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THE CROONIAN LECTURE 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease

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The reversible phosphorylation of tyrosines in proteins plays a key role in regulating many different processes in eukaryotic organisms, such as growth control, cell cycle control, differentiation, cell shape and movement, gene transcription, synaptic transmission, and insulin action. Phosphorylation of proteins is brought about by enzymes called protein-tyrosine kinases that add phosphate to specific tyrosines in target proteins; phosphate is removed from phosphorylated tyrosines by enzymes called protein-tyrosine phosphatases. Phosphorylated tyrosines are recognized by specialized binding domains on other proteins, and such interactions are used to initiate intracellular signalling pathways. Currently, more than 95 protein-tyrosine kinases and more than 55 protein-tyrosine phosphatase genes are known in *Homo sapiens*. Aberrant tyrosine phosphorylation is a hallmark of many types of cancer and other human diseases. Drugs are being developed that antagonize the responsible protein-tyrosine kinases and phosphatases in order to combat these diseases.

Keywords: phosphorylation; protein-tyrosine kinase; protein kinase; signal transduction; protein-tyrosine phosphatase

1. INTRODUCTION

The 1701 bequest which established the Croonian Lecture was to support a lecture at the Royal Society for the advancement of natural knowledge on local motion. It is therefore particularly appropriate that the 1997 Croonian lecture should be on the subject of protein kinases, since kinase is derived from the Greek κινειν, meaning to move. The enzymatic activity of protein kinases results in the 'movement' of a molecule of phosphate from a donor ATP molecule to a protein, leading to the covalent attachment of phosphate to the side chain of a protein amino acid. The existence of phosphate covalently attached to protein was hinted at over 90 years ago, and the presence of the phosphate esters of serine and threonine in proteins was reported in 1933. However, it was not until 1959 that the first enzyme able to attach phosphate to proteins, phosphorylase kinase, was identified. The discovery of the cyclic-AMP-dependent protein kinase in 1968 led to the realization that protein phosphorylation could be regulated by extracellular stimuli, in this case through the second messenger cAMP, which is generated in response to hormones like adrenalin. In the ensuing years it has become apparent that phosphorylation is the most common type of protein modification, and that it is the major mechanism for reversible regulation of protein activity and function. Indeed, it is surprising that this is the first Croonian lecture that has specifically addressed the topic of protein phosphorylation, since it has been estimated that greater than a third of all proteins in a mammalian cell can be modified by phosphorylation and that up to 5% of the genes in a vertebrate genome encode either protein kinases or phosphatases.

2. TYROSINE PHOSPHORYLATION: PAST

The role of serine and threonine phosphorylation and dephosphorylation in the regulation of protein activity became well established during the late 1960s and 1970s. However, the modification of proteins by phosphorylation of tyrosine was not discovered until 1979. Tyrosine phosphorylation was stumbled on serendipitously through the study of tumour viruses, and emerged by virtue of an enzymatic activity able to phosphorylate tyrosine *in vitro* that was found associated with three different tumour virus transforming proteins, polyomavirus middle T antigen (Eckhart *et al.* 1979), the Rous sarcoma virus (RSV) Src protein (Hunter & Sefton 1980), and the Abelson leukaemia virus Abl protein (Witte *et al.* 1980). The association of this activity with viral transforming proteins immediately suggested that tyrosine phosphorylation might play a role in the unregulated growth and morphological changes induced by these proteins in tumour cells. This idea was quickly bolstered by the discovery that the receptors for epidermal growth factor (EGF) (Ushiro & Cohen 1980), platelet-derived growth factor (PDGF) (Ek *et al.* 1982) and insulin (Kasuga *et al.* 1981), all have protein-tyrosine kinase (PTK) activity that is stimulated by binding of their cognate ligands. The PTK activity of v-Src was proved to be intrinsic, and c-Src, the product of the cellular gene that gave rise to the

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v-*src* gene, was also shown to have PTK activity (Collett *et al.* 1980; Hunter & Sefton 1980). Mutants of RSV that are temperature sensitive for transformation were found to have temperature sensitive v-Src PTK activity *in vivo*, thus providing a strong correlation between PTK activity and transforming ability (Sefton *et al.* 1980). Subsequently, site-directed mutagenesis of a key lysine residue in the v-Src catalytic domain proved that PTK activity is essential for transformation (Snyder *et al.* 1985; Kamps & Sefton 1986).

The overall level of phosphotyrosine (P.Tyr) (*ca.* 0.05%) in proteins in normal vertebrate cells is very low compared to the levels of phosphoserine (P.Ser) (*ca.* 90%) and phosphothreonine (P.Thr) (*ca.* 10%) (Hunter & Sefton 1980). This low level of P.Tyr in part accounts for the earlier failure to detect tyrosine phosphorylation by analysis of whole cell proteins. Following the development of new methods to study tyrosine phosphorylation, it was shown that there is a significant increase in phosphorylation of cellular proteins on tyrosine in cells transformed by RSV (Hunter & Sefton 1980; Sefton *et al.* 1980) or Ab-MuLV (Sefton *et al.* 1981*b*) as well as in cells stimulated by EGF (Hunter & Cooper 1981) or PDGF (Cooper *et al.* 1982). v-Src itself and a 50 kDa v-Src associated cellular protein were found to be phosphorylated on tyrosine *in vivo*. Subsequently, a number of P.Tyr-containing proteins were identified in cells transformed by oncogenic PTKs and in cells treated with growth factors whose receptors are PTKs (Erikson & Erikson 1980; Radke *et al.* 1980; Cooper & Hunter 1981; Sefton *et al.* 1981*a*). Serine and threonine phosphorylation were known to regulate protein activity, and therefore one could predict that tyrosine phosphorylation would do the same. However, it took some time before tyrosine phosphorylation was demonstrated to regulate protein activity. The first example was c-Src, which was shown to be negatively regulated through phosphorylation of a Tyr very close to the C-terminus (Courtneidge 1985; Cooper *et al.* 1986; Cooper & King 1986). This Tyr is deleted from v-Src, thus precluding negative regulation of v-Src PTK activity, and leading to constitutive enzymatic activation, which accounts for its transforming activity (Hunter 1987*a*). The second example was phospholipase C-γ (PLCγ), an enzyme which hydrolyses phosphatidylinositol bisphosphate thus generating two second messengers. PLCγ is phosphorylated on tyrosine in response to stimulation with growth factors such as PDGF that activate receptor PTKs (Margolis *et al.* 1989; Meisenhelder *et al.* 1989), and this leads to an increase in its catalytic activity (Nishibe *et al.* 1990; Goldschmidt-Clermont *et al.* 1991; Kim *et al.* 1991). Thus, in the first decade following the discovery of tyrosine phosphorylation its importance in the regulation of cell growth and in oncogenic transformation was well established.

3. TYROSINE PHOSPHORYLATION: PRESENT

An enormous amount of progress has been made in the 18 years since the discovery of tyrosine phosphorylation. The events that I consider to be milestones are listed in table 1. One of the most important events was the discovery that the PTKs and the protein-serine kinases are related and together constitute the eukaryotic protein kinase superfamily. The protein-tyrosine phosphatases (PTP), on the other hand, form a family of enzymes that is unrelated to the major protein-serine phosphatase families. Another breakthrough was the discovery that many substrates possess a related sequence, the Src homology 2 (SH2) domain (Sadowski *et al.* 1986), that

Table 1. *A brief history of tyrosine phosphorylation*

1979	polyoma virus middle T antigen is phosphorylated on tyrosine *in vitro*
1980	v-Src and c-Src have protein-tyrosine kinase (PTK) activity *in vitro*
	v-Src is phosphorylated on Tyr *in vivo* and elevates P.Tyr in cell proteins
	v-Abl has PTK activity
	EGF receptor has EGF-stimulated PTK activity
1981	insulin receptor has insulin-stimulated PTK activity
1982	v-Src related to cAMP-dependent protein kinase catalytic subunit
	PDGF receptor has PDGF-stimulated PTK activity
	c-Abl PTK gene is rearranged in CML
1983	v-ErbB is related to Src
	polyoma virus middle T associates with and activates c-Src
1984	v-ErbB is derived from the EGF receptor
1985	Bcr-Abl fusion occurs in chronic myelogenous leukaemia (CML)
	c-Src is negatively regulated by Tyr phosphorylation
1986	c-Src is constitutively phosphorylated at Tyr527, which is deleted in v-Src
	Neu oncoprotein is related to EGF receptor and has activating point mutation
	trk human tumour oncogene encodes a PTK
1987	c-Src negatively regulated by phosphorylation of Tyr527
	Drosophila Sevenless is a receptor PTK
1988	protein-tyrosine phosphatase (PTP) 1B purified and sequenced
	CD45 lymphocyte receptor is related to PTP1B
	acetyl choline receptor regulated by Tyr phosphorylation
1989	Cdc2 is negatively regulated by Tyr phosphorylation
	phospholipase Cγ has an SH2 domain and is a PTK substrate
1990	SH2 domains bind P.Tyr-containing proteins
	Yersinia virulence plasmid encodes an essential PTP
	activated PDGF receptor associates with PLCγ
1991	Wee1 PTK phosphorylates Cdc2; Cdc25 PTP dephosphorylates Cdc2
	Trk is the NGF receptor
	c-Src knockout causes osteopetrosis
1992	individual receptor PTK Tyr phosphorylation sites bind distinct SH2 proteins
	STAT transcription factors are activated by Tyr phosphorylation by JAK PTKs
	structure of SH2 domain bound to P.Tyr-containing peptide
1994	three-dimensional structure of PTP1B
	three-dimensional structure of insulin receptor PTK domain
1997	three-dimensional structure of c-Src in the inactive state

Table 2. *Protein-tyrosine kinase families*

non-receptor protein-tyrosine kinases

Src family	Src family-related	Abl family	Jak family
c-Src	Frk/Rak (Mkk3)	c-Abl	Jak1
c-Yes	Brk (Sik)	Arg	Jak2
Fyn	Srm		Jak3
Yrk	Sad	Fes/Fps family	Tyk2
Lck	Iyk	c-Fes/Fps	
Lyn		Fer	Fak family
c-Fgr	Btk family		Fak
Blk	Btk (Atk/Bpk/Emb)	Twin SH2 domain family	Pyk2
Hck	Itk (Tsk/Emt)	Zap 70	
	Tec	Syk	Ack family
Csk family	Bmx (Etk)		Ack
Csk (Cyl)	Txk (Rlk)		AckII
Ctk (Hyl/Matk//Ntk/ Lsk/Batk)			

receptor protein-tyrosine kinases

EGF receptor family	FGF receptor family	VEGF receptor family	Ror family
EGF receptor (c-ErbB)	FGF receptor 1 (Flg/Cek1)	VEGF receptor (Flt1)	Ror1
ErbB2 (Neu/HER2)	FGF receptor 2 (Bek/K-Sam Cek3)	VEGF receptor (Flk1/ KDR)	Ror2
ErbB3 (HER3)			
ErbB4 (HER4/Tyro2)	FGF receptor 3	Flt4	DDr family
	FGF receptor 4		DDR (Nep/Cak)
insulin receptor family		Eph family	TKT/Tyro10
insulin receptor	NGF receptor family	EphA1 (Eph)	
IGF-1 receptor	NGF receptor (Trk)	EphA2 (Eck/Sek2/Myk2)	Axl family
insulin receptor-related kinase (IRR)	BDNF/NT-3/NT-4/5 receptor(TrkB)	EphA3 (Hek/Cek4/Mek4/Tyro4)	Axl (UFO/Ark/Tyro7)
		EphA4 (Sek1/Cek8/Hek8/Tyro1)	Rse (Brt/Sky/Tif/Tyro3/ Dtk/Etk2)
c-Ros	NT-3 receptor (TrkC)	EphA5 (Cek7/Bsk/Hek7/Ehk1/Rek7)	
Ltk		EphA6 (Ehk2)	Mer (c-Eyk/Nyk/Tyr12)
Alk	PDGF receptor family	EphA7 (Hek11/Mdk1/Ehk3/Ebk)	
	PDGF receptor α(*patch* locus)	EphA8 (Eek/Ptk4)	Tie family
HGF receptor family		EphB1 (Elk/Cek6/Net)	Tie
HGF receptor (Met)	PDGF receptor β	EphB2 (Cek5/Nuk/Erk/Sek3/Tyro5/Hek5)	Tek/Tie2
Ron	CSF-1 receptor (c-Fms)	EphB3 (Hek2/Cek10/Sek4/Mdk5/Tyro6)	
c-Sea	SCF receptor (c-Kit) (*W* locus)	EphB4 (Myk1/Htk/Hek5)	Ret
		EphB5 (Cek9)	Klg
	Flk2 (Flt3)	EphB6 (Mep)	Ryk (Nyk-r/Vik)
			MuSK (Nsk2)

binds to P.Tyr in a sequence-specific manner. This led to the identification of a novel principle in signalling by protein phosphorylation (see below). On the technical side the development of specific, high affinity anti-P.Tyr antibodies provided a critical impetus to the study of tyrosine phosphorylation and the identification of PTK substrates (Ross *et al.* 1981).

With one exception all known PTKs are members of the eukaryotic protein kinase superfamily, and have a related catalytic domain. The core region of the catalytic domain contains 11 subdomains, and is about 260 residues in length. The PTKs proper are more closely related to one another in their catalytic domains than they are to the protein-serine kinases (Hanks *et al.* 1988). There are no characterized PTKs in prokaryotes, although there are scattered reports of prokaryotic PTK activities. Tyrosine phosphorylation, however, occurs in all eukaryotes. In fission and budding yeasts tyrosine phosphorylation is used for cell cycle regulation and receptor-mediated intracellular signalling pathways. However, the PTKs involved are not closely related to one another or to the PTK family proper. In evolutionary terms, true PTKs did not emerge until the appearance of metazoans. The simplest organisms in which PTK genes proper have been identified are the sponges (Ottilie *et al.* 1992; Schacke *et al.* 1994) and coelenterates (Bosch *et al.* 1989; Chan *et al.* 1994*a*), where both receptor and non-receptor type PTKs have been found. However, tyrosine phosphorylation is a common event in the protist slime mould *Dictyostelium*. Four *Dictyostelium* protein kinases that can phosphorylate tyrosine have been reported (Tan & Spudich 1990; Adler *et al.* 1996; Nuckolls *et al.* 1996). However, although they are all recognizably members of the eukaryotic protein kinase superfamily, none is classified by sequence analysis to the PTK subfamily. Strikingly, a STAT-type transcription factor has recently been identified in *Dictyostelium*, which like vertebrate STATs is regulated by tyrosine phosphorylation (Kawata *et al.* 1997) (see below). The PTK that

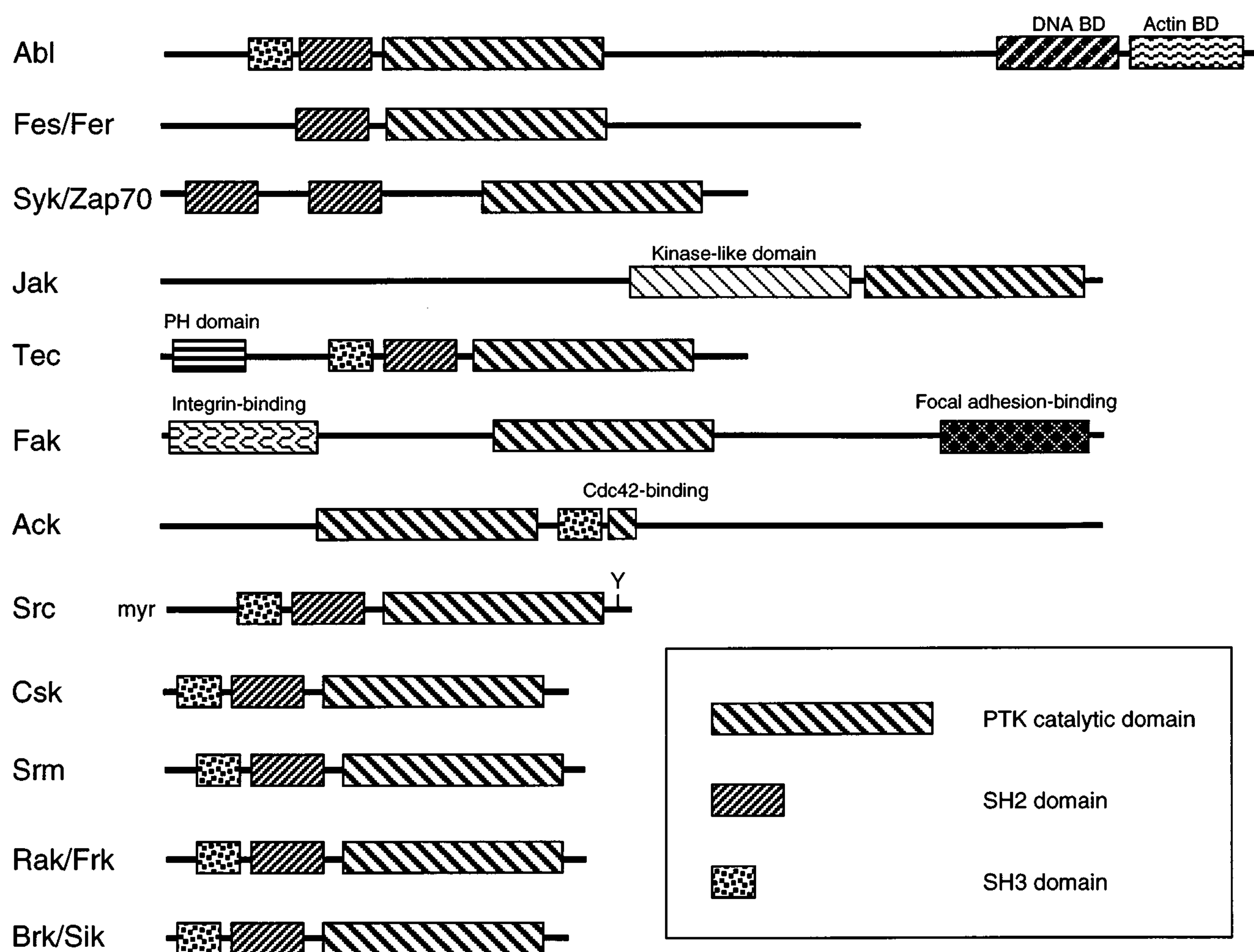

Figure 1. Schematic structures of non-receptor protein-tyrosine kinase families.

phosphorylates the Dd-STAT is unknown, but one of the recently described *Dictyostelium* PTKs has a twin catalytic domain structure reminiscent of the JAK PTK family (Adler *et al.* 1996), which are known to phosphorylate STATs in insects and higher eukaryotes. Intriguingly, the PTP family is more ancient than the PTK family, and true members of this family are present in all eukaryotes including protozoans such as the budding and fission yeasts.

Given the emergence of the PTK family with metazoans, it is no coincidence that a major function of tyrosine phosphorylation is in intercellular communication, a process for which there is limited need in protozoans. The explanation for why tyrosine phosphorylation, as opposed to serine and threonine phosphorylation, evolved as a mechanism of intercellular communication is not immediately obvious. Although P.Tyr is as stable chemically as P.Ser and P.Thr at physiological pH, the turnover numbers for PTPs are very high, and this property, in combination with the generally tight negative regulation of PTK activity, means that most PTK substrates have a very low basal level of tyrosine phosphorylation, which is a critical requirement for an inducible signalling system with maximal gain. One plausible reason for the adoption of tyrosine phosphorylation for intercellular signalling was the successful evolution of high affinity P.Tyr-binding domains, such as the SH2 domain, which recognize P.Tyr in a defined sequence context and thereby facilitate specific inducible protein–protein interactions in signalling pathways. Phosphorylated tyrosine may have been selected for this purpose because the aromatic side chain of P.Tyr affords significantly greater binding energy than the aliphatic side chain of P.Ser or P.Thr.

The existence of signature sequences in the catalytic domain that distinguish the PTKs from the protein-serine kinases has enabled the design and very successful use of PCR primers for the amplification of sequences corresponding to new PTKs. By this and other means, over 95 distinct mammalian PTK genes had been identified by the end of 1996 (table 2) (Hanks & Hunter 1995). There are two main types of PTK. The non-receptor PTKs are proteins that do not span the membrane, although many of them, like c-Src, are associated with cellular membranes (figure 1) (Neet & Hunter 1996). Most non-receptor PTKs are cytoplasmic, but a minority have a dual nuclear and cytoplasmic distribution (e.g. c-Abl). Many of the non-receptor PTKs are known to have roles in cellular signalling. In particular, members of the Src, Jak, and Fak families are directly involved in transmembrane signalling, and act as catalytic subunits of surface receptors that lack intrinsic catalytic activity.

The other major group of PTKs are the receptor PTKs (RPTKs). These are all type I membrane-spanning proteins, with large N-terminal extracellular domains

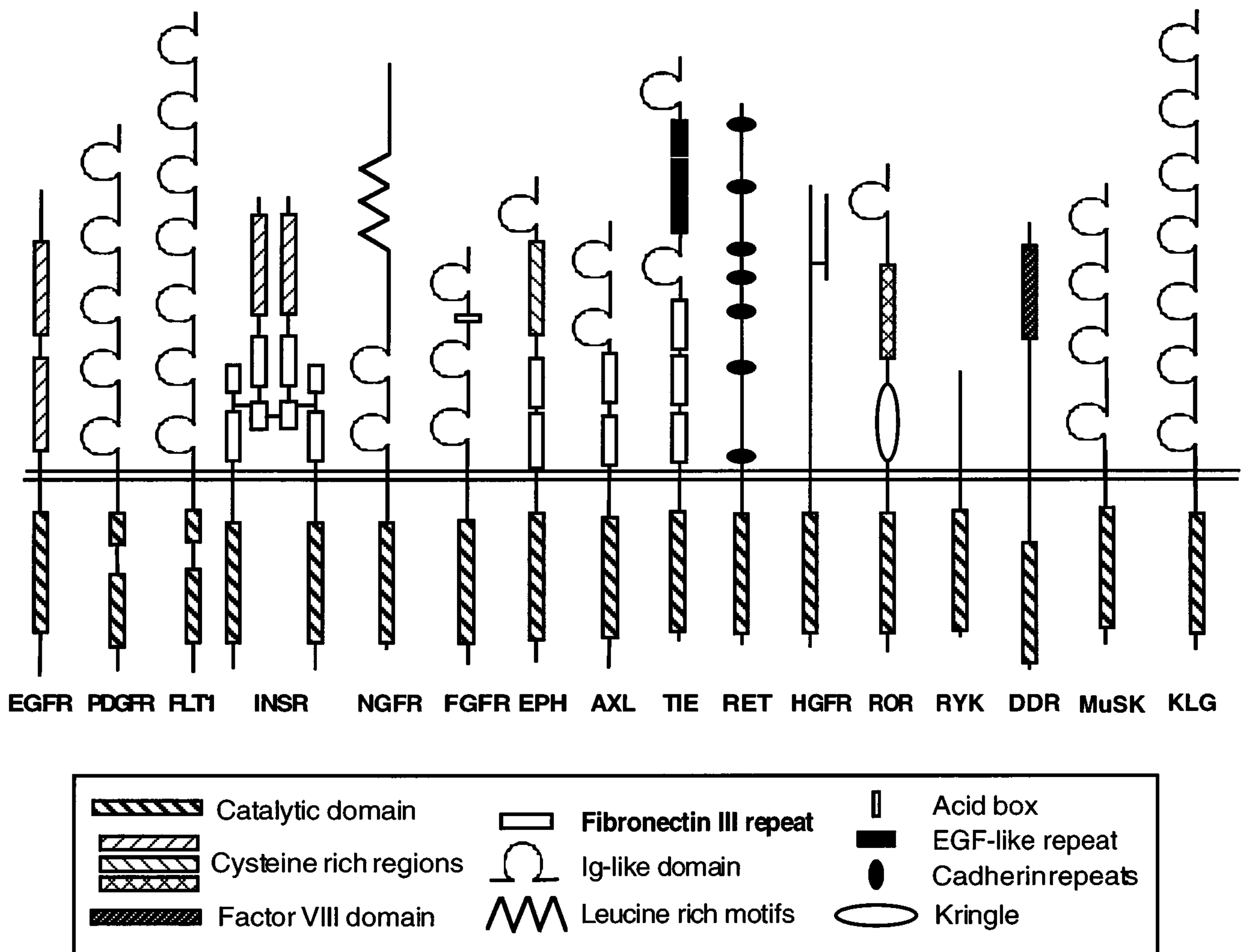

Figure 2. Schematic structures of receptor protein-tyrosine kinase families.

that act as ligand-binding sites (figure 2). Receptor PTK extracellular domains display a wide variety of structural motifs. Intracellularly, they possess a juxtamembrane domain, a canonical protein kinase catalytic domain, and a C-terminal tail. At present 16 distinct RPTK families are known, with several additional individual RPTKs that are not members of families (van der Geer *et al.* 1994). The distinction between the families is most evident in their extracellular domain structures, where each family is characterized by a specific array of structural motifs, but detailed sequence comparison shows that these relationships can be discerned even in the closely related catalytic domains of these receptors. Many of the RPTKs are growth factor receptors. However, RPTKs also play roles in differentiation, as is the case with the NGF receptor family, and in metabolic control, as is the case for the insulin receptor.

Both receptor and non-receptor PTKs have been shown to play a role in cellular responses to extracellular signals (van der Geer *et al.* 1994; Neet & Hunter 1996). Two out of the three major classes of growth factor receptor use tyrosine phosphorylation as a direct transmembrane signalling mechanism (figure 3). One class of growth factor receptors are transmembrane proteins with intrinsic PTK activity that is stimulated following ligand binding. Good examples are the EGF and PDGF receptors. The second group of receptors lack a PTK catalytic domain, but signal through association of one of the receptor subunits via its cytoplasmic domain with a non-receptor PTK (figure 3). This group, which are sometimes known as binary receptor PTKs, includes many of the cytokine receptors, and also the T-cell and B-cell antigen receptors and the F_c receptors. The Src family and the Jak family non-receptor PTKs serve as signalling subunits for these receptors. The third class of growth factor receptors are G protein-coupled receptors. Although these receptors do not use tyrosine phosphorylation as a mechanism of transmembrane signalling, it is emerging that the activation of non-receptor PTKs downstream of the activated G proteins is essential for mitogenic signalling by these receptors as well (van Biesen *et al.* 1996).

Activation of transmembrane RPTKs occurs through ligand-induced dimerization (Schlessinger 1986; Heldin 1995). The receptors normally exist as monomers, which are diffusible in the plane of the plasma membrane. Ligand binding to the extracellular domain either induces or stabilizes receptor dimer formation. Dimerization juxtaposes the catalytic domains, inducing a conformational change, which then allows mutual transphosphorylation of the two receptor molecules to occur. Autophosphorylation of one or more tyrosines in the so-called activation loop of the catalytic domain is essential for generating an active

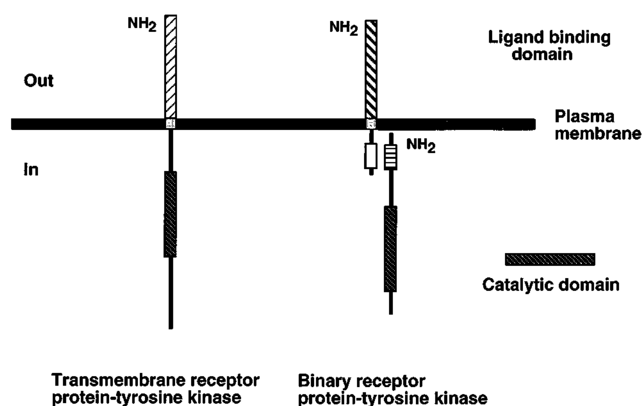

Figure 3. Mechanisms of transmembrane signalling by protein-tyrosine kinases.

dimer that is capable of phosphorylating substrates. The structural basis for RPTK activation has emerged from a comparison of the crystal structures of inactive and active, phosphorylated forms of the insulin RPTK catalytic domain (Hubbard *et al.* 1994). Phosphorylation of residues in the activation loop is necessary to anchor the activation loop to allow access of substrates and to alter the conformation of the N-terminal lobe to allow the correct orientation of catalytic residues. Autophosphorylation, however, is not only important in enzymatic activation, but also provides an essential means of substrate recruitment (see below). Topologically, the binary receptor PTKs are very similar to the transmembrane RPTKs (figure 3), and they also appear to be activated by ligand-induced oligomerization. This results in activation of the associated non-receptor PTK, and phosphorylation of receptor subunits. In both signalling systems, additional types of non-receptor PTKs may be activated secondarily adding diversity to the substrates that can be phosphorylated. In addition to the cytokine and antigen receptors, other types of receptor signal as binary receptor systems. For instance, the integrin cell adhesion receptors localized to focal adhesions associate with and signal via the Fak PTK.

To understand such signalling responses it is essential to know which proteins are targets for the activated PTK in question. An impressive number of substrates for both receptor and binary receptor PTKs has been identified over the past 18 years (van der Geer *et al.* 1994). There are four major types of substrate for signalling PTKs, namely enzymes, adaptors, docking proteins, and structural proteins (table 3).

Substrates in the enzyme and adaptor categories are comprised of a series of discrete domains (figures 4 and 5). In particular, many of these proteins have so-called Src homology 2 (SH2) and Src homology 3 (SH3) domains (Pawson 1995). The SH2 domain is a compact globular domain of about 100 residues that interacts with proteins that contain a P.Tyr embedded in a specific sequence; in general residues between 1 and 5 on the C-terminal side of the P.Tyr are recognized (Songyang *et al.* 1993). Structures of several SH2 domains have been solved together with bound phosphopeptides, and these show that the P.Tyr is buried in a deep pocket, and that the residues on the C-terminal side of the P.Tyr make specific contacts with the surface of the SH2 domain, thus defining its sequence specificity (Waksman *et al.* 1993). The c-Src

Table 3. *Substrates and targets for signalling protein-tyrosine kinases*

substrates	targets
enzymes	
PLCγ 1 and PLCγ 2 (PI-specific phospholipase)	SH2 (2), SH3, split PHD
GAP120 (Ras GTPase activator)	SH2, SH3, PHD
Src family PTKs	SH2, SH3
Zap70/Syk family PTKs	SH2 (2)
Shp1 (PTP1c/SH-PTP1/HC-PTP)	SH2 (2)
Shp2 (PTP1D/SH-PTP2/SYP)	SH2 (2)
PI-3 kinase p85 (p110 regulatory subunit)	SH2 (2), SH3
Ship (inositol polyphosphate-5′-phosphatase)	SH2
Vav (and Vav2) (Rho/Rac/Cdc42 GEF)	SH2, SH3, PHD
adaptors	
Shc (and ShcB and ShcC, Shb)	SH2, PTB
Nck	SH2, SH3 (3)
Crk (and CrkL)	SH2, SH3 (2)
Lnk	SH2
Slp76	SH2
Slap (negative regulator)	SH3, SH2
Grb2 (and Grap)	SH2, SH3 (2) (not phosphorylated)
Gab1 (binds Grb2)	novel P.Tyr-binding domain
docking proteins	
IRS1 (and IRS2)	PHD, PTB
p130Cas (and Efs, Hef1)	SH3
Cbl	novel P.Tyr-binding domain
p62Dok	PHD
FRS2	PTB (myristoylated)
structural proteins	
Annexin I (p35) and annexin II (p36)	
Ezrin (p81) and ezrin family proteins	
Vinculin (focal adhesion)	
Talin (focal adhesion)	SH2
Tensin (focal adhesion)	
Paxillin	
Zixin	
p80/85 (cortactin/EMS1)	SH3
AFAP-110 (F-actin binding protein)	
β-catenin and p120 (β-catenin-related)	
Cadherins	
Connexin43	
Caveolin	
Integrin receptor β- subunits	
others	
STAT1-5 (transcription factors)	SH2, SH3
p190 (Rho GAP)	
Grb7 (Ras GAP related)	SH2, PHD
Grb10	SH2, PHD
Eps8	SH3
Eps15 (α-adaptin/coated pit associated)	
VCP (valosin-containing protein)	
HS1 (helix-loop-helix protein)	SH3

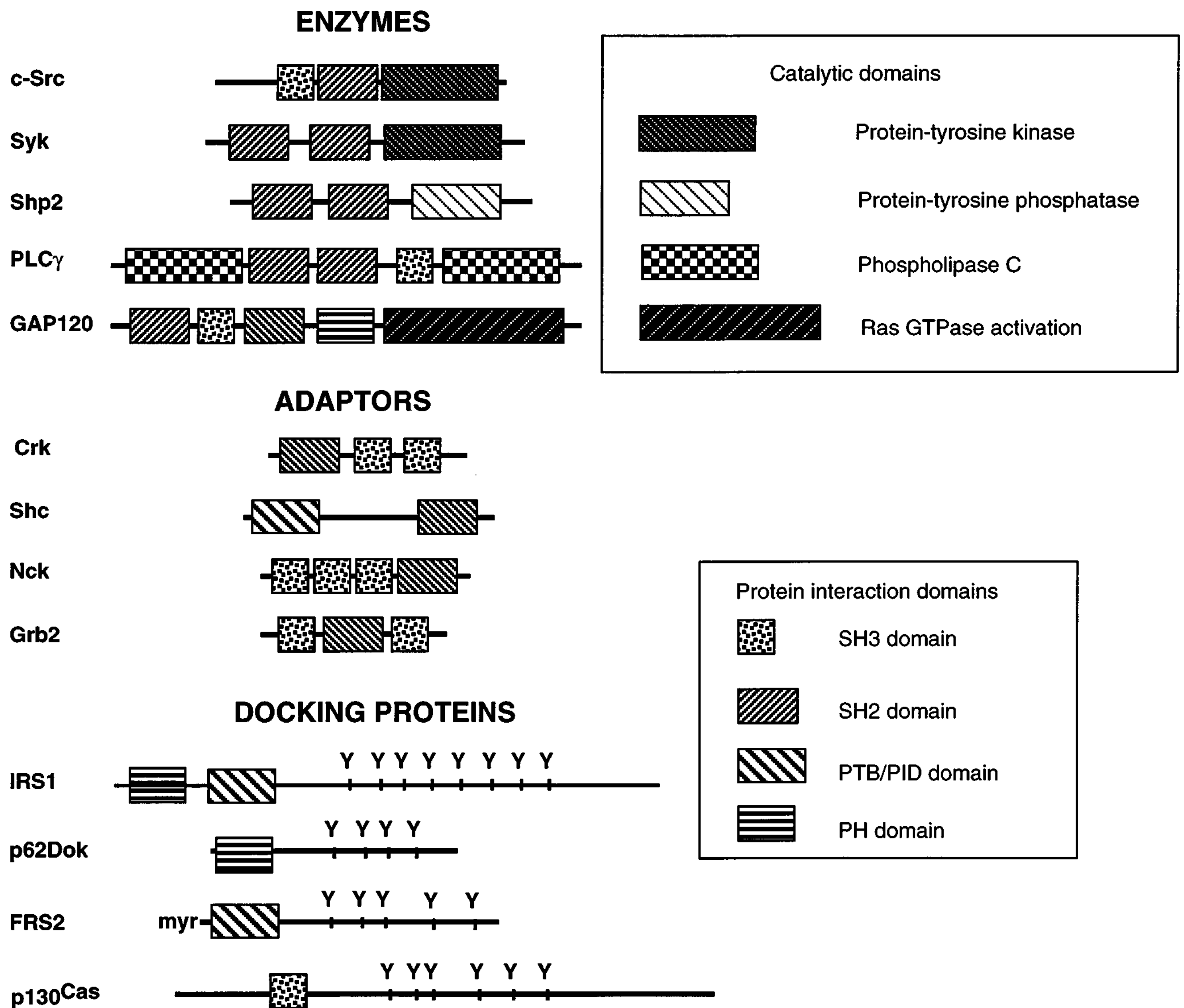

Figure 4. Schematic structures of representative enzyme and adaptor targets for signalling protein-tyrosine kinases.

SH2 domain is essential for negative regulation of its activity. As beautifully shown by the recently reported crystal structure of c-Src in its inactive state (Xu *et al.* 1997), when the C-terminal regulatory tyrosine is phosphorylated the SH2 domain is bound intramolecularly to this P.Tyr on the backside of the catalytic domain. The SH3 domain is also located on the backside of the catalytic domain being held there by an interaction of the SH3 domain ligand binding site with residues in the linker between the SH2 domain-catalytic domain, which adopts a type II polyproline helix conformation. In combination these SH2 and SH3 domain interactions promote contacts between the RT and N-Src loops of the SH3 domain and residues in the N-terminal lobe of the catalytic domain that result in the displacement of the C-helix away from the active configuration.

Recently, a second type of sequence-specific P.Tyr-binding domain has been identified, which is present in Shc and Shc family proteins and IRS1. This domain, known as PTB (phosphotyrosine-binding domain), is also a globular domain of about 150 residues, that is related in structure to the pleckstrin homology domain (Eck *et al.* 1996). In contrast to the SH2 domain, the PTB domain recognizes the sequence of residues between one and eight upstream of the P.Tyr, in the general consensus Asn.Pro.X.Tyr (Pawson 1995). Other PTB domains bind Asn.Pro.X.Tyr motifs, which form tight turns, in a phosphorylation-independent manner. Finally, there is evidence that additional types of P.Tyr-binding domain exist. For instance, the N-terminal domain of c-Cbl binds to the EGF RPTK in a P.Tyr-dependent manner (Lupher *et al.* 1996), and a proline rich domain in Gab1 binds to the tyrosine-phosphorylated bidentate docking site in the HGF RPTK (Weidner *et al.* 1996), but these domains are not related to one another nor to the SH2 or PTB domains in sequence.

The SH3 domain, which is another compact globular domain of about 60 residues, interacts with target proteins in a phosphorylation-independent, sequence-specific fashion, binding to short sequences containing a central Pro.X.X.Pro sequence (Pawson 1995). Interestingly, different protein ligands can be bound to an SH3 domain in either an N-to-C-terminal or a C-to-N-terminal orientation in a type II polyproline helix conformation. The WW domain is another small domain used in signalling

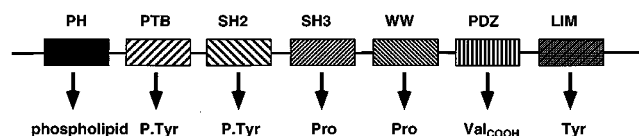

Figure 5. Binding specificities of modular protein domains involved in transmembrane signalling.

proteins, which can also interact with Pro-containing sequences, but with a different sequence specificity to SH3 domains (Sudol 1996). The pleckstrin homology (PH) domain is a globular structure of about 120 residues. PH domain structures suggest that they ought to bind ligands, but despite extensive searches no convincing protein ligands have been identified. Instead, it appears that PH domains may bind phospholipids (Lemmon *et al.* 1997). For instance, the phospholipase C-δ (PLCδ) PH domain binds specifically to inositol-1,4,5-triphosphate (IP_3), and a structure of the PLCδ PH domain bound to IP_3 shows that the 4′ and 5′ phosphates of IP_3 are specifically bound in a pocket. This pocket could also accommodate the head group of phosphatidylinositol-4,5-bisphosphate (PIP_2) in the membrane, thus allowing association of PLCδ with the cytoplasmic face of the plasma membrane via its PH domain. Thus, the best guess is that PH domains in general act as phospholipid-dependent membrane-anchoring domains, and thereby help stabilize the interaction of substrates with the membrane once they are translocated in response to receptor PTK activation (e.g. as in the case of Sos).

Docking proteins also have recognizable domains, but in addition contain many sites of tyrosine phosphorylation and their main function appears to be to act as phosphorylation-dependent docking proteins for SH2 and PTB-containing proteins, thus acting as receptor PTK surrogates. The best characterized example is IRS1, which is the major substrate for the insulin RPTK in insulin treated cells. IRS1 has a PTB domain, which allows its association with P.Tyr960 in the juxtamembrane domain of the activated insulin RPTK, and subsequent phosphorylation at multiple tyrosines. IRS1 contains over 20 potential sites of tyrosine phosphorylation that lie in SH2 binding motifs, and it has been shown to become associated with PI-3 kinase, Nck, Grb2, and Shp2 following its phosphorylation (White 1994). p62Dok and FRS2 are two recently identified docking proteins. p62Dok, which has potential PH domain, is a cytoplasmic protein that is phosphorylated by many activated PTKs, but its downstream effectors are unknown (Carpino *et al.* 1997; Yamanashi & Baltimore 1997). FRS2 is a major substrate for the activated FGF and NGF receptor PTKs (Kouhara *et al.* 1997). A unique feature of FRS2 is that it is myristoylated and is therefore permanently associated with cytoplasmic membranes. FRS2 phosphorylation leads to the recruitment of multiple molecules of Grb2 and thereby activation of the Ras/ERK MAP kinase pathway (see below). Finally, phosphorylation of structural proteins must play a role in the structural changes characteristic of transformation by oncogenic PTKs and also in response to growth factor treatment. In this connection it should be noted that some of the substrates themselves (e.g. Vav) or their effector proteins are activators of small G proteins in the Rho family, whose role is to regulate the actin cytoskeleton.

Although PTKs are often considered rather nonspecific protein kinases, different subfamilies do have distinct primary sequence substrate preferences. In particular, the Src subfamily PTKs prefer Glu.Glu.Ile/Val.Tyr.Gly.Glu. hydrophobic.Glu/Asp, whereas the EGF and PDGF RPTKs prefer Glu.Glu.Glu.Tyr.hydrophobic.X.hydrophobic (Songyang *et al.* 1995). Thus, it turns out the Src family PTKs phosphorylate sequences to which their own SH2 domains prefer to bind (P.Tyr.X.Glu.Ile), and this may allow processive phosphorylation of certain substrates with multiple phosphorylation sites. Furthermore, the preferred sequences (auto)phosphorylated by RPTKs are in contexts that are selected by the group III type SH2 domains found in enzyme and adaptor protein substrates for RPTKs. The consensus sequences for different PTKs can be useful in screening newly identified candidate signalling proteins for possible tyrosine phosphorylation sites.

Once a signalling PTK is activated, it creates a set of binding sites for SH2-containing proteins, either through autophosphorylation or through phosphorylation of an associated receptor subunit. Target proteins are recruited from the cytoplasm, where they exist in an inactive state, to the activated transmembrane RPTK or binary receptor PTK complex through binding of their SH2 domain to specific phosphorylated tyrosines, which is a high affinity interaction (Cantley *et al.* 1991; Pawson 1995). This recruitment mechanism permits several outcomes, which are by no means mutually exclusive. First, translocation to the membrane may be sufficient to initiate a signal (e.g. Grb2/Sos). Second, recruitment may be accompanied by tyrosine phosphorylation which activates (or inhibits) an intrinsic or associated enzymatic activity (e.g. PLCγ). Third, binding of the SH2 domain to its target P.Tyr may result in an allosteric effect on the target protein (e.g. PI-3 kinase).

For substrates in the enzyme category one can readily imagine how tyrosine phosphorylation could regulate activity, based on the numerous precedents where the activity of metabolic enzymes is regulated by serine phosphorylation. A good example is phospholipase Cγ, which is phosphorylated on a single tyrosine following recruitment and this results in activation (Kim *et al.* 1991), and hydrolysis of PIP_2 enerating diacylglycerol and IP_3, which are both second messengers. Diacylglycerol is an activator of the protein-serine kinase, protein kinase C, and IP_3 causes release of calcium from intracellular stores, which among other things can activate calcium/calmodulin-regulated protein-serine kinases.

Proteins in the adaptor class lack enzymatic function, and how they act is less intuitively obvious. However, it has become clear that adaptors function by bringing effector molecules to the plasma membrane. The effector molecules may act on a target at the membrane, or else they may be phosphorylated by the RPTK. A good example is Grb2. This is a highly conserved protein, and functional homologues are known in *C. elegans* and *Drosophila*. Grb2 has a central SH2 domain flanked by SH3 domains (figure 4). Grb2 is constitutively associated via its SH3 domains with an effector protein called Sos (Schlessinger 1994). Sos is a GTP-exchange factor (GEF) for Ras, a plasma membrane-bound small G protein. Sos activates Ras by exchanging GDP on inactive Ras

molecules for GTP, which allows Ras to generate a signal. Thus, following ligand binding, RPTK autophosphorylation creates a binding site for the Grb2 SH2 domain, which binds the Grb2/Sos complex. In this manner, the Grb2/Sos complex is translocated to the membrane, where the Sos PH domain may be able to interact with the plasma membrane phospholipids to stabilize membrane association. Once translocated to the membrane, the Sos catalytic domain is juxtaposed with Ras facilitating GTP exchange. Both transmembrane and binary growth factor RPTKs, and oncogenic PTKs, such as v-Src, can activate the Grb2/Sos system. The system is also activated by the NGF and insulin RPTKs, and by the integrin/Fak binary receptor system. Thus, this signalling system is used to transduce signals from extracellular stimuli that elicit a wide range of responses.

The events downstream of Ras provide a good illustration of how PTK signalling pathways are propagated. In the past few years, it has been shown that a protein kinase cascade lies downstream of Ras (figure 6) (Marshall 1994). Activated Ras binds to the regulatory domain of the protein-serine kinase Raf, thus recruiting it to the membrane where it is activated by an unknown event, which probably involves phosphorylation. Once activated, Raf phosphorylates and activates a second protein kinase, a MAP kinase kinase, called MEK. MEK is a dual-specificity protein kinase that phosphorylates its targets, ERK1 and ERK2, protein-serine kinases in the MAP kinase family, on both a threonine and a tyrosine, which lie in the activation loop of the catalytic domain. Once activated, a fraction of the ERK kinase population is translocated into the nucleus where it can phosphorylate and activate transcription factors (Hill & Treisman 1995; Karin & Hunter 1995). Thus, this constitutes a PTK signalling pathway that can be described in molecular detail all the way from the plasma membrane to the activation of gene expression.

Recently, a second type of transcytoplasmic signalling pathway has been discovered, which involves the Jak PTKs (figure 7) (Darnell *et al.* 1994; Ihle 1995; Leaman *et al.* 1996). The Jak PTKs bind to and are activated by many cytokine receptors, including the interferon receptors. Following activation, the Jak PTKs phosphorylate the cytoplasmic domain of a receptor subunit, and these P.Tyr residues act as binding sites for latent cytoplasmic transcription factors known as STATs, which contain an SH2 domain. The bound STATs are phosphorylated by the Jak PTKs, which allows them to dimerize via their SH2 domains. The dimerized STATs then translocate into the nucleus where they bind specific response elements and activate transcription. Although nuclear PTKs are known, the Jak/STAT transcytoplasmic signalling pathway provides the first example of a nuclear activity that is regulated by tyrosine phosphorylation.

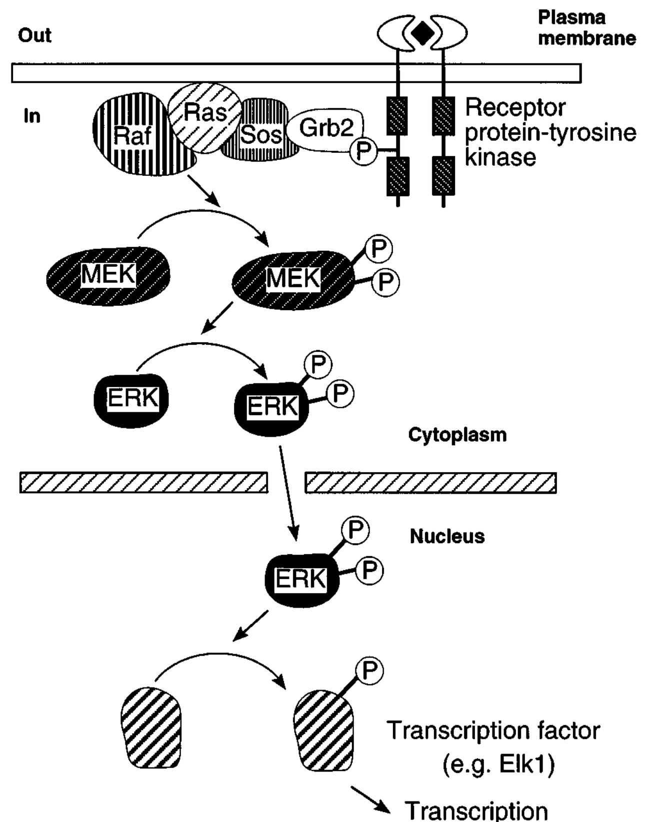

Figure 6. The Ras/MAP kinase transcytoplasmic signalling pathway. The signalling pathway initiated by an activated transmembrane RPTK at the plasma membrane is propagated by binding of Grb2/Sos to the autophosphorylated receptor, which activates Ras, which then activates the Raf protein-serine kinase. Raf phosphorylates and activates MAP kinase kinase (MEK), which phosphorylates and activates the ERK MAP kinase. Activated ERK translocates into the nucleus where it can phosphorylate and activate transcription factors leading to increased transcription of their target genes.

4. TRANSMEMBRANE SIGNAL TRANSDUCTION BY TYROSINE PHOSPHORYLATION

All of the characterized RPTKs have multiple autophosphorylation sites and when activated they bind a subset of the available SH2 (and PTB) containing proteins in the cell type in question in a manner dictated by the precise sequence around each phosphorylation site. For instance, the PDGF β RPTK can bind up to eight different SH2-containing proteins (figure 8) (van der Geer *et al.* 1994). It is not known how many SH2 domain proteins a single RPTK molecule or an activated RPTK dimer can bind simultaneously. There is evidence that two different target proteins can bind to the PDGF β RPTK, but steric constraints will presumably limit the number that can be accommodated, especially when the phosphorylation sites lie close to one another in the monomer. In principle, the signalling potential of the autophosphorylation sites in an RPTK can be defined by testing cellular responses to ligand binding in cells expressing RPTK mutants in which individual phosphorylation sites have been mutated to phenylalanine. In the case of the PDGF β RPTK, mutation of individual autophosphorylation sites has little effect on PDGF β RPTK signalling in tissue culture cells, suggesting that they may have partially redundant functions. A PDGFRβ RPTK mutant in which the five main autophosphorylation sites are mutated to Phe lacks mitogenic activity (Valius & Kazlauskas 1993). Restoration of individual phosphorylation sites in the quintuple mutant PDGFRβ RPTK indicates that the PI-3 kinase and the PLCγ binding site is each sufficient for mitogenic signalling when the mutant receptors are

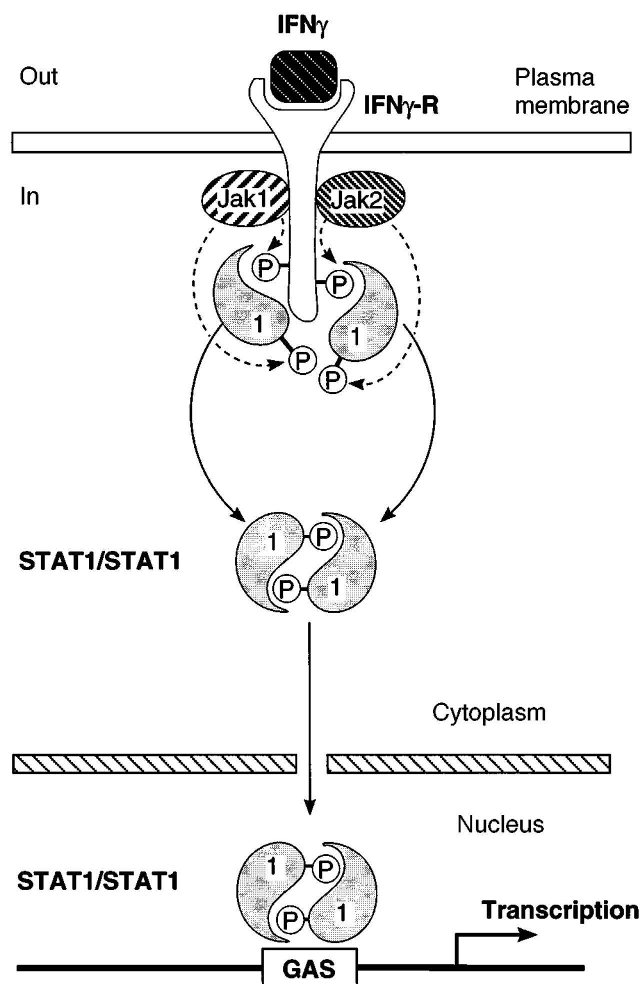

Figure 7. The Jak/STAT transcytoplasmic signalling pathway. In this example, binding of interferon γ (IFNγ) to its receptor induces oligomerization (the oligomer is shown here as a single complex), and the receptor-associated Jak1 and Jak2 PTKs are activated and phosphorylate the cytoplasmic domain of a receptor subunit. These P.Tyr residues act as binding sites for the SH2 domain in a latent cytoplasmic transcription factor known as STAT1. The bound STAT1 molecules are phosphorylated by the Jak's, which allows them to dimerize via their SH2 domains. The STAT1 dimer then translocates into the nucleus where it binds specific response elements (GAS) and activates transcription.

overexpressed in epithelial cells. However, it seems likely that the endogenous PDGFRβ RPTK, which is expressed at lower levels, requires a combination of signals derived from different phosphorylation sites to signal successfully *in vivo*. Tests of which phosphorylation sites are important in the animal are currently being made by mutation of phosphorylation sites individually and in combination in the germ line copy of the mouse PDGFRβ RPTK gene.

The importance of cooperation between signalling pathways initiated by different autophosphorylation sites is illustrated by our own work on the colony-stimulating factor 1 (CSF-1) RPTK, which is a member of the PDGF RPTK family that is expressed in myeloid cells and is needed for proliferation of myeloid precursor cells and their differentiation into macrophages. We identified the major CSF-1-induced phosphorylation sites in the murine CSF-1 receptor as Tyr697, Tyr706, and Tyr721, which lie in the kinase insert, and Tyr807, which lies in the activation loop (van der Geer & Hunter 1990, 1991). Phosphorylation of Tyr807 in the activation loop is required for maximal ligand-stimulated CSF-1 receptor PTK activity, and mutation of this site compromises all CSF-1 RPTK-mediated responses. The autophosphorylation sites in the kinase insert each activate distinct signalling pathways through interaction with SH2 domain proteins. By site-directed mutagenesis and expression of the mutant CSF-1 receptors in Rat2 fibroblasts we found that P.Tyr721 is responsible for the binding of PI-3 kinase via the p85 regulatory subunit SH2 domains (Reedijk *et al.* 1992), and P.Tyr697 for the binding of the Grb2 adaptor protein via its SH2 domain (van der Geer & Hunter 1993). Phosphorylation of Tyr706 is required for the activation of STAT1, apparently because it acts as a binding site for the STAT1 SH2 domain (Novak *et al.* 1996). Phosphorylation of Tyr697, Tyr 706 and Tyr721 is important for CSF-1-induced differentiation of CSF-1 receptor expressing FDC-P1 mouse myeloid cells, although it is not needed for CSF-1-stimulated growth of these cells (Bourette *et al.* 1995). In contrast, phosphorylation of Tyr697 and Tyr721 is required for a maximal growth response to CSF-1 in CSF-1 receptor expressing Rat2 fibroblasts (van der Geer & Hunter 1993). The cooperation between the signalling pathways activated by phosphorylation of Tyr721 and Tyr697 in the CSF-1 receptor would appear to illustrate a general principle of RPTK signalling (figure 9). The net outcome of RPTK signalling depends upon the integration of all the activated pathways and the cellular context, and the specificity of RPTK responses is then a consequence of the combination of different signalling pathways that are activated by a particular receptor and how the cell in question is wired to respond to these pathways.

Binary receptor PTKs also activate multiple signalling pathways generating specific cellular responses. One such system is the integrin/Fak binary receptor system. Normally adherent cells require attachment to the extracellular matrix via integrin receptors to grow (Clark & Brugge 1995). Integrin occupancy somehow generates a permissive signal for progression through late G1, and in some cells provides an anti-apoptotic signal (Meredith *et al.* 1993). Fak, is a non-receptor PTK that is localized to focal adhesions, which are sites where cells attach to the extracellular matrix via surface integrin receptors. Fak was first identified through its tyrosine phosphorylation and association with the v-Src PTK in v-*src* transformed cells. Fak has a central catalytic domain, a C-terminal focal adhesion localization domain, and an N-terminal domain that associates with integrin β subunit cytoplasmic domains (Hanks & Polte 1997). Integrin engagement with the extracellular matrix protein, fibronectin, increases Fak tyrosine phosphorylation and Fak PTK activity. The main Fak autophosphorylation site, Tyr397, lies just upstream of the catalytic domain.

We have shown that adhesion of murine NIH3T3 fibroblasts to fibronectin promotes association of the c-Src PTK and the Grb2 adaptor protein with Fak *in vivo*, and also results in activation of the ERK2 MAP kinase (Schlaepfer *et al.* 1994). In v-Src transformed NIH3T3, the association of v-Src, Grb2 and Sos with Fak is independent of cell adhesion to fibronectin. We have identified the Grb2 binding site both *in vitro* and *in vivo* as P.Tyr925, and

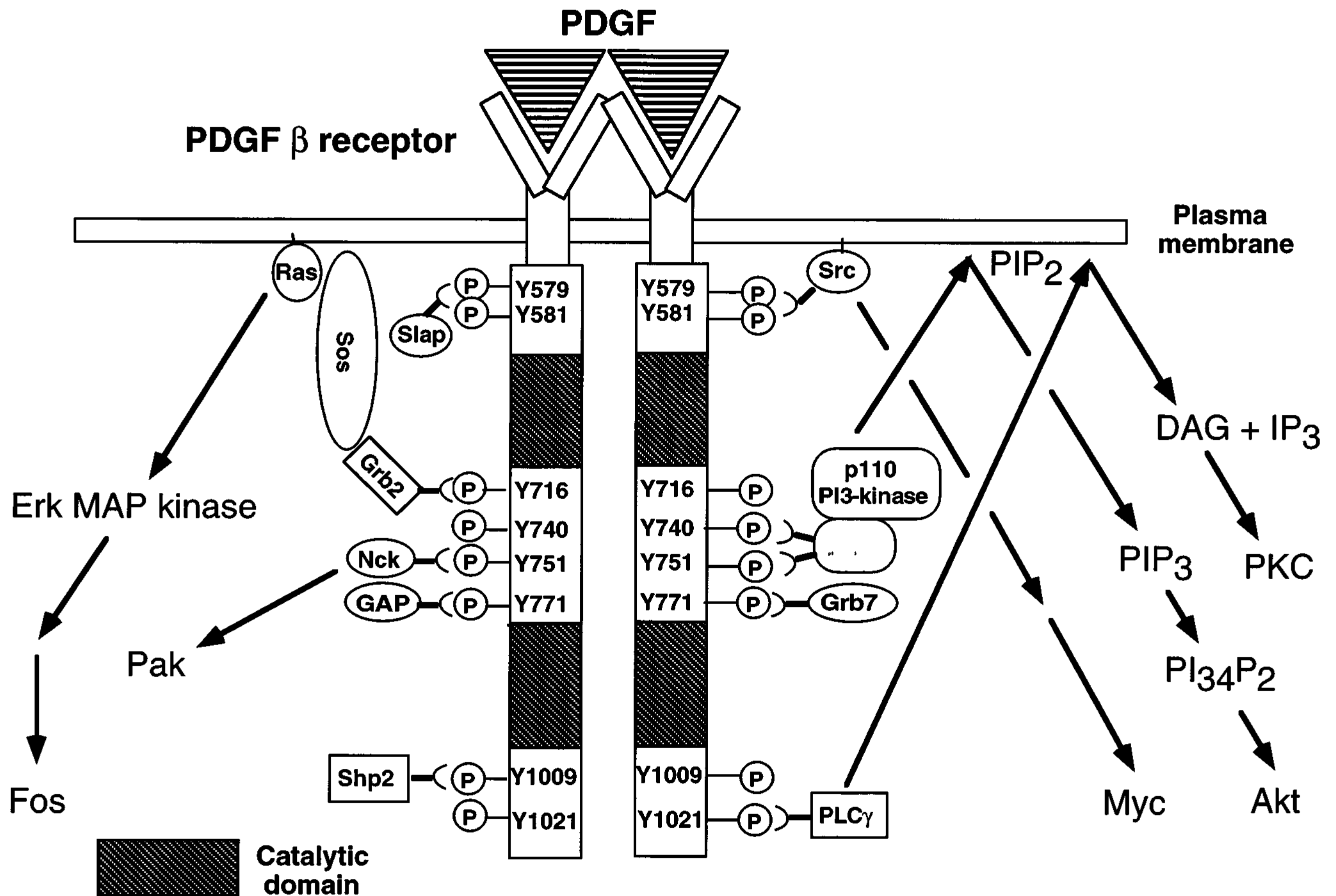

Figure 8. PDGF receptor protein-tyrosine kinase signalling pathways.

shown that Tyr925 is phosphorylated *in vivo* in response to fibronectin binding (Schlaepfer & Hunter 1996). c-Src binds to Fak via its SH2 domain at P.Tyr397, which is the major autophosphorylation site. Mutation of Tyr397 to Phe abolishes c-Src binding and also Grb2 binding (Schlaepfer & Hunter 1996). Therefore, we conclude that fibronectin binding to integrins on NIH3T3 fibroblasts promotes activation of Fak, and binding of c-Src, which then phosphorylates a series of tyrosines in the C-terminal tail of Fak (figure 10). This creates binding sites for Grb2 and other SH2 proteins, such as Nck and PI-3 kinase, thus providing a means through which integrins can signal upon cell attachment. We have shown that activation of ERK2 via FAK requires Ras function (Schlaepfer & Hunter 1997), but is not absolutely dependent on Src function or Grb2 binding to FAK, since it occurs in Src^{-} cells expressing a dominant negative mutant of c-Src, which blocks fibronectin-stimulated Grb2 binding to FAK (Schlaepfer *et al.* 1997). In this circumstance, tyrosine phosphorylation of the docking protein p130Cas by FAK appears to provide an alternative mechanism for the activation of Ras and the ERK MAP kinase. We have shown that fibronectin stimulation leads to association of the Nck adaptor protein with Cas at sites that are phosphorylated by both FAK and c-Src (Schlaepfer *et al.* 1997). In addition, phosphorylation of the Shc adaptor protein and its association with Grb2 is stimulated by fibronectin in these cells providing another pathway to ERK2 activation (Schlaepfer & Hunter 1997). Thus, integrin mediated Fak activation and association with Src triggers multiple signalling pathways that can lead to the activation of the ERK MAP kinases as well as other effector systems (figure 10). These can in turn elicit events such as induction of gene expression, and cell migration.

5. OTHER FUNCTIONS FOR PROTEIN-TYROSINE KINASES

Although first uncovered in the realm of growth regulation and oncogenesis, it has emerged that tyrosine phosphorylation plays a fundamental role in many cellular processes (table 4). Indeed activation of certain RPTKs can induce differentiation rather than growth, and it is clear that many of the functions of PTKs are in differentiated cells that have no potential for further growth. In this regard, a great deal has been learned from the functions of PTKs in simpler eukaryotes, particularly in *Drosophila*, where orthologues of many of the receptor and non-receptor PTKs have been identified. For instance, the Sevenless RPTK is required for the cell fate decision that leads to the differentiation of the R7 photoreceptor cell in each ommatidion of the *Drosophila* compound eye. Likewise, the Torso RPTK is required to establish the anterior–posterior embryonic axis, a polarity that results in the development of terminal structures in the *Drosophila* embryo.

Another important arena in which tyrosine phosphorylation plays an important role is in neural differentiation and function. Receptor PTKs in the NGF receptor family are required for differentiation and survival of specific neurons in the brain (Barbacid 1995). NGF and related

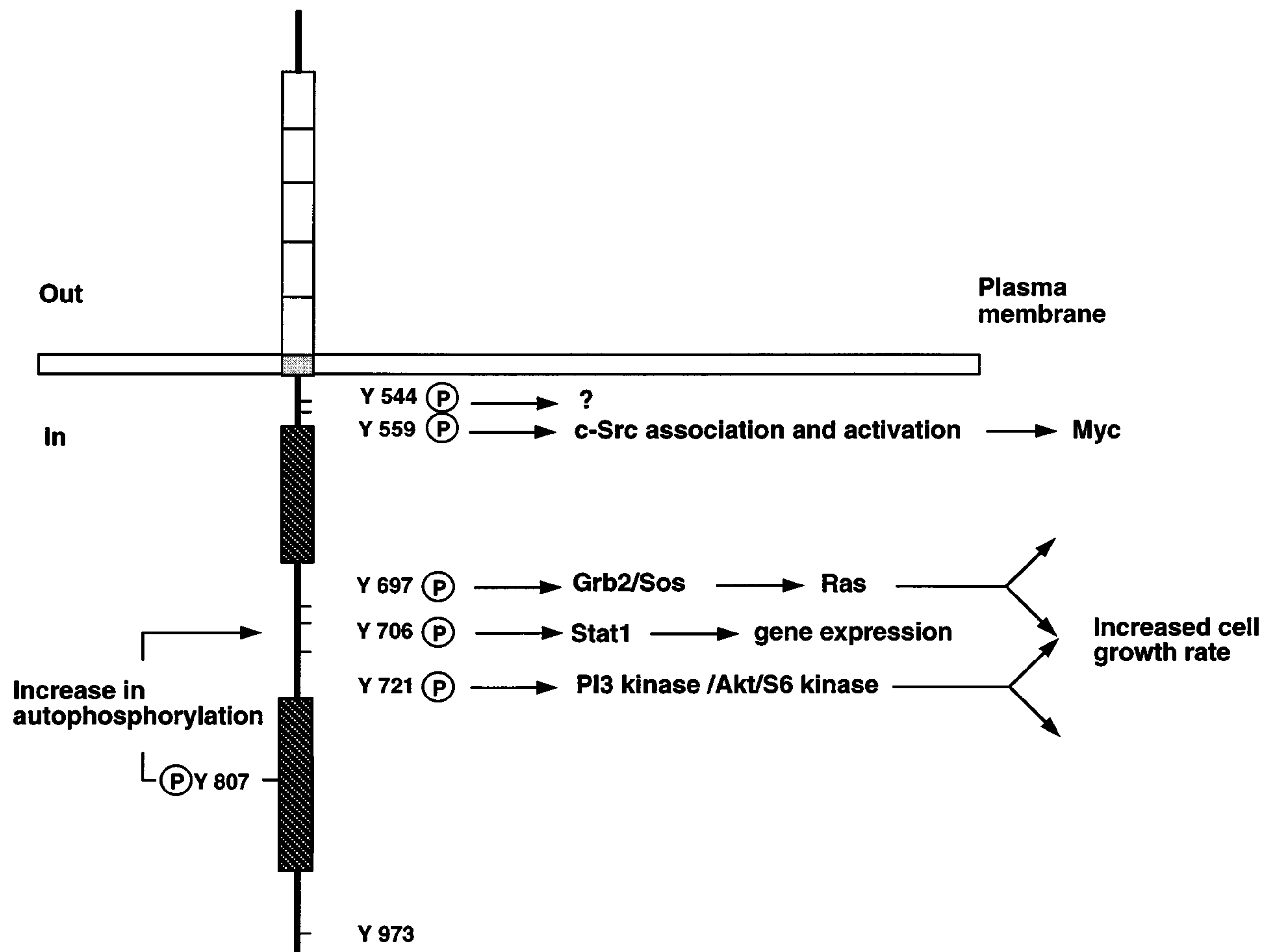

Figure 9. CSF-1 receptor protein-tyrosine kinase signalling pathways. CSF-1-induced autophosphorylation of different tyrosines in the CSF-1 receptor leads to the binding of individual SH2 domain target proteins, including the c-Src PTK, Grb2 and PI-3 kinase. The binding of Grb2/Sos to P.Tyr697, which activates Ras and downstream effectors, and the binding of PI-3 kinase to P.Tyr721, which stimulates formation of 3′ phosphoinositides and possibly leads to activation of the 70 kDa S6 protein kinase, together synergize to provide a maximal growth response.

neurotrophins not only act as survival factors for neuronal cells in culture, but they can also induce neurite outgrowth and differentiation of neuronal precursors and cell lines. Interestingly, RPTKs such as the NGF receptor, which deliver differentiation-inducing signals, activate many of the same signal pathways as growth factor RPTKs, and we are only just beginning to understand how different cells interpret the same signals in different ways (Marshall 1995). At least in part this can be explained by differences in potential nuclear responses, which are predetermined by the constellation of transcription factors expressed in each cell type. There is also growing evidence that signal duration and intensity also play important roles in the specificity of cellular responses (Dikic *et al.* 1994). Another class of receptor PTKs, the Eph family receptors have recently been found to play a critical role in axonal guidance (Friedman & O'Leary 1996; Nieto 1996). For instance, in the eye retinal neurons are guided by gradients of Eph family receptor PTK ligands, which are membrane anchored, such that ligand binding to the cognate receptor causes axonal repulsion, probably through regulation of the actin cytoskeleton in the growth cone preventing its forward migration. In this way retinal axons can be guided to the correct site in the optic tectum allowing a topographic map of the retina to be established in the tectum. Receptor PTKs in the EGF and FGF receptor families are also known to be important in neural differentiation, based on gene disruption experiments in the mouse. Another receptor PTK, MuSK is essential for the establishment of neuromuscular synapses, being required in the muscle cell for the clustering of acetyl choline receptors and organization of the synapse at the the neuromuscular junction (DeChiara *et al.* 1996; Glass *et al.* 1996).

One unexpected function for tyrosine phosphorylation was discovered a few years ago, when it was shown that the Cdc2 cell cycle regulatory protein kinase is negatively regulated by tyrosine phosphorylation (Gould & Nurse 1989; Morla *et al.* 1989). This conserved regulatory phosphorylation evolved in the yeasts, and is critical for properly orchestrated entry into mitosis (Morgan 1995). The regulatory tyrosine is phosphorylated by members of the Weel family of protein kinases, and dephosphorylated by members of the Cdc25 family of dual-specificity protein

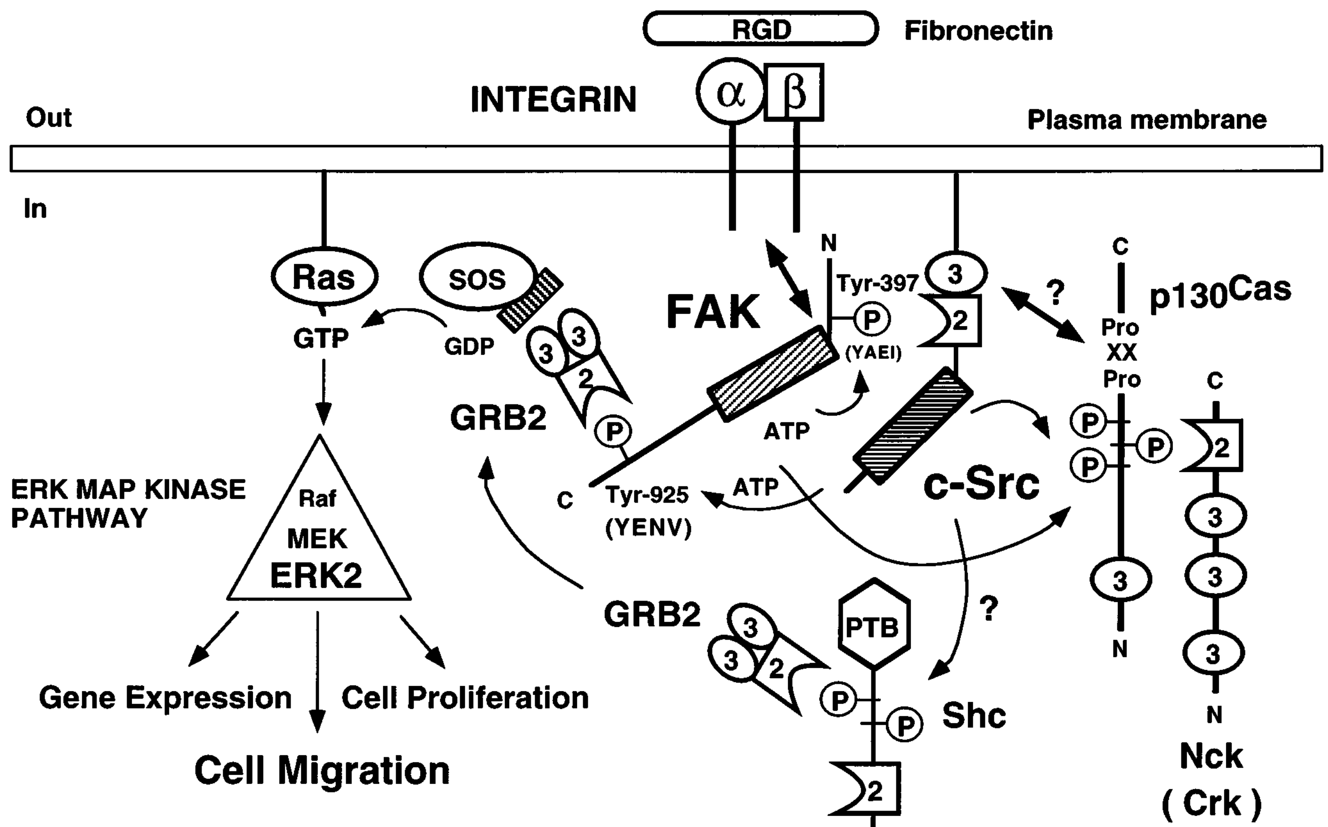

Figure 10. Integrin-mediated Fak signalling pathways. The binding the fibronectin-specific integrins to fibronectin in the extracellular matrix activates Fak and increase autophosphorylation of Tyr397, leading to the binding of the c-Src PTK to P.Tyr397 via its SH2 domain. This activates c-Src, which phosphorylates Tyr925 in the C-terminal domain of Fak. Grb2/Sos binds to P.Tyr925 leading to activation of Ras and the ERK MAP kinase pathway and cellular responses.

Table 4. *Functions of tyrosine phosphorylation*

1.	cell growth (and oncogenesis)
2.	cell shape and attachment
3.	cell differentiation and development
4.	cell cycle control
5.	apoptosis
6.	intracellular signaling pathways
7.	transcription
8.	glucose uptake
9.	platelet activation in blood clotting
10.	angiogenesis (formation of new blood vessels)
11.	regulation of ion channel activity in neural transmission

phosphatases. The regulation of Weel and Cdc25 is complex, involving upstream protein kinases and phosphatases, coupled with a positive feedback loop in which active Cdc2 phosphorylates and stimulates Cdc25 leading to activation of additional Cdc2 molecules (Morgan 1995). In mammals, there are three distinct families of cyclin-dependent kinases, Cdks, that govern distinct cell cycle transitions, and each of them is regulated through phosphorylation of the equivalent tyrosine. The tyrosine in question lies in the ATP-binding site, but exactly how this phosphorylation inhibits Cdk activity is unclear. The most likely possibility is that the phosphate alters the conformation of the N-terminal lobe, thus precluding proper alignment of catalytic residues. Phosphorylation of this regulatory tyrosine is critical for checkpoint control, which prevents a cell from progressing through the cycle before a previous process such as DNA replication is completed or when DNA is damaged.

As described above, cell adhesion through integrins activates tyrosine phosphorylation, and this provides a signal to cells that they are attached. Members of the Src, Fak, Abl, and Ack non-receptor PTK families are involved. Ligand-activated RPTKs may also be recruited to sites of cell-substratum adhesion, thus providing an explanation for the known cooperativity between adhesion and growth factor signalling. Cell–cell interaction can also involve tyrosine phosphorylation. For instance, cell–cell interaction receptors known as cadherins, which interact homotypically between cells, associate with members of the Src family non-receptor PTKs. In addition, PTPs such as RPTPμ, which associate with cadherins, may play a role in cell-cell signalling.

Another unexpected function of tyrosine phosphorylation is its role in regulating the activity of transcription factors. This is illustrated by the Jak/STAT pathway, where tyrosine phosphorylation of STATs is required for their dimerization, nuclear translocation and DNA-binding activity (see above). Tyrosine phosphorylation may also regulate RNA polymerase II, which transcribes the majority of mRNAs in the cell. The repeat sequences in the C-terminal domain (CTD) of the large subunit of RNA polymerase II are phosphorylated in the active form of the enzyme. A number of protein kinases have

been found that can phosphorylate the CTD including an intrinsic activity in TFIIH, a basal transcription factor. However, c-Abl has also been shown to be able to phosphorylate the CTD (Baskaran *et al.* 1993; Baskaran *et al.* 1996), and this may play a role in gene expression during the G1 to S transition, at a time in the cell cycle when c-Abl is activated.

Receptor PTKs also play a role in differentiation of cell types other than neuronal cells. For instance, members of the FGF RPTK family are required during bone development to restrict the growth of chondrocyctes (Webster & Donoghue 1997). VEGF RPTKs and Tie family RPTKs are required in combination for angiogenesis (Sato *et al.* 1995; Shalaby *et al.* 1997), which requires coordinated growth and differentiation of the endothelial cells that line the vasculature and the surrounding smooth muscle cells, which maintain the shape of the blood vessels

PTKs also play a role in the functions of many fully differentiated cells. For example, platelet activation requires activation of a cascade of non-receptor PTKs. Mast cell degranulation upon antibody/antigen complex binding to surface F_c receptors requires Src family PTKs. Osteoclast function in bone resorption is absolutely dependent on the activity of the c-Src PTK, as evidenced by the osteopetrotic phenotype of c-Src knockout mice (Soriano *et al.* 1991). A particularly important role for tyrosine phosphorylation has emerged in the regulation of ion channel function in neuronal and non-neuronal cell types. Several years ago it was shown that tyrosine phosphorylation of the muscle nicotinic acetyl choline receptor increased its rate of desensitization (Hopfield *et al.* 1988). More recently a delayed-rectifier type potassium channel has been shown to be negatively regulated by tyrosine phosphorylation, which is induced by activation of muscarinic type acetyl choline receptors (Huang *et al.* 1993). A candidate for the PTK in questions is Pyk2, a member of the Fak family of non-receptor PTKs, which is activated in response to elevation of intracellular calcium (Lev *et al.* 1995). Pyk2 has been shown to phosphorylate and downregulate the Kv1.2 potassium channel. A voltage-gated cation channel in Aplysia bag-cell neurons, which is activated response to a rise in cAMP, is negatively regulated by tyrosine phosphorylation (Wilson & Kaczmarek 1993). Physiological regulation of channel tyrosine phosphorylation may be achieved by a PKA-stimulated PTP. Most recently, NMDA-type glutamate receptors, which are needed for the long-term potentiation responses in the hippocampus that are believed to be important for memory, have been shown to be stimulated by c-Src PTK dependent tyrosine phosphorylation (Yu *et al.* 1997). Interaction of the NMDA receptor with c-Src requires a short sequence in the unique region of c-Src. Direct association of the Src SH3 domain with the human Kv1.5 potassium channel via a proline-rich motif has been observed. Furthermore, hKv1.5 is tyrosine phosphorylated, and the channel current is suppressed in cells coexpressing v-Src (Holmes *et al.* 1996). Finally, the IP_3 receptor in the endoplasmic reticulum, which effluxes calcium from luminal stores upon IP_3 binding, associates with the Fyn Src family PTK upon activation of T cells, and this triggers tyrosine phosphorylation and activation of the IP_3 receptor leading to calcium release into the cytoplasm (Jayaraman *et al.* 1996).

In summary, although originally thought to be primarily involved in growth regulation, tyrosine phosphorylation turns out to be used in a surprisingly wide variety of cellular processes.

6. PROTEIN-TYROSINE PHOSPHATASES

In any process regulated by phosphorylation the protein phosphatase is as important as the protein kinase, and this is equally true for tyrosine phosphorylation. Although the first protein-tyrosine phosphatase (PTP) was not identified molecularly until 1988 (Tonks *et al.* 1988), a large family of PTPs has now been found (Stone & Dixon 1994; Tonks 1996), and many of them appear to play a critical role in regulation (Sun & Tonks 1994). Including dual-specificity phosphatases, like Cdc25 and MKP1, which are related to the PTPs proper, over 55 distinct mammalian PTP genes had been identified by the end of 1996. As is the case for the PTKs, there are two types of PTP, non-receptor PTPs and receptor-like PTPs (RPTP) (figure 11). The non-receptor PTPs have a single catalytic domain, and are localized either in the cytoplasm or the nucleus by discrete targetting domains. Two of the non-receptor PTPs, Shp1 and Shp2, have twin SH2 domains, which serve to regulate PTP activity negatively, and also target these PTPs to activated RPTKs and binary receptor PTKs. Shp1 is a negative regulator of cytokine receptor signalling, but, although PTPs in general are negative regulators of tyrosine phosphorylation based signalling, the closely related Shp2 is required for productive signalling by some RPTKs (Streuli 1996).

The RPTPs are all type I membrane spanning proteins. There are currently seven RPTP families, most readily distinguished based on their extracellular domain sequences (figure 11). All except one of the families have two catalytic domains in their cytoplasmic regions; in general, the membrane proximal domain has activity, whereas the membrane distal domain is either inactive or has very weak activity. The functional significance of this twin catalytic domain arrangement is unknown. In most cases ligands for the RPTPs, if they exist, have not been identified, and it is unclear whether they truly serve as receptors. However, contactin, which is a GPI-linked cell surface protein, has recently been identified as a ligand for RPTBβ/ζ (Peles *et al.* 1995), and this coupled with the fact that the extracellular domain of one the RPTP families resembles that of N-CAM, a cell adhesion molecule, has led to the suggestion that RPTPs may in general be involved in cell-cell communication. This notion is reinforced by the finding that RPTPμ associates with cadherin (Brady-Kalnay *et al.* 1995), a cell surface receptor that is known to be involved in the formation of cell–cell junctions. While such a function for RPTPs seems reasonable, it is not clear whether ligand binding regulates RPTP activity or rather localizes them in the vicinity of their substrates.

Clues to RPTP function have been slow in coming. CD45, the first RPTP to be identified, is essential for T-cell activation upon antigen binding to the T-cell receptor (Frearson & Alexander 1997). CD45 is probably required to activate members of the Src family of non-receptor PTKs, through dephosphorylation of the C-terminal negative regulator tyrosine. It is still unclear, however,

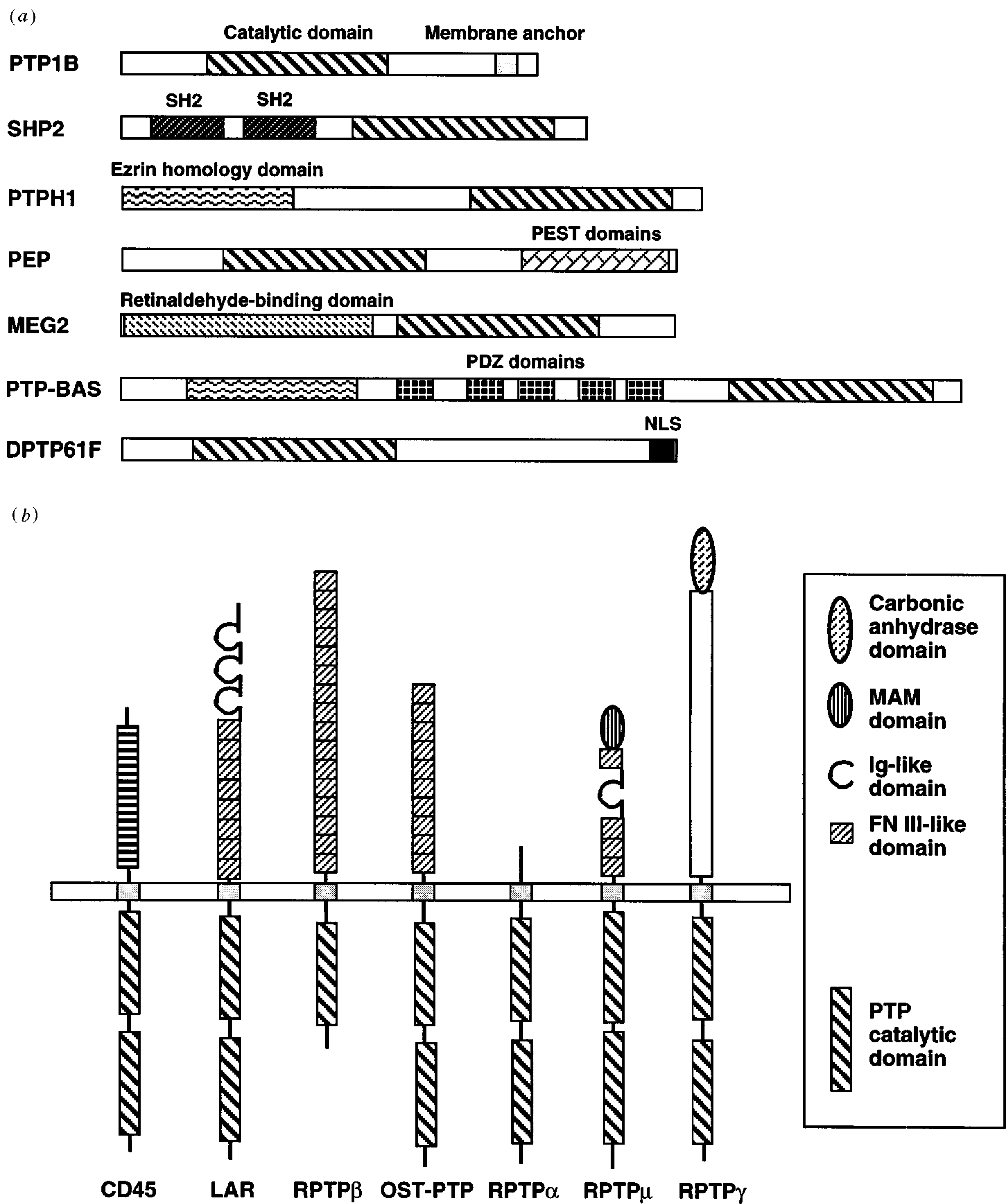

Figure 11. Schematic structures of (*a*) non-receptor and (*b*) receptor-like protein-tyrosine phosphatase families.

whether CD45 has a physiological ligand. RPTPs are particularly common in the brain suggesting that they may have a neural function. This idea is supported by the finding that combinations of RPTPs play critical roles in motor axon guidance in *Drosophila* embryogenesis (Desai *et al.* 1997). Mouse knockouts of several RPTPs have been reported, with little sign of a phenotype. However, there is significant structural redundancy in some of the RPTP families, and it seems likely that targeted disruption of multiple members of a family will be needed to uncover a function.

Because of the obvious importance of PTPs in the regulation of signals using tyrosine phosphorylation we have been studying these enzymes. In particular, we have characterized a receptor-like PTP, RPTPα, which has twin cytoplasmic catalytic domains and a rather small extracellular domain of 123 residues that lacks Cys, is rich in Ser and Thr, and contains several potential sites for

N-linked glycosylation. We have shown that RPTPα is constitutively phosphorylated on Ser180 and Ser204, which are protein kinase C phosphorylation sites (Tracy *et al.* 1995). We have found that RPTPα is also constitutively phosphorylated on tyrosine in NIH3T3 mouse fibroblasts, and identified the single Tyr phosphorylation site as Tyr789, located five residues from the C terminus (den Hertog *et al.* 1994). The sequence on the C-terminal side of Tyr789 fits the consensus binding site for the adaptor protein Grb2, and we have found that wild-type RPTPα, but not a Tyr789Phe RPTPα mutant, binds to Grb2 *in vitro* and *in vivo* (den Hertog *et al.* 1994). However, the Ras-GTP-exchange factor, Sos, which associates with Grb2 via its SH3 domains, is not associated with RPTPα *in vivo* nor can it bind *in vitro*. The C-terminal SH3 domain is needed for stable association of Grb2 with P.Tyr789 RPTPα, and this SH3 domain interacts with the membrane proximal catalytic domain (den Hertog & Hunter 1996), leading us to suggest that the binding of Grb2 may be a means of regulating RPTPα activity.

One of the most intriguing features of the receptor-like PTPs is the presence of twin catalytic domains. In many RPTPs the C-terminal domain (domain II) lacks catalytic activity, but may still be required structurally for the function of the N-terminal catalytic domain (domain I), as is true for CD45. However, in the case of RPTPα, domain II has about 5% of the activity of the domain, and is not required for the activity of domain I (Wang & Pallen 1991). To investigate the significance of this twin domain structure, we set out to determine the three-dimensional structure of the entire cytoplasmic domain of RPTPα in collaboration with Alex Bilwes and Joe Noel. We have begun by obtaining crystals of the two domains separately, and we have solved the crystal structure of domain I. Unexpectedly, domain I exists as a symmetric dimer in the crystal, in which a wedge-shaped loop composed of a sequence just upstream of the catalytic domain proper is inserted into the catalytic cleft of the opposing monomer (Bilwes *et al.* 1996). Were such a dimer to exist in the cell, one would predict that neither catalytic domain would be active because active site residues essential for hydrolysis of the P.Tyr phosphate ester are masked as a result of the interactions between the loop of one monomer and the catalytic cleft of the other. However, we are now attempting to obtain formal proof that the dimer is indeed inactive, using recombinant forms of domain I that can be induced to dimerize artificially. The organization of this domain I dimer is such that it could form between two molecules of RPTPα in a cell membrane, and we have obtained preliminary evidence that RPTPα can form dimers in intact cells. On this basis, we propose that RPTPα can undergo reversible dimerization, which leads to its inactivation. This mechanism of regulation would be the exact opposite of that for RPTKs, where dimerization is required for their activation. What is not clear is whether dimerization is a ligand-mediated event, as it is for the receptor PTKs. In principle dimers could exist constitutively in the absence of ligand, and ligand binding could result in dimer dissociation. The identification of ligands will be required to determine exactly how ligand binding might regulate RPTP activity.

7. TYROSINE PHOSPHORYLATION AND DISEASE

From the outset it was clear that unscheduled tyrosine phosphorylation could play a role in cancer. However, tyrosine phosphorylation plays many roles in cellular physiology, and functional perturbation of PTKs and PTPs underlies many diseases. The cardinal role that constitutively activated PTKs play in oncogenesis, first brought to light by the discovery of the v-Src PTK, has been underscored by the large number of viral and tumour oncogenes that encode PTKs (currently more than 17). At least ten PTKs have been implicated in human cancer, having been found either overexpressed or expressed in a mutationally activated form in one or more tumour types (table 5). For instance, an activated mutant form of the NGF RPTK (Trk) was identified as an oncogene in a human colon carcinoma in 1986 (Martin-Zanca *et al.* 1986), and *TRK* oncogenes have subsequently been found to occur in a fraction of papillary thyroid carcinomas. These Trk-derived oncoproteins are generated through chromosomal translocations, and consist of a domain derived from a protein that can dimerize appended to the C-terminal part of the NGF receptor (Greco *et al.* 1992). The Trk oncoproteins are constitutively dimerized and by analogy with NGF-induced NGF RPTK dimers, the oncoprotein dimers have constitutive PTK activity, accounting for their transforming potential. Mutant forms of the GDNF RPTK (Ret) are causal in the inherited predisposition to cancer syndromes, known as multiple endocrine neoplasia type 2A and B (MEN) and familial medullary thyroid carcinoma (FMTC) (van Heyningen 1994). In MEN2A a variety of mutations in the Ret extracellular domain commonly involving Cys have been found (e.g. Cys634arg). Such mutations lead to constitutive activation, presumably through dimerization mediated by unpaired Cys. In MEN2B the mutation is in the catalytic domain (Met918Thr), and this not only activates Ret but also alters its substrate specificity, making it more similar to that of the Src family PTKs (Songyang *et al.* 1995).

In more than 95% of chronic myelogenous leukaemias (CML), an activated form of the c-Abl non-receptor PTK is expressed. In these cases, the N-terminus of the Bcr protein is fused to most of c-Abl, generating Bcr-Abl; this fusion occurs as a result of a translocation of the *cABL* gene on chromosome 9 with the *BCR* gene on chromosome 21 (Rabbitts 1994). The Bcr domain provides an oligomerization domain for Abl, and results in constitutive activation of the Abl PTK. Chromosomal translocations in which a protein with a dimerization domain is fused to an RPTK lead to the oncogenic activation of at least two other RPTKs in human leukaemia. The PDGF β RPTK is activated through a fusion with Tel, a dimeric Ets family transcription factor in chronic myelomonocytic leukaemia (Golub *et al.* 1994), and the Alk RPTK is activated through fusion with nucleophosmin, a dimeric nuclear protein, in anaplastic large cell lymphoma (Morris *et al.* 1994). In addition, amplification and consequent overexpression of the EGFR and ErbB2 RPTK genes are common characteristics of glioblastomas and mammary carcinomas respectively. Thus, it is abundantly clear that unregulated PTK activity can play an important role in human cancer.

Table 5. *Protein-tyrosine kinases and phosphatases in human disease*

receptor protein-tyrosine kinases	
EGF receptor	mutated, amplified and overexpressed in cancer
ErbB2	amplified and overexpressed in breast cancer
PDGF receptor β	activated by chromosome translocation in leukaemia ectopic expression in cancer
CSF-1 receptor	inactivated by mutation in hereditary piebaldism
Kit (SCF receptor)	activated by mutation in leukaemia
FGF receptor 1 FGF receptor 2 FGF receptor 3	activated by mutation in craniosynostosis syndromes that affect skull/bone development (e.g. Crouzon's and Pfeiffer's syndromes and thanatophoric dysplasia) and in multiple myeloma
Tie2 (angiopoietin receptor)	activated by mutation in hereditary vascular dysmorphogenesis disease
Met (HGF receptor)	activated by mutation, amplified and overexpressed in cancer
Axl	overexpressed in myeloid leukaemias
Rse/Sky	overexpressed in breast cancer
Ret (GDNF receptor)	inactivated by mutation in hereditary intestinal disease activated by mutation in hereditary cancer syndromes
insulin receptor	inactivated by mutation in non-insulin dependent diabetes
Alk	activated by chromosome translocation in leukaemia
non-receptor protein-tyrosine kinases	
c-Src	activated and overexpressed in cancer
c-Yes	activated and overexpressed in cancer
c-Abl	activated by chromosome translocation in chronic myelogenous leukaemia
Btk	inactivated by mutation in hereditary agammaglobulinemia
ZAP70	inactivated by mutation in severe combined immune deficiency disease (SCID)
Jak2	activated by chromosome translocation in leukaemia
Jak3	inactivated by mutation in SCID disease
protein-tyrosine phosphatases	
Shp1 (mouse)	inactivated by mutation in immune hyperproliferative disease
MMAC1	putative phosphatase inactivated by mutation in sporadic breast and prostate cancer and in Cowden's hamartoma syndrome
YopH1 (bacterium)	PTP encoded by the virulence plasmid of *Yersinia* (plague bacterium) that is required for pathogenesis

Activating mutations in RPTKs can also result in developmental dysfunction. For instance, mutations in three of the four members of the FGF RPTK family are causative in craniosynostosis syndromes that affect skull and bone development (Webster & Donoghue 1997). In general, the activating mutations are point mutations that occur in the extracellular domain or the transmembrane domain; they all result in constitutive dimerization and PTK activation. In the case of FGFR3 there is also a mutation in the activation loop of the catalytic domain that results in PTK activation. FGF RPTK mutations can either occur spontaneously or be inherited. Although activation of FGF RPTKs in fibroblasts results in cell proliferation, the craniosynostosis disease phenotype indicates that FGF RPTKs are playing an inhibitory role in chondrocyte growth *in vivo*; this is borne out by the phenotype of mice lacking FGFR3, which have increased bone length due to chondrocyte hypertrophy. An inherited activating mutation in the catalytic domain of Tie2, the receptor for angiopoietin, causes a dominant venous malformation defect due to failure to form a proper smooth muscle coating of the venous endothelial cell tube (Vikkula *et al.* 1996). Tie2 is expressed in endothelial cells, and apparently it is critical for communication with the overlying mesenchymal smooth muscle cells, which make angiopoietin, the Tie2 ligand.

Inactivating mutations in RPTKs also play an important role in disease. Inactivating mutations in the stem cell factor RPTK, c-Kit, cause a deficiency in stem cell proliferation, resulting in defects in melanocyte formation, haematopoiesis, and germ cell development (Lev *et al.* 1994). This is a heritable mutation that results in piebaldism in humans, and the so-called white-spotting phenotype in mice. These mutations act in a dominant fashion in heterozygotes, because the inactive c-Kit protein that is expressed can dimerize with the wild type in response to ligand thus forming an inactive dimer. Assuming the the wild type and mutant forms of c-Kit form dimers equally well, one can predict that in a cell expressing equal levels of wild type and mutant c-Kit the signal elicited by ligand binding will be only 25% of that in a cell with two wild-type c-*kit* alleles. This suggests that there is a critical threshold for the the response of stem cells to the c-Kit signal, and that when the signal drops 75% it falls below this threshold. Another RPTK that is mutated in human disease is the insulin RPTK; *ca.* 5% of patients with non-insulin dependent diabetes (NIDDM), have inactivating mutations in one or both alleles of the insulin RPTK gene, which may prevent surface expression for a variety of reasons, or which may impair PTK activity (Taylor 1992).

Deficiencies in immune system function are linked to mutations in PTK genes. Two forms of severe combined immunodeficiency diseases are caused by inactivating mutations in non-receptor PTK genes. Mutational inactivation of both copies of the ZAP70 PTK occurring as a result of a cross between carriers of different recessive inactivating mutations in ZAP70, results in SCID (Chan *et al.* 1994*a*). Likewise individuals carrying two mutationally inactivated Jak3 genes evince SCID symptoms (Russell *et al.* 1995). ZAP70 is essential for T-lymphocyte development, being activated downstream of the the T-cell receptor. Interleukin signalling via the

Table 6. *Cancer targets for anti-protein-tyrosine kinase drugs*

1. chronic myelogenous leukaemia (activated Abl PTK)
2. papillary thyroid carcinoma (activated Trk RPTK)
3. multiple endocrine neoplasia (activated Ret RPTK)
4. colon, mammary, and ovarian carcinomas (increased ErbB2 and Src PTK activity)
5. neuroblastomas and glioblastomas (increased EGF and PDGF RPTK activity)
6. acute lymphoblastic leukaemia (constitutive Jak2 PTK)
7. resistance of tumour cells to apoptosis (IGF-1 RPTK required)
8. tumour angiogenesis (VEGF RPTK activity required)

Table 7. *Types of anti-protein-tyrosine kinase drugs*

(Given the number of different roles that PTKs play in the body, the problem is to devise drugs with sufficient specificity that they target only the PTK of interest.)

1. specific chemical PTK inhibitors
2. antisense oligonucleotides and ribozymes
3. dominant negative growth factors and receptor PTKs
4. neutralizing antibodies to receptor PTKs
5. antibody-PTK inhibitor conjugates
6. designer DNA-binding proteins against gene fusion junctions (e.g. Bcr-Abl)

cytokine receptor γ chain that is a shared, essential component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 requires Jak3, and the lack of Jak3 results in a failure of B and T lymphocytes to develop (Nosaka *et al.* 1995). X-linked agammaglobulinaemia is also caused by a mutation in the *Btk* non-receptor PTK gene (Tsukada *et al.* 1993; Vetrie *et al.* 1993). Btk is required for the development of cells in the B-cell lineage, and it appears to act downstream of the B-cell antigen receptor and other haematopoietic receptors. Btk has a PH domain, SH3 and SH2 domains as well as a PTK catalytic domain. Mutations in all of these domains have been found to result in loss of Btk function (Mattsson *et al.* 1996). The outcome is a failure of mature B cells to develop, resulting in a lack of immunoglobulin synthesis and a severe immunodeficiency.

Mutations in PTPs can also play a role in disease (table 5). The first example was the moth-eaten mutant mouse, which exhibits hyperproliferation of cells in several different haematopoietic cell lineages. This phenotype was tracked down to an inactivating mutation in the Shp1 PTP gene (Shultz *et al.* 1993; Tsui *et al.* 1993). Shp1 is required for negative regulation of several cytokine binary PTK receptors and also the stem cell factor RPTK (Streuli 1996), and the hyperproliferative response is a direct consequence of overstimulation of such receptors. So far, however, mutations in this gene have not been identified in humans. An interesting example where a PTP plays a critical role in disease is in the pathogenesis of *Yersinia pestis*, the cause of the black death and the plague. *Yersinia* grows through attachment to integrins on the surface of cells, but cannot survive if phagocytosed. *Yersinia* carries a virulence plasmid which encodes several proteins that are secreted into the cytoplasm of infected cells, by a specialized mechanism. One of these proteins, YopH, is a PTP, and it has recently been shown that YopH specifically dephosphorylates Fak and $p130^{Cas}$, an important downstream target of Fak, and thereby blocks adhesion-mediated internalization of the attached bacterium (Black & Bliska 1997; Persson *et al.* 1997).

Given the preponderance of oncogenes that encode activated PTKs, one might have expected PTP genes to play a critical role as tumour suppressor genes counteracting the effect of PTKs. However, although every newly discovered PTP gene has been checked to see whether it is located at the position of a known or suspected tumour suppressor gene, up until very recently, no such connection had been made. The *MMAC1/PTEN* gene may now provide the first example. The *MMAC1/PTEN* gene is found to be mutated in a wide variety of sporadic cancers, including brain, breast and prostate cancer, and it also carries inactivating point mutations in patients with Cowden disease, an inherited autosomal dominant cancer predisposition syndrome associated with breast and thyroid cancer (Li *et al.* 1997; Liaw *et al.* 1997; Steck *et al.* 1997). This gene encodes a putative dual-specificity protein phosphatase, which would in principle be able to dephosphorylate P.Tyr residues. If this turns out to be true, then the discovery of the *MMAC1/PTEN* gene may presage the existence of additional tumour suppressor genes that encode PTPs.

8. DEVELOPMENT OF DRUGS THAT TARGET PTKS AND PTPS

The realization that tyrosine phosphorylation plays a critical role in triggering the unbridled growth of some types of cancer cells raises the hope that it will be possible to develop specific inhibitors of PTKs implicated in the relevant types of human cancer (table 6) (Levitzki & Gazit 1995; Levitzki 1996). There are a number of approaches for developing drugs that target PTKs and PTPs (table 7). The most popular is the development of chemical inhibitors of PTK and PTP enzymatic activity, but there are a number of other possibilities. Considerable progress has already been made in this direction. For instance, a potent inhibitor of EGF RPTK kinase activity active in the picomolar range has been developed, which may be useful in treating cancers where the EGF receptor is overexpressed (Fry *et al.* 1994). Another PTK inhibitor has been shown to be effective in causing regression of a tumour expressing high levels of the EGF receptor in a mouse model (Buchdunger *et al.* 1994). In addition, specific *in vivo* targetting and elimination of a human B-cell precursor leukaemia has been achieved by treatment of tumour-bearing SCID mice with an immunoconjugate, in which the PTK inhibitor genistein is covalently coupled to a monoclonal antibody specific for the CD19 surface receptor on these cells (Uckun *et al.* 1995). Monoclonal antibodies (MAbs) that recognize the extracellular domains of ErbB2 and the EGF receptor are already in clinical trials for breast cancer. Specific chemical inhibitors for Jak2 and Bcr-Abl have been reported, and derivatives of these compounds may prove useful in treating leukaemias involving the activation of these PTKs (Meydan *et al.* 1996).

Table 8. *Noncancer targets for anti-protein-tyrosine kinase and phosphatase drugs*

1. platelet activation (Src family PTKs required)
2. lymphocyte activation (Lck, ZAP70 and Syk PTKs required)
3. osteoporosis (c-Src PTK required for osteoclast function in bone resorption)
4. septic shock (tyrosine phosphorylation required)
5. apoptosis – IGF-1 mimetics (IGF-1 receptor PTP inhibitors)
6. psoriasis (EGF RPTK inhibitors)
7. atherosclerosis/restenosis (PDGF RPTK inhibitors)
8. diabetes – insulin mimetics (insulin receptor PTP inhibitors)
9. nerve regeneration – neurotrophic factor mimetics (Trk and Ret PTP inhibitors)
10. HIV and papilloma virus infection

Specific inhibitors against the PTKs involved in angiogenesis, including the VEGF RPTK Flk1 have been developed, and these may be effective anti-cancer drugs. The use of a retrovirus expressing a truncated, dominant negative form of Flk1, which abolishes tumour growth in mice, has validated Flk1 as a target for anti-tumour drugs. The three-dimensional structure of FGFR1 bound to a potent inhibitor, SU5402, has revealed why this compound is such a specific inhibitor of the FGF RPTK family, even though, like most chemical PTK inhibitors reported to date, it binds into the ATP binding site (Mohammadi *et al.* 1997). SU5402 interacts with unique residues in the activation loop of FGFR1 and induces a large conformational change in this region of the catalytic domain. Such structural information can be used for rational design of improved anti-FGFR1 inhibitors and also to develop inhibitors specific for other RPTKs.

A number of noncancer-related diseases are suitable targets for drugs that antagonize PTKs and PTPs (table 8). These include important human diseases affecting a large number of people, such as osteoporosis and diabetes. Progress in developing specific inhibitors against the relevant PTKs and PTPs has been made in several of these cases (table 9). The successful development of drugs designed to abrogate PTK signalling pathways that are aberrantly activated in a variety of different human diseases is a very satisfying outcome of nearly 20 years of research into the function of tyrosine phosphorylation in eukaryotic organisms.

Table 9. *Progress in developing protein-tyrosine kinase and phosphatase antagonists*

1. PTK inhibitors	EGF receptor (AG1478[a]; PD153035; RG13022[b]; ZD1839; SU5271[b])
	ErbB2 (DAPH[b]; AG879)
	PDGF receptor (AG17[b]; AG1295[b]; CGP53716[b]; SU101[b])
	VEGF receptor/Flk1 (AG1433[b]; SU5416[c])
	FGF receptor 1 (SU5402)
	Src (PPI)
	Abl (AG1112; AG957)
	Jak2 (AG490[b])
	nonspecific (genistein, herbimycin A)
2. PTP inhibitors	insulin receptor PTP (bpV(pic)[b]; SU54348)
	nonspecific (peroxovanadium compounds)
3. oligonucleotides and ribozymes	Bcr-Abl antisense and ribozyme against mRNA junction
4. dominant negative RPTKs	truncated form of VEGF receptor/ Flk1[b]
5. anti-receptor antibodies	anti-EGF receptor monoclonal antibodies (MAb225[c])
	anti-ErbB2 monoclonal antibodies (MAb4D5[c])
	intracellular single chain antibodies against EGFR (scFv-225) and ErbB2 (scFv-FRP5)
6. MAb-inhibitor conjugates	anti-CD19-genistein conjugate[b]
7. repressors of chimeric genes	Bcr-Abl junction Zn finger DNA-binding protein

[a] AG, tyrphostins.
[b] Drugs have effects in *in vivo* models.
[c] Drugs in clinical trials.

9. TYROSINE PHOSPHORYLATION: FUTURE

One important question is how many different PTK genes there are in a vertebrate genome. A few years ago I made the prediction that the human genome might contain as many as a thousand protein kinase genes (Hunter 1987*b*). At the time this seemed far-fetched, but surprisingly it may have been an underestimate. One can now begin to make some real predictions about how many protein kinase genes there might be based on genomic sequencing. For instance, based on the complete genome sequence, budding yeast is now known to have 113 conventional protein kinase genes (Hunter & Plowman 1997). The *C. elegans* genome is about 60% sequenced, and so far *ca.* 280 protein kinase genes have been found; of these 35 are PTKs. By extrapolation *C. elegans*, which is is believed to have about 16 000 genes. should have about 475 protein kinases genes. *H. sapiens* probably has about four times as many genes as *C. elegans*, and so the human genome should have approximately 2000 protein kinase genes. If, as in *C. elegans*, 10% of these are PTK genes, the total number of human PTKs will be *ca.* 200. Only 97 PTK genes are known so far, but with the completion of the human genome project in a few years time, the accuracy of these predictions will be apparent.

Undoubtedly new functions for tyrosine phosphorylation will be found in the coming years. For instance, it has recently been shown that tyrosine phosphorylation facilitates nuclear entry of the HIV core when it is uncoated upon infection of nonproliferating cells, which is a prerequisite for its replication (Gallay *et al.* 1995). The plethora of PTKs and PTPs expressed in the brain, coupled with the emerging evidence that ion channel and neurotransmitter receptor function is regulated by tyrosine phosphorylation, presages a major role for tyrosine phosphorylation in neural function. One novel type of PTK has already been reported (Beeler *et al.* 1994), and it is likely that others will be uncovered. Likewise, additional types of P.Tyr-binding domain will probably be

identified. Structures of isolated PTK and PTP catalytic domains, and intact PTKs and PTPs will give us essential insights into the regulation of these enzymes and their interactions with targets. Finally, it seems likely that highly specific drugs that antagonize PTKs and PTPs will be developed, and will prove useful for the treatment of human disease involving aberrant tyrosine phosphorylation.

I would like to thank my collaborators over the years who have contributed to the study of tyrosine phosphorylation. In particular, my colleagues Walter Eckhart and Mary Ann Hutchinson helped me in the initial discovery that polyoma virus middle T antigen is phosphorylated on tyrosine. Bart Sefton and Karen Beemon provided invaluable expertise and assistance in the studies of the protein-tyrosine kinase activity of the RSV v-Src protein. Over the years many other people in my group have contributed to the identification and study of protein-tyrosine kinases and their substrates, but I owe a particular debt of gratitude to Jon Cooper who helped me identify some of the first protein-tyrosine kinase substrates.

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