining fairly constant over the next 6 hours.

Figure 4.7.

Induction of TYPs and CL100 mRNA by serum in HKK fibroblast cells.



Northern analysis: HKK cells were grown in 0.5% serum for 60 hour to render then quiescent. The cells were then stimulated with 10% serum for the indicated periods of time before isolation of total RNA. 1mg/ml total RNA was used to prepare poly (A) mRNA, and 10 µg samples were electrophoresed overnight through a 1% agarose gel. The RNA was transferred to Hybond N+, and the membranes sequentially probed with TYP 1, TYP 2, and CL100. Membranes were stripped from radioactivity between each hybridisation, refer to methods for details.

4.6. Effects of TGFβ treatment on TYP and CL100 mRNA expression

In many mammalian cell types, transforming growth factor-β treatment promotes growth arrest in G1 (Massague, 1990), by inhibiting G1 cyclins and their associated kinase activities, thereby suppressing *Rb* phosphorylation, as introduced in section 1.1.2. This inhibition is mediated in part by the activities of cyclin-dependent kinase inhibitors (Li *et al.*, 1995, Reynisdottir *et al.*, 1995). However, protein phosphatases have also been demonstrated to play a role in cell-cycle regulation, such as Cdc25 (Kumagai and Dunphy, 1991), and possibly Kap/Cdi1 (Hannon *et al.*, 1994; Gyruris *et al.*, 1993).

The expression levels of TYP 1 and 2, and CL100 as a consequence of TGFβ treatment, were therefore investigated in SCC cell lines known to be sensitive to this growth factor (Mailliri, A. *et al.*, submitted). After treating the cell lines BICR6 and MS2 with TGFβ for 24 hours, both TYP 1 and CL100 transcripts are induced compared to the control levels in growing cells, although the induction of CL100 mRNA is greater than that seen for TYP 1, shown in figure 4.8, p128. The levels of TYP 2 mRNA are extremely low in BICR6 cells, however, evidence demonstrates that TYP 2 mRNA levels decrease as a consequence of TGFβ treatment in MS2 cells.

Following this preliminary result, phosphatase expression levels were investigated in MS2 cells treated with TGFβ over a 24 hour period. TGFβ induces and represses the transcription of a variety of genes, and whilst there is a rapid decrease in c-Myc expression (Pietenpol *et al.*, 1990), there is a much slower reduction, between 12 and 24 hours, in the hyperphosphorylated form of pRb (Laiho *et al.*, 1990), cyclin E (Geng and Weinberg, 1993) and CDK4 (Ewen *et al.*, 1993). Figure 4.9, p129 demonstrates that TYP 1 mRNA levels substantially increase between 8 and 12 hours, and remain elevated for at least 24 hours following TGFβ treatment. The expression of CL100 is possibly biphasic following treatment of MS2 cells with TGFβ, with a significant increase after 1 hour, which declines to below the basal level seen in growing cells by 2 hours. The CL100 mRNA levels then increase a second time, returning to basal levels by 8 hours. It is difficult to interpret whether the CL100

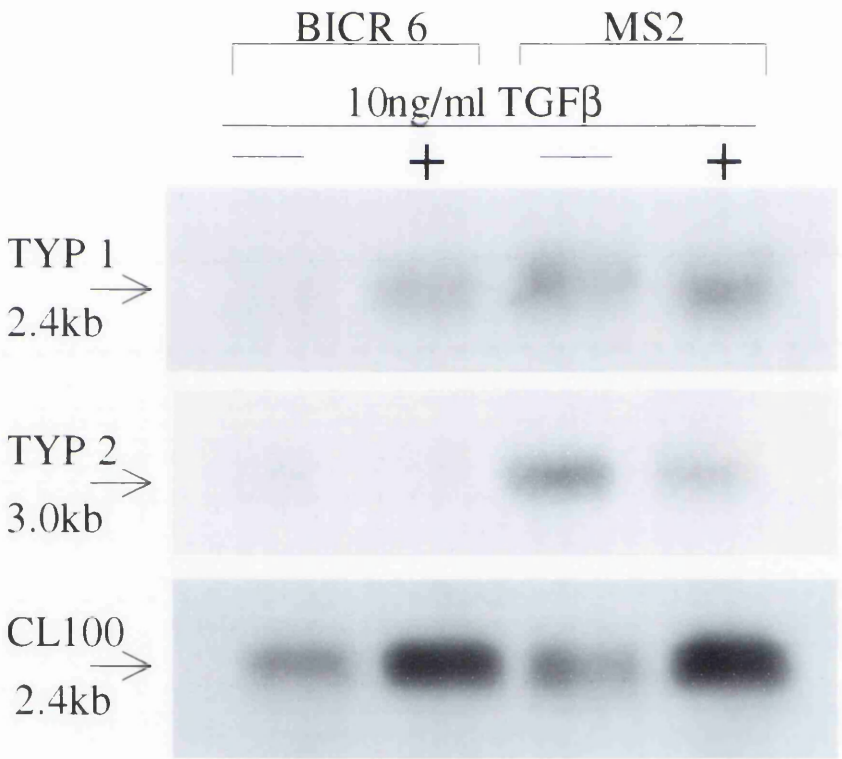
message increases above the basal level between 8 and 24 hours, as the poly (A)+ RNA on the membrane would appear to be slightly under loaded for the last four time points, as shown by the EGF receptor mRNA expression level. However, this does suggest that the increase seen in TYP 1 message after 8 hours, is more exaggerated than the figure would suggest. TYP 2 mRNA levels could not be detected by northern analysis using this membrane.

The squamous cell line SCC12F, is resistant to the anti-proliferative effects of TGFβ. The loss of sensitivity to TGFβ has been reported to occur late during the development of carcinomas, but in most cases, the mechanisms of resistance to TGFβ remain unclear. As demonstrated in figure 4.10, p130, the mRNA levels of TYP 1, TYP 2 and CL100 remain unchanged following TGFβ treatment over 24 hours. This confirms that the changes in mRNA expression levels seen in MS2 and BICR6 cell lines is a true response to the growth factor TGFβ.

In summary, the above expression studies demonstrate that whilst TYP 2 is regulated in an immediate-early fashion reminiscent of CL100, and other documented CL100-like phosphatase genes, TYP 1 is unusual, showing a differential pattern of expression from CL100 and other family members, suggesting that TYP 1 is unique in terms of its regulation and cell-type specificity.

Figure 4.8.

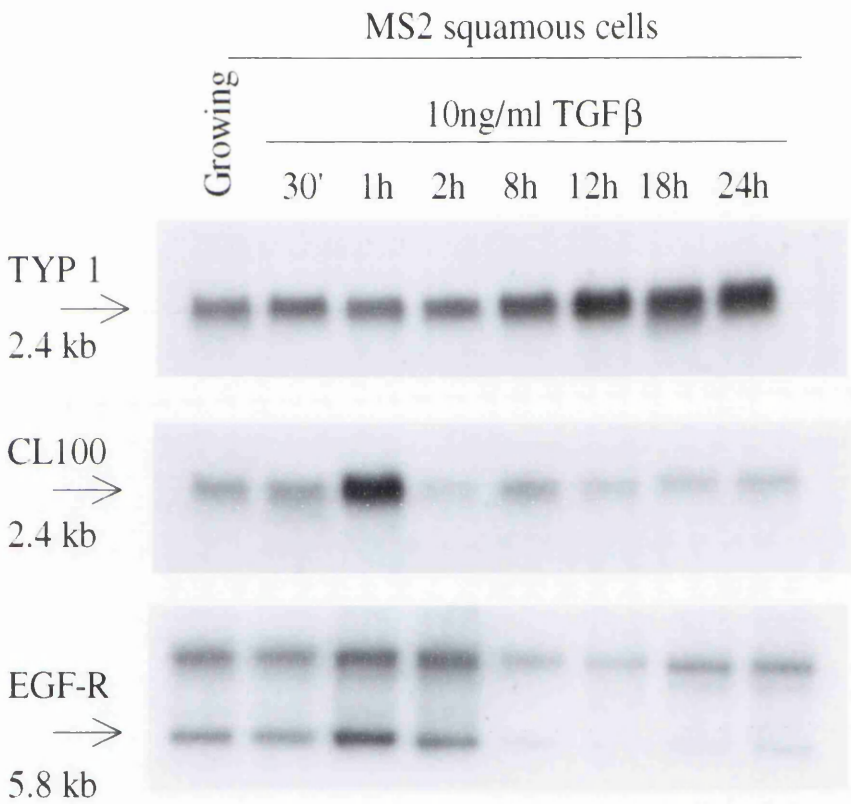
Induction of TYPs and CL100 in response to TGFb treatment in BICR6 and MS2 squamous cell lines.



Northern analysis: Growing BICR6 and MS2 cells were incubated in 10 % serum with and without the presence of 10ng/ml TGFβ. After 24 hours, total RNA was isolated and 1mg/ml samples were used to prepare poly (A) mRNA. 10 µg of poly (A) mRNA was loaded onto a 1% agarose gel and electrophoresed overnight. RNA was transferred to Hybond N+, and probed with TYP 1, TYP 2 and CL100 as indicated. Membranes were stripped from radioactivity between each hybridisation.

Figure 4.9.

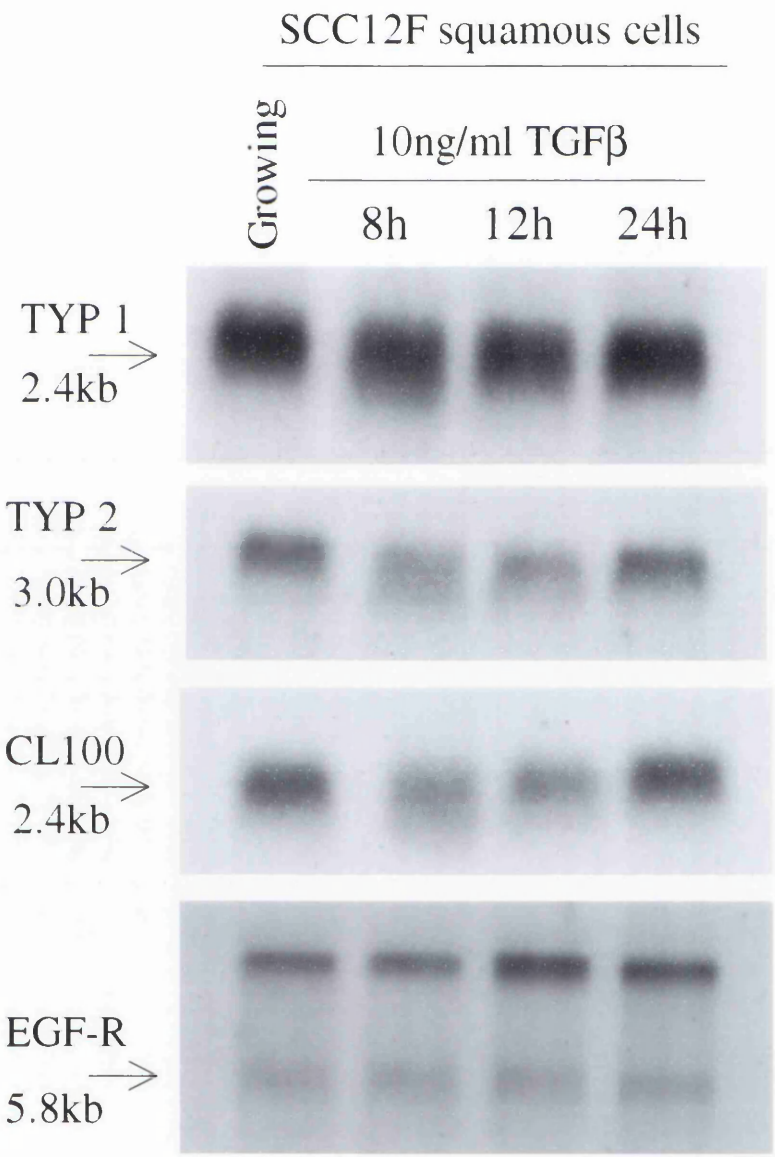
Induction of TYPs and CL100 in response to TGFβ treatment of MS2 squamous carcinoma cells.



Northern analysis: Growing MS2 cells were incubated in 10% serum supplemented with 10ng/ml TGFβ for the indicated times, prior to total RNA isolation. 1mg/ml total RNA was used to prepare poly (A) mRNA, and 10μg samples were loaded onto a 1% agarose gel and electrophoresed overnight. RNA was transferred onto Hybond N+, and the membranes were sequentially probed with TYP 1, TYP 2, CL100, and the EGF receptor as a loading control. Membranes were stripped from radioactivity between each hybridisation. See Methods for details.

Figure 4.10.

Induction of TYPs and CL100 in response to TGFβ in SCC12F squamous carcinoma cells.



Northern analysis: Growing SCC12F cells were treated with 10% serum supplemented with 10ng/ml TGF β for the indicated periods of time prior to total RNA isolation. Poly (A) mRNA samples were prepared from 1mg/ml total RNA, and electrophoresed overnight through a 1% agarose gel. RNA was transferred onto Hybond N+, and the membrane sequentially probed with TYP 1, TYP 2, CL100, and the EGF receptor as a loading control. Membranes were stripped from radioactivity between hybridisations, refer to Methods for details.

CHAPTER FIVE

RESULTS

Characterisation of TYP 1 activity

5. Characterisation of TYP 1 activity

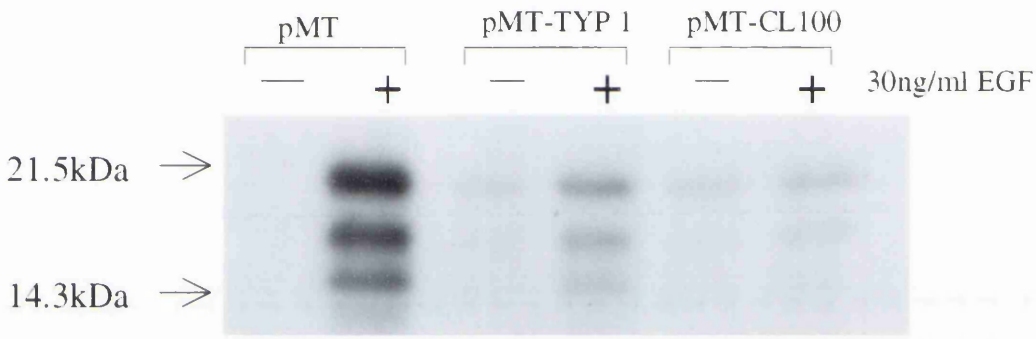
5.1. TYP 1 can inactivate MAP kinase

The TYP 1 catalytic domain shows extremely high sequence identity to the VH1-like dual specificity phosphatases, and furthermore, CL100 has been demonstrated to specifically dephosphorylate and inactivate ERK 1 and 2 MAP kinase isoforms (Alessi *et al.*, 1993a). More recently, other family members have also shown substrate specificity towards the ERK MAPK isoform both *in vitro* and *in vivo*, such as PAC-1 (Ward *et al.*, 1994), HVH2 (Guan and Butch, 1995), and MKP-2 (Misra-Press *et al.*, 1995).

In order to establish whether TYP 1 exhibits a similar phosphatase activity towards the MAP kinases, a transient expression system was used. Cos cells were transfected with either 0.5μg pMT-TYP 1 or pMT-CL100. The activation state of endogenous ERK2 was examined by immunoprecipitation with a polyclonal ERK2 antibody, from cells that were either serum deprived or re-stimulated with EGF. The immunoprecipitates were then tested for MAP kinase activity by an *in vitro* assay which utilises myelin basic protein (MBP) as a substrate for ERK2 in the presence of [γ-^{32}P]ATP. As shown in figure 5.1, p133, the first two lanes demonstrate that under control conditions where cells are transfected with parental vector only, the phosphorylation of MBP by immunoprecipitated ERK2 is dependent upon EGF stimulation (+). However, EGF stimulation of cells which are constitutively expressing TYP 1, reduces the phosphorylation status of MBP by 73% compared to the control cells. As previously reported, constitutive expression of CL100 reduces the phosphorylation status of MBP by 87% compared to the control levels. The percentage figures are calculated on the basis that the control shows 100% MBP phosphorylation, and relative percentages are estimated using densitometry. Extensive TYP 1 inactivation of ERK2 also occurs when a sample of cell lysate from TYP 1 transfected Cos cells (i.e. lane 3) is pre-incubated with immunoprecipitated ERK2 from stimulated cells (lane 2), and then assayed for activity towards MBP, data not shown.

Figure 5.1.

Effect of transient transfection of TYP 1 and CL100 on ERK2 MAP kinase activity.



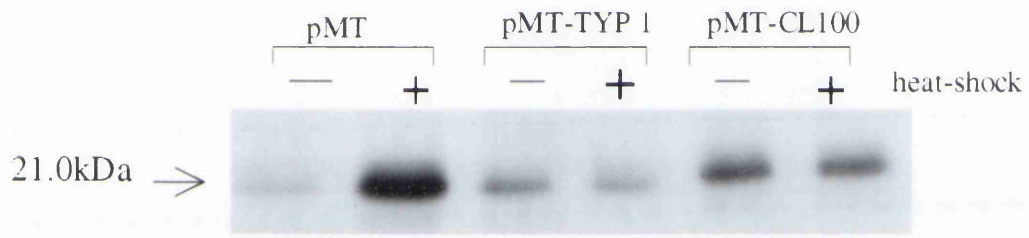
Cos cells were transfected with 0.5 µg of pMT-TYP 1, pMT-CL100 or control pMT vector, and treated with 30ng/ml EGF. Lysates were prepared as described in the Methods. The activation state of ERK2 was examined by immunoprecipitation. Cell lysates (either serum deprived or stimulated with EGF for 5 min, as indicated) were incubated overnight at 4°C with 2 µl of anti-ERK2 antibody, pre-bound to protein A-sepharose. The antibody/protein complex was pelleted, washed and incubated with MBP in the presence of [γ-32P]ATP for 20 min at 30 °C. The reactions were terminated with SDS loading buffer and proteins resolved on a 15% SDS-polyacrylamide gel. Molecular mass markers (bio-rad) are indicated. See methods for more detail. MBP runs at 21.5 kDa as indicated, with degradation products running below.

CL100 was initially identified as a gene whose mRNA was rapidly induced in response to cellular stress (Keyse and Emslie, 1992). It has also been demonstrated that CL100 inactivates the stress-induced MAP kinase isoform, p38, (Rouse *et al.*, 1994). Further stress-activated protein kinases which are involved in the phosphorylation of c-Jun include the SAPK, and the JNK family members, see section 1.2.1. To establish if stress-activated protein kinase activity is compromised in the presence of TYP 1, Cos cells were transfected with TYP 1, and subjected to heat shock at 44°C in order to induce the stimulation of endogenous SAPKs/JNKs. The activation state of endogenous, immunoprecipitated p54JNK was then assayed against recombinant c-Jun protein in the presence of [γ-^{32}P]ATP. As illustrated in figure 5.2, p135, heat shock for 30 minutes at 44°C is sufficient to activate endogenous p54JNK, as demonstrated by the ability of immunoprecipitated p54JNK to phosphorylate recombinant c-Jun, see lane 2. However, constitutive expression of TYP 1 inhibits the phosphorylation of recombinant c-Jun protein by activated p54JNK, by more than 90%, as compared to the mock-transfectants. Identical results were obtained for CL100 expression, which similarly inhibits c-Jun phosphorylation more than 90% compared to control levels. These results indicate that MAP kinase activity is compromised in the presence of TYP 1.

To demonstrate that MAP kinase inactivation was a direct consequence of TYP 1, the ability of TYP 1 protein to inactivate recombinant ERK2 was examined. TYP 1 cDNA was cloned into baculovirus in order to express a GST fusion protein, and purified by Alan Ashworth. For this assay, recombinant ERK2 protein was activated and ^{32}P-labelled by phosphorylation with purified MAP kinase kinase (EE mutant, Alessi *et al.*, 1993a), and then incubated for 20 minutes at 30°C with 20ng purified TYP 1 protein. As demonstrated in figure 5.3, p136, loss of ERK2 activity was observed, as analysed by the reduced ability of ERK2 to phosphorylate MBP in the presence of [γ-^{32}P]ATP, lanes 3 and 4. As an internal control, activated ERK2 was also incubated with recombinant Myb protein, lanes 5 and 6, and as expected, ERK2 remains active, phosphorylating MBP as efficiently as active ERK2 alone. These three reactions were performed in duplicate.

Figure 5.2.

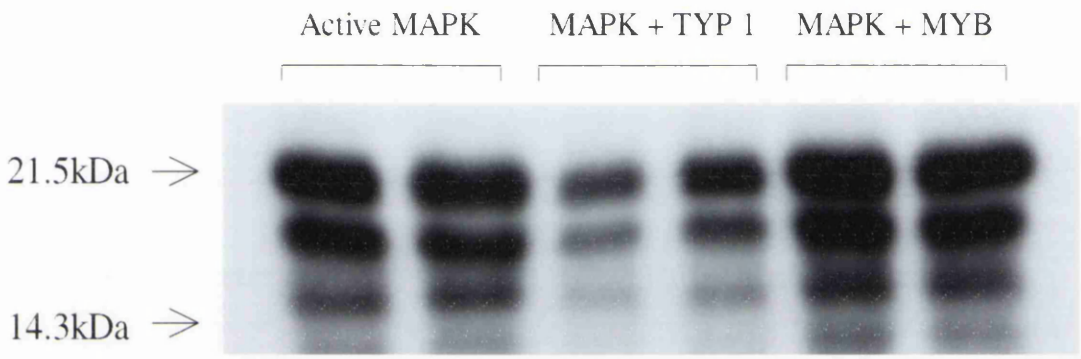
Effect of transient transfection of TYP 1 and CL100 on stress-activated MAP kinase activity.



Cos cells transfected with 0.5 μg of pMT-TYP 1, pMT-CL100 or control pMT vector were heat shocked at 44 °C, and lysates prepared as described in the Methods. The activation state of p54JNK was examined by immunoprecipitation. Cell lysates were incubated overnight at 4 °C with 1 μl anti-p54 antibody, pre-bound with protein A sepharose beads. The antibody/protein complex was pelleted, washed, and incubated with recombinant c-Jun protein in the presence of [γ-32P]ATP for 20 min at 30 °C. The reactions were terminated by the addition of SDS loading buffer and the proteins resolved on a 15% SDS-polyacrylamide gels. Although the translation of recombinant c-Jun yields a full length protein, in this assay system a truncated c-Jun protein, running at 21kDa, is preferentially phosphorylated.

Figure 5.3.

Inactivation of recombinant ERK2 by TYP 1.



Baculovirus expressed TYP 1 (20 ng) was incubated with activated, phosphorylated recombinant GST-ERK2 protein (200ng) as described in the Methods. The activity of ERK2 towards MBP was assayed in the presence of [γ-32P]ATP for 20 min at 30° C. The reactions were terminated by the addition of SDS loading buffer, and the proteins resolved on a 15% SDS polyacrlamide gel. Recombinant Myb protein was used as a control, and each reaction is shown in duplicate. Molecular mass markers (Bio-rad) are indicated by the arrows.

5.2. TYP 1 is a dual specificity Tyr/Thr protein phosphatase

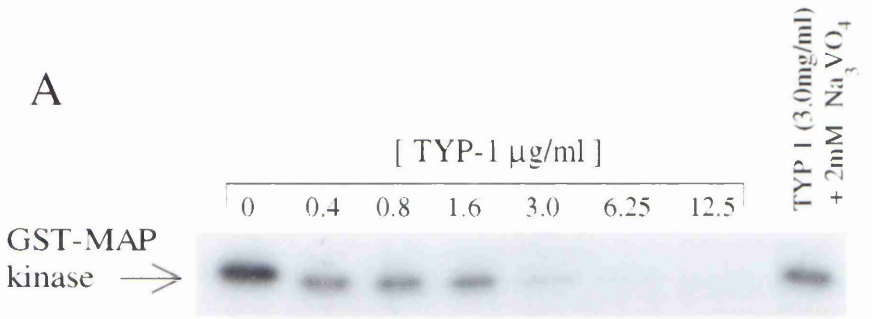
ERK2 autophosphorylates on serine and threonine residues, while phosphorylation of both threonine 183 and tyrosine 185 by MAP kinase kinase is required for its activation. It has been demonstrated previously that CL100 inactivates MAP kinase by a parallel dephosphorylation of both tyrosine and threonine residues (Alessi *et al.*, 1993a).

To determine the molecular mechanism of TYP 1 inactivation of ERK2, increasing concentrations of TYP 1 protein were incubated for 10 minutes at 30°C with activated, ^{32}P-labelled ERK2, and the reaction products were analysed on an SDS-PAGE gel, see figure 5.4a, p138. Consistent with the previous observation that TYP 1 can directly inactivate ERK2, TYP 1, like CL100, is capable of dephosphorylating MAPKK-activated ERK2 in a concentration dependent manner. Indeed, incubation of activated ERK2 with 3.0μg TYP 1 results in almost all the labelled phosphate being removed from ERK2. At this particular concentration of TYP 1, co-incubation with sodium vanadate, an inhibitor of protein tyrosine phosphatases and CL100-like phosphatases, effectively blocks dephosphorylation of ERK2. Furthermore, aliquots of ^{32}P-labelled ERK2 treated with TYP 1 protein, and subjected to one dimensional phosphoamino analysis, demonstrates that both threonine and tyrosine residues are effectively dephosphorylated, illustrated in figure 5.4b. As expected, no loss of phosphate is seen in the presence of sodium vanadate. Analysis of the autoradiograph by densitometry shows that TYP 1 inactivates ERK2 with 50% dephosphorylation occurring in 10 minutes (the incubation time), with a concentration of approximately 14nm TYP 1. This is comparable to CL100 which has been shown to inactivate ERK2 at a concentration of approximately 7nm (Alessi *et al.*, 1993a).

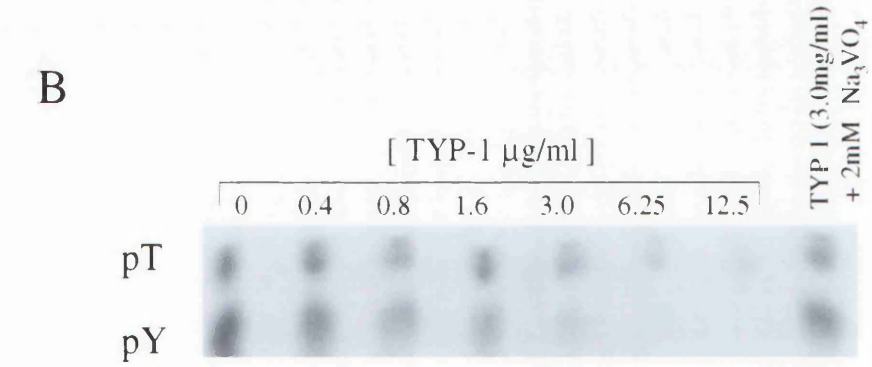
In conclusion, these studies demonstrate that TYP 1 acts as a dual specific threonine/tyrosine phosphatase, rapidly inactivating and dephosphorylating ERK2. Phosphoamino acid analysis suggests that TYP 1 dephosphorylates the tyrosine residue more efficiently than the threonine.

Figure 5.4.

Dephosphorylation of recombinant ERK2 by TYP 1



Autoradiogram of an SDS polyacrylamide gel. Recombinant GST-ERK2 was ^{32}P-labelled and activated with MKK as described in Methods. Activated ERK2 was treated with buffer alone, or increasing concentrations of recombinant TYP 1 protein in the presence or absence of 2mM sodium vanadate, as indicated. Reactions were initiated at 30°C for 10 min, terminated by the addition of SDS loading buffer, and analysed by 10% SDS-polyacrylamide gel. See Methods for details.



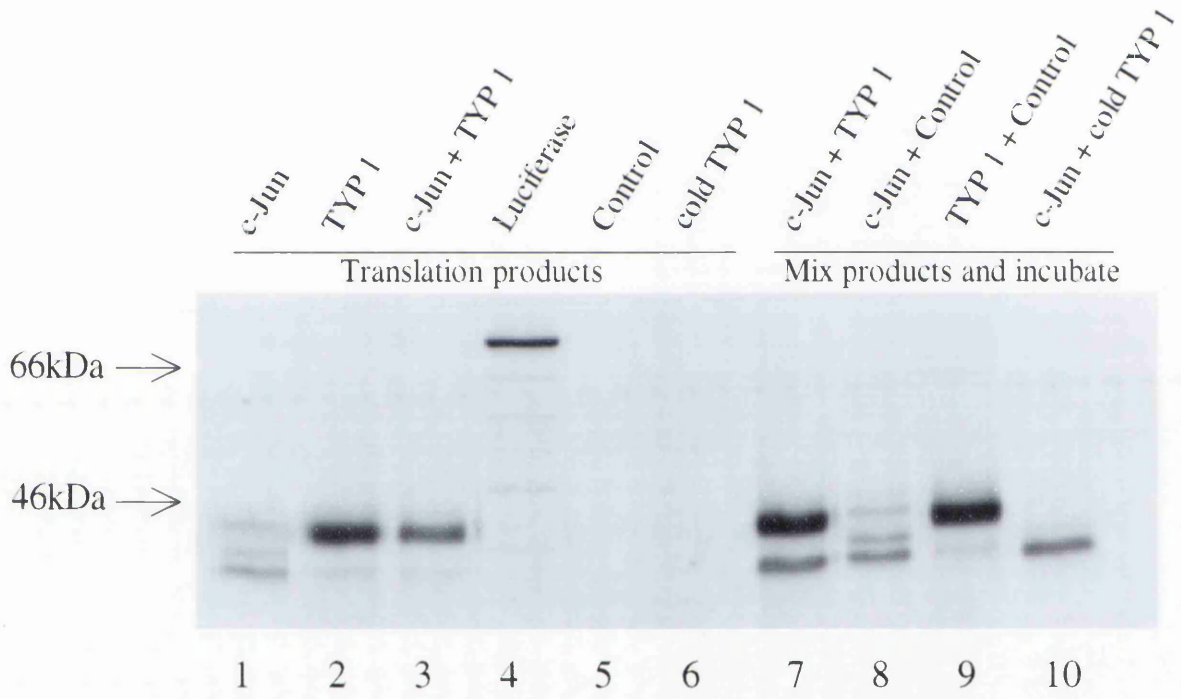
Phosphoamino acid analysis. 10µl aliquots of ^{32}P-labelled GST-ERK2 samples, treated as above, were subject to one dimensional phosphoamino acid analysis, see Methods for details. pT and pY denote positions of phosphothreonine and phosphotyrosine.

5.3. TYP 1 phosphatase activity towards c-Jun

The proto-oncogene c-Jun is phosphorylated at a number of sites at both the amino- and carboxyl-termini of the protein, which alters both DNA binding and transcriptional activity of the gene. Many research groups have demonstrated that the phosphorylation at the amino-terminus of c-Jun is carried out by a family of MAP kinases, known as the JNK/SAPKs subfamily, see section 1.5.2.1. The enzymes which are involved with phosphorylating the carboxy-terminus of the c-Jun protein are presently not known. As demonstrated in figure 5.5, p140, when c-Jun is translated *in vitro* using a reticulocyte lysate containing ^{35}S-labelled methionine, there are a minimum of four sites which are differentially phosphorylated, therefore conferring different mobilities of these phospho-proteins through an SDS-PAGE gel, lane 1. It was therefore investigated whether it was possible to co-translate TYP 1 and c-Jun, and alter the pattern of c-Jun phosphorylation. Translation of TYP 1 results in a protein running at the predicted molecular mass of 43 kDa, lane 2. However, although co-translation of TYP 1 and c-Jun appears to inhibit the phosphorylation states of c-Jun, as demonstrated by the loss of some of the c-Jun phospho-proteins, lane 3, both TYP 1 and c-Jun proteins run at approximately the same size, and consequently the TYP 1 protein masks the four phosphorylation products of c-Jun. In view of this, TYP 1 was translated in the absence of radioactive label, lane 6. An aliquot of translated ^{35}S-labelled c-Jun protein was then incubated with cold-translated TYP 1 protein, and this clearly results in the inhibition of the upper phosphorylation states of c-Jun, lane 10. However, the protein kinase and phosphatase activities in the lysate are undefined, and therefore this assay does not determine whether TYP 1 inactivates a c-Jun kinase, or activates a c-Jun phosphatase, which may be present in the lysate, or indeed directly dephosphorylates c-Jun. Prior to incubation, the protein products were treated with emetine, to prevent any further translation. The luciferase gene, lane 4, was used as a control for the translation reaction, and reticulocyte lysate alone, lane 5, was used to demonstrate that the protein products visualised are due to transcription of the DNA added, and not a component of the lysate itself. Reticulocyte lysate was also used as an incubation control. (see Black et al. 1991. Oncogene 6: 1949-58)

Figure 5.5.

TYP 1 phosphatase activity towards c-Jun.



In vitro translation was carried out using the TNT Coupled Reticulocyte Lysate System from Promega. 1 µg DNA template (pCITE-TYP 1 and/or pSPT19-c-Jun) was used for each translation reaction, and incubated for 2 hours at 30 °C. The control (retic lysate alone) and cold TYP-1 were non-radioactive. Following translation, the reactions were terminated by the addition of 0.1mM emetine. To determine TYP 1 phosphatase activity towards c-Jun, 10 µl samples of translated TYP-1 and c-Jun were mixed and incubated at 30°C for 10 min. All samples were mixed with SDS loading buffer, and proteins resolved on 10% SDS polyacrylamide gel, as indicated. The gel was dried under vacuum and exposed to Kodak film. Molecular mass markers are shown. See Methods for details.

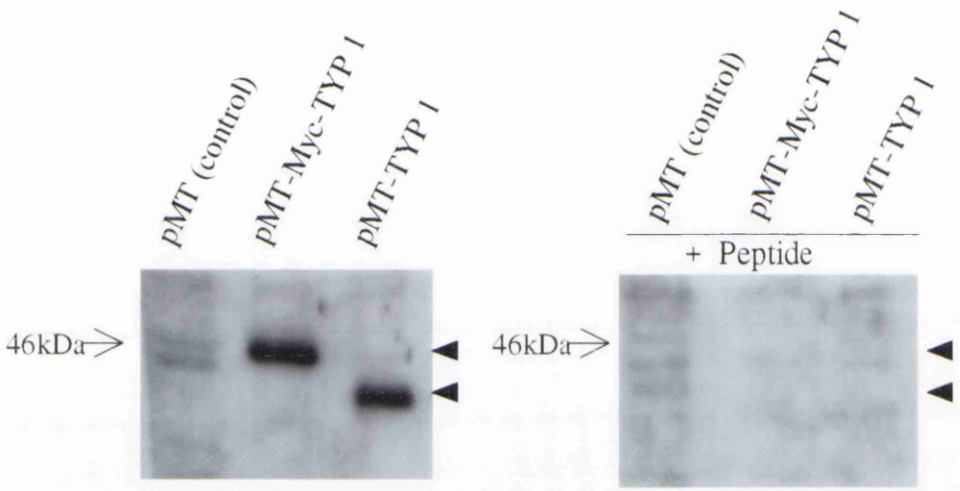
5.4. Expression of the TYP 1 protein

In order to investigate the regulation of TYP 1 protein expression, several peptides were designed to the amino-terminal sequence of TYP 1. This region is preferable for raising antibodies against, considering that the amino-terminal sequences in TYP 1, and indeed the other CL100-like phosphatases, are the most divergent. Several polyclonal rabbit antisera were raised against the TYP 1 peptides, and sequentially screened for the detection of TYP 1 protein using lysates from Cos cells transfected with pMT-TYP 1. One antibody (B) specifically recognises a 43 kDa protein, and the corresponding peptide is illustrated in table 2, p72. The TYP 1 cDNA has also been tagged at the amino-terminus with the c-Myc epitope, see table 2. This was so that TYP 1 protein could be detected and immunoprecipitated with a c-Myc antibody, prior to the TYP 1 antisera having been produced. However, all the applications utilising the c-Myc antibody have since been repeated using TYP 1 antisera, and are therefore not presented in this thesis. However, the Myc-tagged TYP 1 construct is used as an additional control for the detection of TYP 1 protein, and is recognised by the antisera as a 46 kDa protein. The 43 and 46 kDa single protein bands are identified as TYP 1 based on mobility and competition by the addition of excess peptide, as shown in figure 5.6, p142.

Since TYP 1 mRNA is induced upon mitogenic stimulation of squamous carcinoma cell lines, the protein expression was investigated in the SCC line A431, following induction by EGF. As demonstrated in figure 5.7, p143, TYP 1 protein in growing A431 cells is barely detectable, time point 0. However, after stimulation the protein is induced at 4 hours, and peaks at 8 hours. This is consistent with the kinetics of mRNA accumulation, which show an induction after 1 hour and peak at 4 hours, see section 4.3. In A431 cells, unlike transfected Cos cells, background protein bands are also detected by the TYP 1 antisera, however, the TYP 1 protein can be clearly recognised due to the positioning of the control protein bands from transfected Cos cells. Additionally, the TYP 1 protein detected in A431 cells can be entirely competed by an excess of peptide, lower diagram shown in figure 5.7.

Figure 5.6.

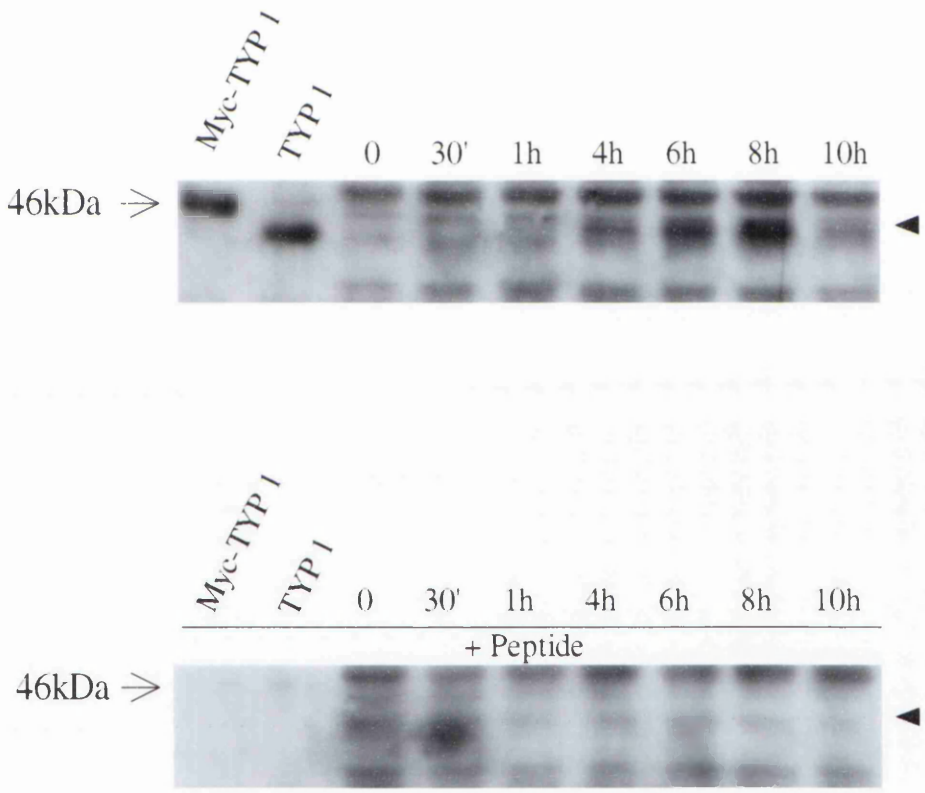
Immunoblot analysis of TYP 1 protein.



50µg samples of extracts from Cos cells transfected with either 0.5µg parental pMT vector, pMT-Myc-TYP 1 or pMT-TYP 1 were resolved by electrophoresis on a 10% SDS polyacrylamide gel, and protein transferred to nitrocellulose as described in the Methods. The filters were probed with rabbit anti-TYP 1 antibody (1:1000) in the presence and absence of excess competitor peptide, as indicated, and proteins detected by immersing the filters in ECL reagent for 1 min, followed by exposure to Kodak film. The positions of Myc-TYP 1 and TYP 1 are indicated by arrow heads, the molecular mass marker (Bio-rad) is indicated on the left.

Figure 5.7.

Immunoblot analysis of a mitogen induced time course of TYP 1 protein.



Growing A431 cells were stimulated with 30ng/ml EGF for the indicated periods of time prior to cell lysis, as described in the Methods. 50µg lysate samples were resolved on a 10% SDS-PAGE gel, and proteins transferred to nitrocellulose overnight. The filters were probed with rabbit anti-TYP 1 antibody (1:1000) in the presence and absence of an excess of competitior peptide, and proteins were detected by immersing the filters in ECL reagent for 1 min, followed by exposure to Kodak fim. Transfected Cos cell extracts were used as internal markers, and the TYP 1 protein is indicated by the the arrow heads. The position of the molecular mass marker (Bio-rad) is shown on the left.

5.5. Analysis of the stability of TYP 1 protein

Following the detection, and the induction of TYP 1 protein upon mitogenic stimulation by western analysis, the stability of the protein was investigated using the technique of pulse-chase, where cells are initially pulsed with [^{35}S]methionine for a period of time sufficient to radioactively label proteins during their synthesis. The cells are then chased for a longer period of time in cold-methionine media, such that newly synthesised proteins, which are not labelled, gradually compete out the radiolabelled-proteins, thus enabling the stability of the radiolabelled proteins to be determined.

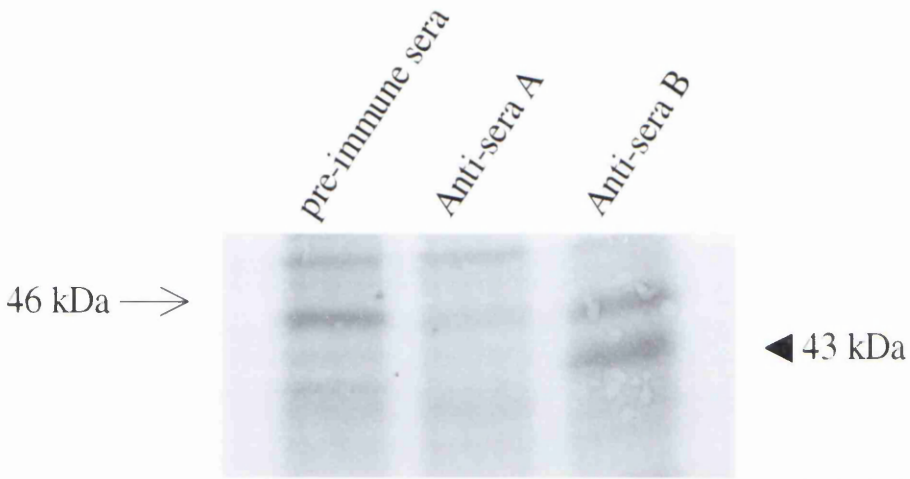
The TYP 1 antibody was initially tested for the ability to immunoprecipitate protein from extracts of [^{35}S]methionine-labelled A431 cells. Since the level of TYP 1 protein is too low to be detected in growing cells, see figure 5.7, the cells were stimulated for 4 hours with EGF prior to labelling with [^{35}S]methionine for one hour. Cell lysates were prepared, and the TYP 1 protein immunoprecipitated using two different antibodies: A and B, (although antibody A is not able to detect TYP 1 protein by western analysis). As demonstrated in figure 5.8a, p145, TYP 1 protein was immunoprecipitated by antibody B only, lane 3. Pre-immune sera was used a control. Immunoprecipitation analysis of TYP 1 protein was then performed using A431 cells which had been stimulated with EGF for 4 hours prior to labelling with [^{35}S]methionine, and then chased over a period of 6 hours. However, no specific protein signal for TYP 1 could be detected, due to the high number of non-specific proteins which also immunoprecipitate with the TYP 1 antibody B in A431 cells, data not shown. Therefore, the stability of the TYP 1 protein cannot be investigated by this method.

Since TYP 1 protein is detectable in transfected Cos cells, with little background interference, pulse-chase labelling was repeated in Cos cells which were transfected with 0.5μg pMT-TYP 1. Post-transfection, the cells were labelled with [^{35}S]methionine for one hour, and chased over a 6 hour period. As indicated in figure 5.8b, TYP 1 protein is readily detectable by immunoprecipitation with antibody B.

Figure 5.8.

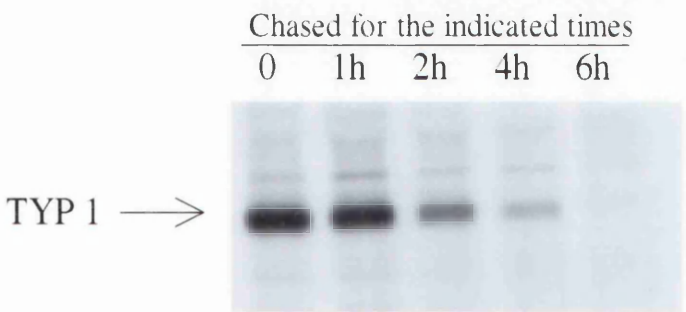
Immunoprecipitation of TYP 1 protein following 35S-methionine labelling

A



A: Growing A431 cells were stimulated with EGF for 4 hours prior to labelling with [35S]methionine for 1 hour. Cell lysates, which had been pre-cleared, were incubated overnight at 4°C with 20µl TYP 1antibody. 50µl protein A-sepharose beads were added and incubated for a further hour at 4°C. The immunoprecipitates were washed, mixed with SDS-loading buffer and TYP 1 protein resolved on a 10% SDS-PAGE gel. The gel was then fixed in acetic acid/methanol, dried under vacuum and exposed to Kodal film. The control antibody is pre-immune sera. See Methods for more detail.

B



B: Cos cells were transfected with 0.5 µg pMT-TYP 1. Post-transfection cells were labelled with [35S]methionine for 1 hour, and then chased in media with cold methinine for the indicated times prior to cell lysis. Immunoprecipitation was carried out exactly as described above using 20µl antibody B. Samples were resolved on a 10% SDS-PAGE gel, the gel dried and exposed to Kodak film.

A decrease in protein is detected by 2 hours, and by 6 hours TYP 1 protein is barely detectable, indicating that the protein is relatively unstable.

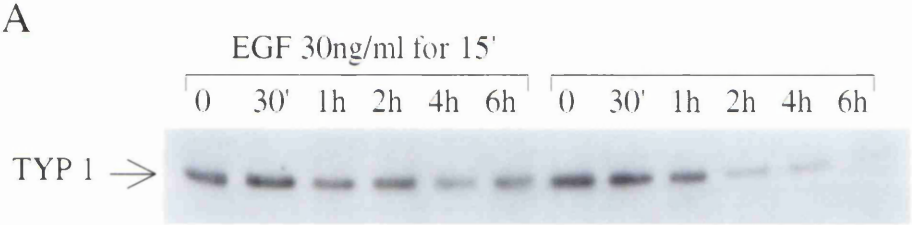
This experiment was approached in a different way by using cycloheximide, which inhibits protein synthesis. Cos cells were transfected with TYP 1 and grown for 24 hours prior to treatment with 10μg/ml cycloheximide at time 0. Cell lysates were then harvested over a period of 6 hours. In this instance, a decrease in TYP 1 protein was detected in the first hour, confirming that the protein is considerably unstable, as shown in figure 5.9a, p147. Interestingly, treatment of the cells with EGF prior to cycloheximide addition appears to stabilise the TYP 1 protein, with protein still detected 6 hours after cycloheximide treatment. However, in cells not treated with EGF, TYP 1 protein is barely detectable at 6 hours. In this regard, the density of each band in the figure was measured by molecular dynamics laser densitometry using the PDI image analysis software. The density is measured in pixel units (OD x MM^2) which is a function of spot intensity and the number of pixels covered. These measurements, which are a composite from three separate experiments, were then converted into a percentage of the control, with the control showing 100%, and plotted against time, see figure 5.9b. The graph demonstrates that EGF treatment stabilises the TYP 1 protein considerably. The half-life of TYP 1 is estimated to be 90 minutes, which is similar to that reported for CL100 (Noguchi *et al.*, 1993). Upon stimulation with EGF, the half-life of TYP 1 protein is extended to just over 3 hours. Although cycloheximide has been shown to prolong the half-life of many immediate early genes, the previous pulse chase experiment results in a similar calculated half-life for TYP1.

To ensure that cycloheximide treatment of Cos cells inhibited protein synthesis, growing Cos cells were treated with 10μg/ml cycloheximide for 30 minutes, and then labelled with [^{35}S]methionine for 1 hour. Cell lysates were then prepared and precipitated with TCA as described in the Methods. The precipitate was collected on a Whatman GF-C filter, the filter was then washed, dried and counts per minute calculated. The control cells were not treated with cycloheximide. Protein synthesis in cells treated with cycloheximide is inhibited by more than 96%.

Figure 5.9.

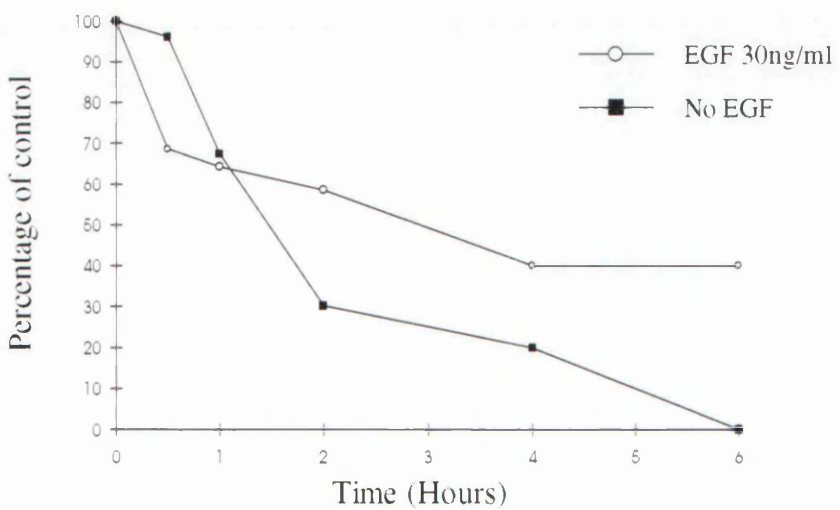
Effect of cycloheximide on TYP 1 protein levels.

A



Cos cells were transfected with 0.5µg pMT-TYP 1, and stimulated with 30ng/ml EGF for 15 min as indicated. Cells were then treated with 10µg/ml cycloheximide for the indicated periods of time prior to cell lysis, see Methods. 50µg protein samples were resolved by electrophoresis on a 10% SDS polyacrylamide gel, and proteins transferred to nitrocellulose overnight. The filter was probed with anti-TYP 1 antibodies (1:1000), and protein detected by immersing the filter in ECL reagent for 1 min, followed by exposure to Kodak film. The position of the TYP 1 protein is indicated.

B



Bands from the above gel were quantitated using a densitometer, and plotted as a percentage of the control, ie. growing cells without cycloheximide treatment.

Cos cells	5.6×10^6 cpm	100 % protein synthesis
Cos cells + cycloheximide	2.2×10^5 cpm	3.9% protein synthesis

5.6. Subcellular localisation of TYP 1 protein

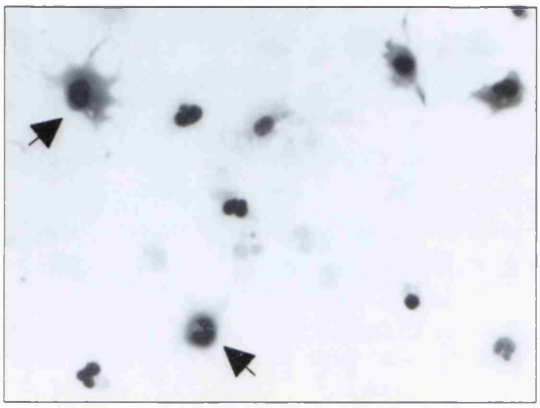
The subcellular localisation of TYP 1 was investigated using a biotin-labelled secondary antibody and a streptavidin-horseradish peroxidase conjugate, see Methods for details. Cos cells were transiently transfected with either pMT-TYP 1 or parental vector, and 24 hours post-transfection were transferred onto glass slides, and grown for a further 24 hours. The slides were then stained with TYP 1 antibody B, or rabbit pre-immune sera as a control. Figure 5.10, p149 panel A demonstrates that cells transfected with TYP 1 show a strong positive staining in the nucleus. A few cells show a slightly higher than background staining in the cytoplasm, presumably due to TYP 1 being transfected in these cells, and consequently overexpressed. The control cells, panels B and C, do not show any nuclear staining above background levels. These results are in agreement with data demonstrating that PAC-1 (Rohan *et al.*, 1993), CL100 and HVH3 (Kwak and Dixon, 1995), HVH2 (Guan and Butch, 1995) and MKP-2 (Misra-Press *et al.*, 1995) also show nuclear staining, suggesting that this phosphatase family functions primarily in the nucleus. The implications of this are discussed later.

Figure 5.10.

Subcellular localisation of TYP 1

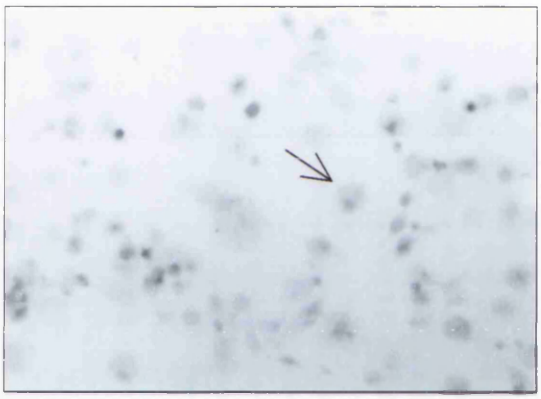
A

TYP 1 Antibody



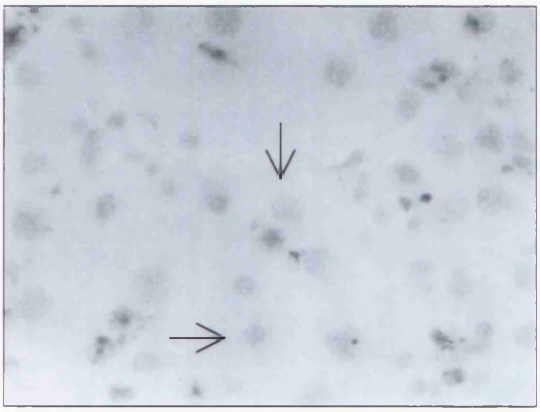
B

Negative Control Antibody



C

Negative Control Transfection



Cos cells were transfected with either 0.5µg pMT-TYP 1 (Panel A and B) or 0.5 µg parental pMT (Panel C), transferred to glass chamber slides and grown for a further 24 hours. Slides were washed, fixed and stained with anti-TYP 1 antibody (1:4000), and a secondary antibody conjugated to biotin, as described in the Methods. Control antibodies were pre-bleed normal rabbit serum. The thick arrow heads indicate TYP 1, which is localised primarily in the nucleus. The thin arrows indicate cells with background staining only.

CHAPTER SIX

DISCUSSION

6. Discussion

Protein phosphatases are generally classified according to their phosphoamino acid specificity, and as such, until presently, have been divided as tyrosine or serine/threonine phosphatases. Unlike the protein kinases, the serine/threonine phosphatases share no sequence identity with the tyrosine phosphatases. However, recently a newly identified group of proteins have been demonstrated to function as dual specific phosphatases, showing activity towards both phosphoserine/threonine and -tyrosine residues. The prototype for this new subfamily of phosphatases is the vaccinia virus protein VH1 (Guan *et al.*, 1991), and interestingly, all the novel proteins which have been identified in this group show sequence homology to the protein tyrosine phosphatases across the PTPase catalytic motif: (I/V)HCXAGXGR(S/T)G.

6.1. Sequence analysis of novel dual specific phosphatases

The results presented in this thesis describe the isolation of further members of this subfamily of dual specific phosphatases, and specifically the characterisation of one protein, TYP 1 (threonine-tyrosine phosphatase 1). Using the technique of degenerative PCR with primers designed across two conserved regions found in the dual specific phosphatase genes CL100 (Keyse and Emslie, 1992), and PAC-1 (Rohan *et al.*, 1993), related DNA sequences were amplified from the squamous cell line A431. Sub-cloning and sequencing resulted in the identification of five groups of clones that show sequence homology to the PTPase catalytic motif. This would suggest that the clones are phosphatase genes, and indeed PCR group A was identified as CL100. The remaining four clones proved to be novel sequences, and of these, the two most frequently isolated sequences were used as a mixed hybridisation probe to screen two cDNA libraries. This resulted in the identification of five cDNA clones which show sequence homology both to the PTPase catalytic motif, and a region conserved only in the CL100-like phosphatases, see figure 3.2. As such, it is proposed that these genes encode proteins with similar functions to CL100, and have therefore

been designated threonine/tyrosine phosphatases (TYP 1-5). The three clones isolated from the liver cDNA library range between 1-2.4 kb in length, the smaller of which possibly represent only partial cDNA clones. The two largest clones were isolated from the brain cDNA library, and are more than 2.7 kb in length. With the knowledge that CL100 mRNA is approximately 2 kb long, it was reasonable to assume that these two clones were more than likely full length. Efforts were concentrated on obtaining full sequence data from these clones, TYP 1 and TYP 2. Full sequence analysis of TYP 1 was completed, and demonstrates that TYP 1 encodes a 394 amino acid, non-transmembrane protein, figure 3.4. Although a great deal of sequence information was collected for TYP 2, an open reading frame running through the entire sequence could not be determined. The occurrence of stop codons throughout the sequence leads to the conclusion that the TYP 2 clone is in fact a pseudogene, a non-functional sequence which is highly homologous to the normal gene. There is no strong selective pressure to eliminate pseudogenes, since at least one copy of the functional gene remains in the genome. However, over time the pseudogene accumulates additional mutations. Consequently, only some regions of the pseudogene are recognised as "normal". However, the PTPase domain and a region upstream of this, which is highly conserved in the CL100-like phosphatases, are clearly present in TYP 2, figure 3.2. Other regions in the sequence of TYP 2 also show similarity, but are not identical to the CL100 gene, therefore suggesting that TYP 2 is a novel gene belonging to the class of dual specific phosphatases. Since the sequence of TYP 2 could be identified as homologous to, but distinct from CL100, the regulation of expression of TYP 2 was investigated by northern analysis. A specific probe for TYP 2 was chosen from the 5' end of the gene, as this region demonstrates near sequence homology to CL100 without including the PTPase motif. As can be seen from the northern analysis, TYP 2 is not hybridising to the CL100 transcript, confirming that there is presumably, a novel functional gene represented by TYP 2.

At the time of isolation, the clones TYP 1-5, were used to search the Genbank/EMBL database, and whilst showing significant homology to conserved regions in CL100 and PAC-1, and the PTPase domain of protein tyrosine

phosphatases, they proved to be novel sequences. The full length of TYP 1 exhibits the highest degree of homology to CL100, 62.8%, confirming that this gene is most likely a member of the dual specific phosphatases rather than the tyrosine phosphatases. More recently, a number of research groups have identified further members of this novel subfamily of phosphatases, and at present the mammalian genes include B23 (Ishibashi *et al.*, 1994), HVH3 (Kwak and Dixon, 1995), HVH2 (Guan and Butch, 1995), and MKP-2 (Misra-Press *et al.*, 1995). TYP 5, which was isolated from the liver cDNA library, shows sequence identity with HVH3. In addition, HVH2, which was cloned from human placenta, shows sequence identity with TYP 1. Misra-Press and colleagues (1995) have also reported the cloning of MKP-2 from PC12 cells which, excluding a few amino acid changes, is extremely homologous to TYP 1 and HVH2. Interestingly the mRNA transcript of MKP-2 is 6.0 kb, and could represent the larger mRNA species which Guan and Butch (1995) identify during northern analysis, using an HVH2 probe. Additionally, expression studies performed in this thesis using a probe for TYP 1, also identify an approximate 7.0 kb transcript, which presumably represents this same larger species of mRNA.

A common evolutionary origin of this family is demonstrated by the presence within each isoform of the PTPase motif, a defining feature of the protein tyrosine phosphatases. The dual specific phosphatases are also highly conserved between species, having been identified in vertebrates, yeast, and baculo- and orthopox viruses, indicating that these phosphatases play an important regulatory role within the cell. Indeed, the dual specific phosphatase Cdc25, is implicated in cell cycle control by virtue of its interaction with the cyclin-dependent kinases, and more recently Galaktionov and co-workers (1995b), have suggested that Cdc25 may function as an oncogene. Two domains present in Cdc25 known as the CH2 domains are also present in TYP 1, and the CL100-like phosphatases. It is thought that these domains are involved in substrate specificity *in vivo*, although presumably they do not confer the property of dual specificity, since the VH1 protein demonstrates activity towards tyrosine and serine residues, but lacks these CH2 domains.

6.2. Regulation of TYP 1 and TYP 2 expression

Analysis of the regulation of TYP 1 and CL100 mRNA levels in squamous cells, demonstrated a difference in expression between CL100 and TYP 1. Whilst CL100 and TYP 2 are significantly induced by 30 minutes upon mitogenic exposure of the squamous cell line A431, TYP 1 mRNA expression is not maximal until 4 hours following mitogen stimulation, figure 4.4. This difference in induction is also seen when quiescent SCC12F squamous cells are stimulated with serum. Although in SCC12F cells, TYP 1 expression is maximal at 2 hours, as opposed to 4 hours as seen in A431 cells. However, the TYP 1 transcript remains up-regulated for a longer period of time in SCC12F cells. Indeed, by 8 hours, when both CL100 and TYP 2 expression have returned to basal levels, TYP 1 mRNA is still induced, figure 4.5. Thus, under all conditions tested here, the temporal difference in mRNA expression between TYP 1 and CL100 is maintained. Interestingly, Guan and Butch (1995) find that HVH2, which is identical to TYP 1, is not significantly induced in a liver cell line upon stimulation with EGF. However, in these experiments cells were treated with EGF for 1 hour, which is perhaps too short a period for TYP 1 induction. Evidence presented here would suggest that TYP 1 does not behave as an immediate early gene, unlike TYP 2, CL100 and other family members identified so far. The late functioning of TYP 1 is further substantiated by the finding that protein expression is not maximal until 8 hours following mitogenic stimulation, and in fact is not detected at all until 4 hours after stimulation, figure 5.7, consistent with the induction of mRNA expression.

A further difference between TYP 1 and previously isolated CL100-like phosphatases, is that TYP 1 is not induced by heat shock. Thus, it would seem that the dual specific phosphatase genes are differentially regulated, demonstrating varying kinetics in mRNA expression. This is clearly shown with TYP 1, and also the phosphatase HVH3, which behaves as an immediate early gene being induced by 30 minutes, but is up-regulated for 6 hours (Kwak and Dixon, 1995). Presumably the discriminate regulation demonstrated by the dual specific phosphatases will impart

unique roles on their function, reflecting their co-expression with specific substrates, such as the particular isoforms of the MAP kinase family. Indeed, evidence presented here demonstrates that the dual specific phosphatases are differentially regulated in response to cellular stress, mitogens such as EGF, and the growth factor TGFβ, suggesting these genes may have distinct roles in separate signal transduction pathways. Although physiologically, as recognised with MAP kinase regulation, it is almost impossible to define "separate pathways" in signal transduction. Rather, there is a co-ordination of "cross-talk" between the signalling cascades.

It is becoming evident that these phosphatases are not only differentially regulated as a consequence of specific stimuli, but they are also expressed in a tissue specific manner. Results in this thesis demonstrate that TYP 1 expression appears to be both tissue and cell-type specific. In particular, TYP 1 is not expressed in human fibroblasts, even upon mitogenic stimulation, figure 4.7. Whilst both TYP 1 and 2 are highly expressed in placenta, CL100 appears to be equally expressed in placenta, lung, liver and skeletal muscle. It was also noticed that TYP 1 mRNA was expressed at a level significantly lower than CL100 and TYP 2. As previously mentioned, PAC-1 is specifically hematopoietic (Rohan *et al.*, 1993), whilst MKP-2 is strongly expressed in the central nervous system (Misra-Press *et al.*, 1995). Interestingly, ERK3 tissue expression is restricted to neuronal cells (L'Allemain, 1994). These subtle differences will undoubtedly be important in determining the precise function and specificity of these phosphatases within the cell.

6.3. The involvement of TYP 1 in the MAP kinase cascade

It has been established from both *in vitro* and *in vivo* experiments that the dual specific phosphatases specifically dephosphorylate and inactivate the extracellular-regulated MAP kinases (Alessi *et al.*, 1993a; Sun *et al.*, 1993; Zheng and Guan, 1993; Ishibashi *et al.*, 1994; Ward *et al.*, 1994; Kwak and Dixon, 1995, Guan and Butch, 1995). Using a transient expression system, results presented in this thesis demonstrate that TYP 1 is capable of inhibiting both growth factor-stimulated ERK2

activity, and heat shock-induced p54JNK activity, figures 5.1. and 5.2. In these experiments, TYP 1 activity is comparable and equivalent to CL100. In spite of TYP 1 and CL100 being over expressed, these observations strongly suggest that TYP 1 is likely to be a component of the MAP kinase signal transduction pathways. This is further substantiated by the findings that TYP 1 directly inactivates and dephosphorylates ERK2 in a concentration dependent manner, and that this is due to the concomitant dephosphorylation of both the threonine and tyrosine residues, which are phosphorylated by MAPK kinase, figures 5.3. and 5.4. These results are in agreement with previous studies that other phosphatase family members also demonstrate substrate specificity towards the MAP kinase isoforms. Such substrate specificity is further demonstrated by the fact that CL100 fails to dephosphorylate and activate $p34^{cdc2}$, the cyclin dependent kinase which is the *in vivo* substrate for Cdc25, although both Cdc25 and CL100 share sequence identity across the catalytic domain (Alessi *et al.*, 1993a). It would be interesting to investigate whether TYP 1 demonstrates activity towards other substrates, such as $p34^{cdc2}$.

It was observed that TYP 1 appears to dephosphorylate the phosphotyrosine residue more efficiently than the phosphothreonine, as demonstrated in the phosphoamino acid analysis, figure 5.4b. This preferential dephosphorylation of the tyrosine residue over the threonine has also been confirmed by studies with PAC-1 (Ward *et al.*, 1994) and HVH2 (Guan and Butch, 1995). Despite the growing number of dual specific phosphates identified, little is known about the biochemical mechanisms employed by this family of enzymes. Denu and co-workers (1995) investigated the catalytic properties of the dual specific phosphatases, using the prototypic human VHR protein. In order to explore substrate specificity, diphosphorylated peptides were used as substrates. This is because phosphorylation of MAP kinase by MAPK kinase is not stoichiometric on both the tyrosine and threonine residues, thus making comparative determination of the rates of dephosphorylation ambiguous. Their results confirm that phosphothreonine hydrolysis is approximately three orders of magnitude slower than the hydrolysis of phosphotyrosine. Perhaps this is not so surprising, since the dual specific phosphatases contain the PTPase catalytic

motif. This raises questions as to the mechanism that confers the function of dual specificty on these phosphatases.

It will be interesting to explore whether the potentially different MAP kinase proteins of a cell regulate their own specific phosphatase for their inactivation. Indeed, the question of specificity of these phosphatases for the various isoforms of MAP kinase still remains. It is worth noting that CL100 was first isolated as a stress-inducible gene, and *in vitro*, CL100 is able to dephosphorylate and inactivate a stress-induced MAP kinase, p38 (Rouse *et al.*, 1994). In some cell-types, the physiological targets of CL100 may not be the classical ERK1 and ERK2 isoforms. This may explain why in PC12 cells, CL100 induction is not accompanied by the inactivation of ERK2 (Alessi *et al.*, 1995). It is also demonstrated here that TYP 1 can alter the phosphorylation pattern of c-Jun, figure 5.5. Although, these translation experiments do not distinguish whether TYP 1 dephosphorylates c-Jun directly, or inactivates a kinase present in the reticulocyte lysate. It is possible that TYP 1 activates a serine/threonine phosphatase such as PP2A, which subsequently dephosphorylates c-Jun. In this regard, it has been reported that PP2A is inhibited *in vitro* by the phosphorylation of a carboxy-terminal tyrosine residue, catalysed by receptor protein tyrosine kinases, or *v-src*. Furthermore, PP2A is phosphorylated at this residue *in vivo* (Chen *et al.*, 1994c). Could PP2A be activated by removal of this inhibitory tyrosine residue by TYP 1? Whether PP2A is responsible for dephosphorylating c-Jun could be investigated through the use of specific phosphatase inhibitors, such as okadaic acid. However, transient transfection studies show that TYP 1 inactivates the stress-induced MAP kinase, p54JNK, suggesting that the observed inhibition of c-Jun phosphorylation maybe mediated through the inactivation of p54JNK, a kinase which phosphorylates c-Jun at the amino-terminus. Since TYP 1 is not induced upon heat shock, this is most likely acting through the mitogen stimulated pathway. Although other stimulants of cellular stress which may activate TYP 1 are yet to be investigated, such as protein synthesis inhibitors, UV irradiation and inflammatory cytokines.

As discussed in the introduction, there is now evidence for parallel MAP kinase cascades, and numerous studies support the contention that at least two signal transduction cascades exist in mammalian cells, that is the stress-related responsive MAP kinases and the mitogenic-mediated MAP kinase response. Whilst there is considerable cross-talk between the components of these MAP kinase pathways, it is anticipated that the dual specific phosphatases will be able to distinguish between the MAP kinase proteins, enabling the differential regulation of these enzymes through the existence of both specific MAP kinase kinases and MAP kinase phosphatases. The possibility of distinct phosphatase targets is perhaps highlighted by the variant tissue and cell-type specificity observed between the immediate early CL100-like genes and TYP 1. Additionally, it has been proposed that the temporal activation of MAP kinases is sufficient for cells to enact proliferative or differentiation responses (Cowley *et al.*, 1994). Therefore, it is possible that the TYP 1 protein may play a more significant role in the regulation of MAP kinase activity during cell differentiation. In these situations, MAP kinase activity is sustained for several hours, concordant with the delayed kinetics of the TYP 1 protein, as opposed to the immediate early induction of the CL100-like phosphatases. It can be envisaged the latter phosphatases are perhaps more involved in regulating the transient activation of MAPK, observed during cell proliferation. The results in this thesis demonstrate that TYP 1 readily inhibits EGF-induced ERK2 activity, and heat shock-induced p54JNK activity. Similar comparisons of MKP-1 activity towards MAP kinase isoforms have been investigated, and have demonstrated that whilst MKP-1 has the capacity to efficiently inactivate ERK2 activity, UV-induced JNK1 activity is largely unaffected (Sun *et al.*, 1994). This data indicates that there is a degree of selectivity of MKP-1 for ERK2. However, cell-type-specific differences also exist in the relative regulation of the MAP kinase pathways, and as such, studies by Zinck *et al.* (1995), suggest that MKP-1 is more efficient towards the stress-activated MAP kinases. Zinck and co-workers demonstrate that in Hela cells, under normal conditions, SAPK activity is down-regulated within 60 minutes, however full inhibition of protein synthesis by treatment with cycloheximide blocks the down-regulation of SAPKs, suggesting that the component

responsible for SAPK inactivation seems to be newly synthesised. Conversely, efficient down-regulation of ERKs is seen within 60 minutes irrespective of the status of cellular protein synthesis. This indicates that the components involved in ERK down-regulation are probably pre-existing and do not require *de novo* synthesis. Concordant with this, the rapid down-regulation of ERK activity has been ascribed to the inactivation of Raf-1 by protein kinase A (Cook and McCormick, 1993; Hafner *et al.*, 1994). Furthermore, as discussed in section 1.8, ERK2 inactivation in some cell types seems to depend on protein phosphatase 2A and another unidentified tyrosine phosphatase (Alessi *et al.*, 1995). Hence, the observations by Zinck *et al.* (1995), would indicate that SAPKs require protein synthesis for down-regulation, raising the possibility that *in vivo*, the dual specific phosphatases such as MKP-1, act more efficiently on SAPKs than on ERKs. However, it is worth noting that the immediate early genes CL100 and 3CH134 are actually super-induced within the first hour of cycloheximide treatment (Sun *et al.*, 1993; Kwak *et al.*, 1994), and it is only after prolonged treatment that CL100 protein synthesis is repressed, since CL100 protein has a half-life of 40-60 minutes (Alessi *et al.*, 1995). Interestingly, Kwak and Dixon (1995) report that regulation of HVH3 during hepatoma cell stimulation differs from CL100 in that it has a component that is dependent on protein synthesis, as cycloheximide addition does not enhance, but partially blocks the induction of HVH3 mRNA.

Activation of the MAP kinase signal transduction cascade subsequently results in the expression of several immediate early genes. As expected, this rapid rise in tyrosine phosphorylation needs to return to normal levels in order to avoid abnormal growth. It is not by coincidence that the stimulation of MAP kinases by mitogens and cellular stress also leads to the induction of the dual specific phosphatases, which specifically inactivate MAP kinase isoforms, and whose patterns of induction are consistent with them playing a role in mitogenic cell cycle progression. However, a fine balance between the activities of the MAP kinases, and the activities of the dual specific phosphatases is crucial, if the same stimuli are to induce and activate both enzymes. Perhaps the maintenance of such an antagonistic system is aided through the

activities of post-translational modification. Considering that so many kinases are themselves regulated by phosphorylation, such as the MAP kinases, it is entirely feasible that the dual specific phosphatases may also be regulated in a similar manner. There is accumulating evidence for the regulation of protein tyrosine phosphatases by phosphorylation. For example, the stimulation of cells transiently expressing RPTPα with TPA, a direct activator of protein kinase C, increases RPTPα serine phosphorylation, concomitant with an enhancement in PTPase activity (den Hertog *et al.*, 1995). The finding of putative MAP kinase phosphorylation sites at the carboxy-terminus of TYP 1, suggests that MAP kinase may itself regulate the activity of the TYP 1 protein. It is interesting that EGF leads to the stabilisation of the protein, increasing its half-life from approximately 1 hour, to more than 3 hours, figure 5.9. Although extremely hypothetical, it is possible that the TYP 1 protein is indirectly stabilised by EGF, as a consequence of EGF stimulating MAP kinase, which in turn functions to phosphorylate TYP 1. It will be exciting to investigate whether MAP kinase does indeed phosphorylate TYP 1, and whether this modification alters the activity of the TYP 1 protein. Krautwald and colleagues (1995) have observed that the stable transfection of activated *raf* in a macrophage cell line suppresses MAP kinase activation, and furthermore, this correlates with the constitutive expression of a number of immediate early genes, including the MAP kinase phosphatase, MKP-1. Northern blot analysis demonstrated that MKP-1 was constitutively expressed at low levels in v-Raf-expressing macrophages, and CSF-1-induced expression of MKP-1 persisted for longer in these cells compared to the parental macrophages. Importantly, a revertant cell line does not express detectable levels of MKP-1 mRNA, and MAPK is activated as normal. This raises the intriguing possibility that in macrophages, Raf may be feeding-back on the MAP kinase pathway by participating in the control of MKP-1 expression.

The kinetics of activation and inactivation of MAP kinases appears to be cell type-specific. As discussed in the introduction in section 1.8, it has been demonstrated that the rapid inactivation of MAP kinase in several cell-types is not catalysed by CL100 (Alessi *et al.*, 1995; Wu *et al.*, 1994). In these situations, CL100 is not induced

until after MAP kinase is completely inactivated, and such a transient activation of MAP kinase does not lead to translocation to the nucleus, where CL100-like protein phosphatases have been localised. As previously mentioned, the proposed model for MAP kinase inactivation in the cytoplasm is through PP2A and an as yet uncharacterised PTP, although such a PTP has been purified and characterised from *Xenopus* (Sarcevic *et al.*, 1993). The potential to dephosphorylate just one residue by class specific phosphatases, as in the above model, is sufficient to inactivate MAP kinase, and allows the possibility of the rapid reactivation of this enzyme, maybe through a route independent of MAPK kinase. In support of this, in certain cell-types MAP kinase activity is biphasic, suggesting that there is a reactivation of MAP kinase (Seth *et al.*, 1992). It has also been demonstrated that MAP kinase activation in chromaffin and endothelial cells, after certain stimuli, is sustained for at least 2 hours with a slow inactivation of up to 6 hours, despite an increase in CL100-like activity after 1 hour. This may be due to a possible regulation exerted on the dual specific phosphatases by MAP kinase through the MAPK phosphorylation sites in the carboxy-terminal of these phosphatases, or indeed, through the possible interaction with a specific phosphatase inhibitor. However, in such a situation the delayed inactivation of MAP kinase may be due to TYP 1, or a similarly late-induced phosphatase, since results presented in this thesis demonstrate that TYP 1 mRNA and protein expression is not maximal until 4 and 8 hours respectively, by which time the immediate early CL100-like phosphatases have returned to basal levels. Furthermore, it is observed that the mitogen, EGF, stabilises the TYP 1 protein in transfected Cos cells. In addition, it is also shown that TYP 1 expression is nuclear, figure 5.10, as is MAP kinase following sustained activation.

MAPK kinase is a cytosolic protein (Lenormand *et al.*, 1993), therefore, MAP kinase activation must be a cytosolic event. However, the site of MAP kinase inactivation in some instances must occur in the nucleus, since this is where the dual specific phosphatases are localised. The mechanism by which MAP kinase enters the nucleus is not understood, as the gene does not contain a classical nuclear localisation signal. Whether MAP kinase relies on a chaperone molecule is not clear. It has been

demonstrated that a single point mutation in the catalytic domain of MKP-1, Cys-258-Ser, is sufficient to exclude MKP-1 from the nucleus (Brondello *et al.*, 1995). This mutant MKP-1CS, although less potent than wild-type MKP-1, was able to partially inhibit MAP kinase sensitive gene induction, presumably through retarding the translocation of MAP kinase to the nucleus. It has also been shown that MKP-1CS forms a physical complex with the phosphorylated form of MAP kinase, and augments MAP kinase phosphorylation. However, the activity of MAP kinase was not tested under these conditions (Sun *et al.*, 1993). Thus, mutants of MKP-1 are able to associate with MAP kinase, and although MAP kinase may still undergo phosphorylation by MAPK kinase, the activity of MAP kinase is abrogated, by virtue of the fact that MKP-1CS remains cytosolic, thus preventing MAP kinase from activating transcription factors in the nucleus. Additionally, a mammalian mutant of MAP kinase, analogous to the dominant gain of function mutation in *Drosophila* known as sevenmaker (rl^{sem}), shows an increased sensitivity to low levels of signalling *in vivo*. However, this mutation does not lead to an elevated basal kinase activity, but rather demonstrates a decreased sensitivity to CL100 (Bott *et al.*, 1994). In relation to the above studies, the mutant MAPK may be less sensitive to CL100 due to an alteration in the sub-cellular localisation of the protein compared to the wild-type, i.e. an inhibition of translocation to the nucleus. Thus, the compartmentalisation of these kinases and phosphatases will play an important role in determining their regulation of activation and substrate availability. It is intriguing that all the dual specific phosphatases identified so far are localised to the nucleus. Whilst there is evidence that the ERK isoforms are translocated to the nucleus, it is still to be demonstrated that the JNK isoforms are definitively nuclear, even though they have been demonstrated to phosphorylate c-Jun.

The dual specific phosphatases may have some involvement in negative cell cycle progression. Results presented here demonstrate that CL100 and TYP 1 mRNA expression is significantly induced after TGFβ treatment, and that this induction is clearly sustained over 24 hours as regards TYP 1 expression. The mechanism of TGFβ signal transduction in cells remains unclear, although there is evidence that TGFβ

inhibition is not a consequence of one single pathway, but may impinge on several co-ordinated pathways, each of which can result in growth inhibition. For example, alterations are seen in the expression levels of c-Myc, CDK4, cyclin D and CDK inhibitors, and the phosphorylation status of pRb. One or more of these pathways tends to be lost in tumour cells. It has been reported that TGFβ exerts its antiproliferative effects at a level that is distal from those of receptors for growth factor-activating factors (Like and Massague, 1986). However, more recent data has shown that TGFβ addition to proliferating cultures of epithelial cells, which are sensitive to the inhibitory effects of TGFβ, show a rapid activation of MAP kinase which is sustained for at least 90 minutes, although these cells are still growth inhibited (Hartsough and Mulder, 1995). It has been reported that in some carcinoma cell lines that are sensitive to TGFβ inhibition, the early effects of TGFβ are the enhancement of the expression of both *jun* proteins (Pertovaara *et al.*, 1989), and c-Fos (Hsu *et al.*, 1994). An important property of TGFβ is its ability to activate its own mRNA expression and thereby increase its own secretion. Two distinct regions of the promoter of TGFβ are responsive to autoregulation, and in both of these regions, autoinduction is mediated by the binding of the AP-1 complex (Kim *et al.*, 1990). With regard to the observed activation of MAP kinase, in association with an inhibition of cell growth (Hartsough and Mulder, 1995), a possible explanation is that activated MAP kinase leads to the efficient phosphorylation and activation of both c-Jun and c-Fos, via the phosphorylation of transcription factors, see section 1.5.2.1. The subsequent AP-1-dependent gene transcription ultimately leads to the autoinduction of TGFβ. In cell types that have the potential to activate the latent, secreted TGFβ, the growth inhibitory effects of TGFβ would be amplified. The overlap between sets of genes stimulated by TGFβ and mitogens is interesting. Through *jun* and *fos* families of proteins, their complexes, and AP-1-binding sites, both mitogens and TGFβ could act by a similar mechanism, but with different kinetics and magnitudes. Thus, evidence would suggest that cells which are sensitive to the inhibitory effects of TGFβ, are able to stimulate AP-1 induction, but these cells do not progress to the S phase of the cell cycle, as demonstrated by growth inhibition. Further evidence for the involvement of

MAP kinases in the inhibition of the cell cycle has been demonstrated in *S. cerevisiae.* The FAR1 protein, which is an inhibitor of G1 cyclin-dependent kinases, is a direct target of the MAP kinase FUS3. Indeed, phosphorylation of FAR1 induces its binding to, and consequent inhibition of the CDK-cyclin complexes (Herskowitz, 1995). In turn, it would be interesting to investigate if MAP kinases are able to activate CDK-inhibitors in a mammalian system, since these cell cycle regulators have been implicated as effectors of TGFβ-induced cell cycle arrest.

Evidence would therefore suggest that the MAP kinases not only play a significant role in cellular differentiation and proliferation, but may also be involved in an additional pathway which leads to growth inhibition, via TGFβ. The induction of TYP 1 and CL100 in response to TGFβ, in cells sensitive to the growth inhibitory effects of TGFβ, suggests a role for these phosphatases in the tight regulation of TGF β-induced MAP kinase activation. This is substantiated by the finding that expression of MKP-1 inhibits serum stimulated transcriptional events, as well as the entry into S phase (Brondello *et al.*, 1995). In a similar study, it has also been demonstrated that Ras-induced DNA synthesis is inhibited by micro-injection of MKP-1 (Sun *et al.*, 1994). It is proposed that TYP 1 plays a role in the growth inhibitory effect of TGFβ by regulating MAP kinase activity in the nucleus, such that cell cycle progression is prevented. Agonists which induce the long term induction of dual specific phosphatases, such as TGFβ, which are distinct from the overlapping mitogenic signals that induce both MAP kinases and MAP kinase phosphatases, provide a suitable mechanism for the attenuation of MAP kinase activity throughout the phases of the cell cycle.

6.4. Future prospects

Experiments are currently underway to generate cell lines stably or conditionally expressing wild-type TYP 1, to determine the long term effects of TYP 1 expression on cell proliferation or transformation. Transfections have been initiated in fibroblasts which do not express TYP 1, and in epidermal cells for which the

expression pattern of TYP 1 has been determined. The expression vector is based on pBABEneo, directed by the MLV LTR, which will ensure reasonable constitutive expression, and can be introduced either by transfection, or by infection as a retrovirus. A further expression vector that will be used is the tetracycline responsive promoter vector. This is co-transfected with a plasmid expressing the hybrid tetR-VP16 transactivator protein (bacterial tetracycline repressor fused to the activation domain of the herpes simplex virus VP16), which regulates expression of the test construct. The tight regulation afforded by this system will enable control over the level of TYP 1 expression via the concentration of tetracycline in the media. These vectors will be of considerable use if continual expression of TYP 1 proves toxic for the cells.

It will be interesting to investigate the downstream consequences of TYP 1 expression on AP-1 DNA binding activity. It has been demonstrated in our laboratory that AP-1 DNA binding activity is biphasic in growth factor stimulated fibroblasts (Hawker *et al.*, 1993). The DNA binding activity of AP-1 is regulated by phosphorylation, at least in part, by a MAPK-like activity. It will be interesting to investigate if the expression of TYP 1 effects the DNA binding capacity of AP-1 in these cells in response to mitogen activation.

Results in this thesis did not examine whether TYP 1 specifically associates with MAP kinase, as has been demonstrated for MKP-1 (Sun *et al.*, 1993). This could be judged by GST-fusion experiments, and through the use of the two hybrid yeast system, which is currently being investigated by Alan Ashworth in his laboratory.

Site-directed mutagenesis will also be used to replace the cysteine-280 with a serine in the TYP 1 catalytic domain, to create a phosphatase dead mutant. This may show a dominant negative phenotype, and if so, will allow the inhibition or enhancement of TYP 1 activity by expression of the wild-type or mutant protein. It will be interesting to determine if the mutant TYP 1 protein displays a different subcellular localisation to the wild-type, and/or alters the activity of MAP kinase.

The growth and differentiation of epidermal cells is very much dependent upon the activation of the EGF receptor. This is highlighted by the finding that most SCC

derived cell lines over-express the EGF receptor, and that inhibition of EGF receptor inhibits proliferation, and in some cases promotes differentiation (Modjtahedi *et al.*, 1993). Results in this thesis have shown that EGF and TGFβ induce TYP 1 mRNA expression. Clearly, the generation of stably transfected cell lines will prove useful in ascertaining the role of MAP kinase pathways in regulating the growth and differentiation of SCCs. It will be interesting to determine if TYP 1 expression alters the antiproliferative response of SCC cells to TGFβ, and to investigate the role of MAP kinase-like activities in the TGFβ response.

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APPENDIX

Figure A

Predicted nucleotide sequences of the five groups of clones isolated by PCR using the degenerative oligonucleotides.

Group A : 745- CAG GGT GGC CCG GTG GAA ATC CTG CCC TTT CTG
(CL100) TAC CTG GGC AGT GCG TAT CAC GCT TCC CGC AAG GAC
ATG CTG GAT GCC TTG GGC ATA ACT GCC TTG ATC AAC
GTC TCA GCC AAT TGT CCC AAC CAT TTT GAG GGT CAC
TAC CAG TAC AAG AGC ATC CCT GTG GAG GAC AAC CAC
AAG GCA GAC ATC AGC TCC TGG TTC AAC GAG GCC ATT
GAC TTC ATA GAC TCC ATC AAG AAT GCT GGA GGA AGG
GTG TTT GTC CAC TGC CAG GCA GGC -1018

Group B: GAG ATC CTT CCC TCC CTC TAC CTT GGA AGT GCC TAC
(TYP 5) CAT GCA TCC AAG TGC GAG TTC CTG GCC AAC TTG CAC
ATC ACA GCC CTG CTG AAT GTC TCC CGA CGG ACC TCC
GAG GCC TGC ATG ACC CAC CTA CAC TAC AAA TGG ATC
CCT GTG GAA GAC AGC CAC ACG GCT GAC ATT AGC TCC
CAC TTT CAA GAA GCA ATA GAC TTC ATT GAC TGT GTC
AGG GAA AAG GGA GGC AAG GTG CTG

Group C: GAG ATC CTT CCC TTC CTC TAC CAT GCT AGT GCC TAC
CAT GCT GCC CGG AGA GAC ATG CTG GAC GCC CTG GGC
ATC ACG GCT CTG TTG AAT GTC TCC TCG GAC TGC CCA
AAC CAC TTT GAA GGA CAC TAT CAG TAC AAG TGC ATC
CCA GTG GAA GAT AAC CAC AAG GCC GAC ATC AGC TCC
TGG TTC ATG GAA GCC ATA GAG TAC ATC GAT GCC GTG
AAG GAC TGC CGT GGG CGC GTG CTG

Group D: GAG GGA CCG ATA AGA TTC CTC TAT CTT CTA AAG CTT
TAC TCT CCC CGA AAA GTC CTC TAC CGC TCC TCC GCC
CGG CTC CTC GGT CTG AAG ACA CCG AGA CTC GAC CAG
ACT CGC CAA CTC CTG AAA GAC TAA

Group E: GAG ATC TTG CCC TAC CTG TTC CTG GGC AGC TGC AGT
CAC TCG TCA GAC CTG CGG GGC TGC AGG CCT GTG CGA
TCA CAG CCC GTC CTC AAC GTG TCC GCC AGC TGC CCC
AAC CAC TTT

Figure B

Predicted nucleotide sequences of the TYP cDNA clones.

TYP 1: Refer to results section.

TYP 2: GAG ATC CTT TCC TCC CTC TAC CTT GGA GGT GCT TAC CAT
GCA TCC AAG TGG AAG TTC CTC GAT AAG CTA TAT ATC ACA
GCC CAG CTG AAT GTC CCC ----- ATC CTT GTG GAA GAA GGC
CAC ATG GCT GAC ATT AGC TCT CAC TTT CAA GAA GCA ATA
GAC TTC ATT GAC TGT GTC AGA GAA AAG AAA GGC AAG GTC
CTG GTC CAC TGT GAA GCT GGG TTC TCC TGT TCA CCC ACC

TYP 3: GAG ATC TTG CCC TAC CTG TTC CTG GGC AGC TGC AGT CAC
TCG TCA GAC CTG CAG GGG CTG CAG GCC TGT GGC ATC ACA
GCC GTC CTC AAC GTG TCC GCC AGC TGC CCC AAC CAC TTT
GAG GGC CTT TTC CGC TAC AAG AGT ATC CCT GTG GAG GAC
AAC CAG ATG GTG GAG ATC AGT GCC TGG TTC CAG GAG GCC
ATA GGC TTC ATT GAC TGG GTG AAG AAC AGC GGA GGC CGG
GTG CTG

TYP 4: GCT GAC ATT AGC TCC CAC TTT CAA GAA GCA ATT GAT TTT
ATT GAC TGC GTC AGG GAA GGA GGA GGC AAG GTC CTA GTC
CAC TGT GAG GCT GGG GTC TCG AGG TCA CCC ACC ATC TGC
ATG GCG TAC CTC ATG AAG ACC AAG CAG TTC CGC CTG AAG
GAG GCC TTC GAC ATC GTC AAG CAG AGG AGG AGC GTG ATC
TCT CCC AAC TTT GGC TTT ATG

TYP 5: See Group B