SIGNALING BY INSULIN RECEPTORS AND RELATED PROTEIN TYROSINE KINASES

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SUMMARY

• *The insulin receptor is a member of the largely expanding family of plasma membrane receptors. In general, ligand binding induces receptor dimerization leading to activation and tyrosine phosphorylation of the cytoplasmic catalytic domain of the receptor. Activation of receptor tyrosine kinases leads to several cellular responses like proliferation, differentitation and survival. The insulin receptor family distinghuishesfrom the other receptor tyrosine kinases in that it also mediates a metabolic response like stimulation of glucose uptake and glycogen synthesis. In this review the general principles of signaling by receptor tyrosine kinases are discussed. Besides, we point out the signaling pathways used by the insulin receptor itself.*

INTRODUCTION

• Many cellular processes in eukaryotes such as proliferation, differentiation and apoptosis are regulated by external signals. An important group of external signals is represented by polypeptide growth factors. Some polypeptide growth factors bind with high affinity to a group of cell surface receptors with intrinsic tyrosine kinase (trk) activity, the receptor tyrosine kinases (Rtrk). Ligand binding to these receptors leads to activation of the Rtrk and of a whole array of early signaling intermediates, such as phospholipases, various serine/threonine kinases, small GTP-binding proteins, non-Rtrk, and protein tyrosine phosphatases. The activation of early signaling intermediates precedes the activation of several phosphorylation cascades, leading to stimulation of gene expression, changes in intracellular calcium levels, and the generation of phospholipid metabolites. Despite striking similarities in

postreceptor signaling, Rtrk often exert their own specific effects. These pleiotropic responses can be partially explained by structural characteristics of the Rtrk, and tissue-specific expression of their ligands. Alternatively, the array of signaling intermediates activated by a specific Rtrk in a certain tissue may contribute to the nature of the response. In this review, the activation of early signaling pathways by Rtrk, and the occurrence of cross-talk between signaling pathways are discussed.

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF RECEPTOR TYROSIHE KIHASES

• **Overall structure**

All Rtrk are transmembrane proteins (1,2). The extracellular domain is the most distinctive feature in Rtrk. It contains a characteristic array of structural motifs, such as cysteine-rich stretches, and sequences homologous to immunoglobulin or fibronectin type III. The extracellular domain is involved in ligand binding and is often modified by N-linked glycosylation and O-linked sugars. The single membrane spanning region is composed of a stretch of 20-25 generally hydrophobic amino acids, followed by several basic residues that represent the stop transfer signal anchoring the protein in the plasma membrane. The cytosolic domain contains a highly conserved trk catalytic domain. Carboxy- (C-) terminus to the catalytic domain resides a stretch with a length varying from a few residues up to 200 residues. The length and the primary structure of this C-terminal stretch varies among members of the Rtrk family. Based on sequence similarities and distinct structural characteristics, Rtrk have been classified in at least 15 subfamilies (Fig.l)(l-4)
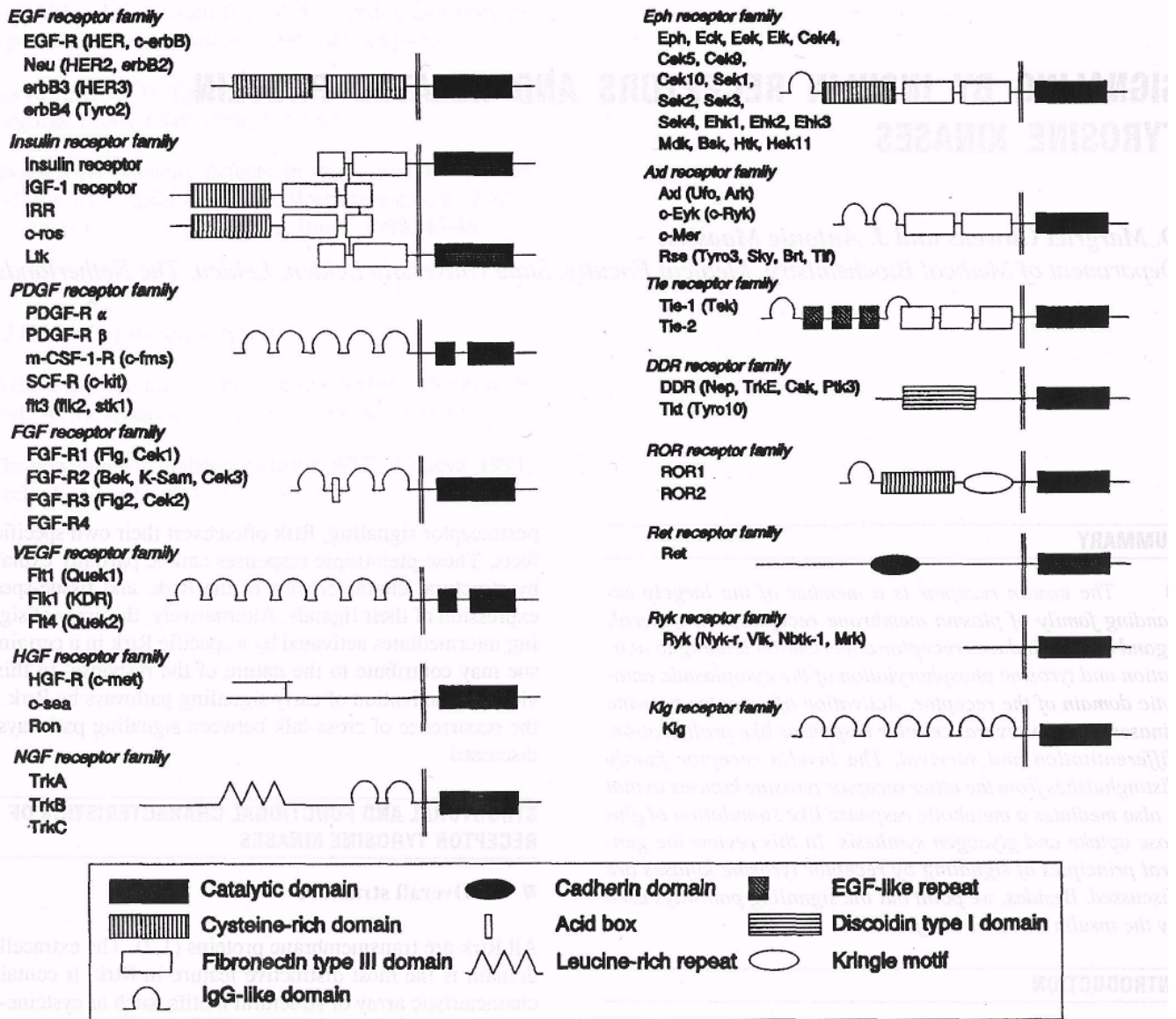
Figure 1, *Structural topology of the subfamilies of receptor tyrosine kinases. Based on sequence similarities and distinct structural characteristics, receptor tyrosine kinases have been classified into 15 subfamilies (1,4,25,26). The abbreviations are defined in the text.*

- **Function and classification**

- *Epidermal growth factor receptor family*

The epidermal growth factor receptor (EGFR), also known as HER or c-ErbB, shares close sequence similarity with the transforming protein of avian erythroblastosis virus (v-ErbB) (5,6). The EGFR is a monomeric Rtrk. The extracellular domain is

621 amino acids long and is characterised by the presence of two cysteine-rich clusters. In v-ErbB this domain is truncated, with only 65 amino acids at the external portion. The extracellular domain is separated from the cytoplasmic domain by a transmembrane region of 23 amino acids. The cytoplasmic domain contains an uninterrupted trk domain (Fig.l). Other members of the EGFR family include the p!85neu trk, also known as ErbB2, or HER2, ErbB3 (HERS), and ErbB4 (Tyro2)

(2,4). The EGFR is expressed on a variety of cell types, and activation of the receptor is implicated in cell proliferation and differentiation.

- *Insulin receptor family*

Members of the insulin receptor family are heterotetrameric proteins consisting of two oc-subunits that are disulfide-linked to two p-subunits (Fig. 1). The extracellular ligand binding domain contains one cysteine-rich cluster and three fibronectin type III repeats. The insulin receptor is derived from a precursor which is proteolytically cleaved to yield a mature receptor (7,8). The CC-subunit is involved in ligand binding, whereas the transmembrane p-subunit contains the cytoplasmic trk domain. Activation of the insulin receptor induces a pleiotropic response which can be divided into metabolic and growth-promoting effects (9). The stimulation of metabolic effects, that include glucose transport, glycogen synthesis and lipid synthesis, is a unique characteristics of the insulin receptor. Mutations in the insulin receptor and defects in the postreceptor signaling pathways are implicated in syndromes of insulin resistance and may contribute to the pathogenesis of non-insulin dependent diabetes mellitus (NIDDM) (9,10). Other members of the insulin receptor family include the insulin-like growth factor-1 (IGF-1) receptor (11), the insulin receptor-related receptor (DRR), *c-ros,* and Ltk (2,4). Activation of the IGF-1 receptor also induces a pleiotropic response (12). Targeted disruption of the IGF-1 receptor gene causes severe growth deficiency and the knock out mice die at birth (13).

- *Platelet-derived growth factor receptor family*

The platelet-derived growth factor receptors (PDGFR) are characterized by the presence of five immunoglobulin repeats in their extracellular domain and a cytoplasmic trk domain that is interrupted by a large sequence (-100 amino acids) termed the kinase insert region (Fig.. 1) (14). Activation of the PDGFR is implicated in the proliferation of mesenchymal cells. Other members of the PDGFR family include the macrophage-colony stimulating factor-1 (M-CSF-1, *c-fins*) receptor, the steel receptor (SCF receptor, *c-kit),* and the fms-like trk 3 (flt3), also known as fetal liver kinase 2 (flk2) or stem cell trk 1 (stkl) (2,4).

- *Fibroblast growth factor receptor family*

The extracellular domain of the fibroblast growth factor receptor (FGFR) family is composed of three immunoglobulin-like repeats. In addition, the extracellular domain harbours an acid box, consisting of eight acidic amino acids, which is located between the first and the second immunoglobulin domain (Fig. 1). The catalytic trk domain is interrupted by a small (14 amino acids) kinase insert sequence. The prototype for the

FGFR family, FGFR1, was originally identified as a Fms-like gene (Fig) (15). Subsequently, a receptor for basic FGF that was purified from chicken embryo, chicken embryo kinase 1 (Cekl), was found to be the chicken Fig product (2,4,15). Homologsfor Fig have been isolated from a variety of species, including human, mouse, and chicken (2,4,15). Other members of the FGFR family include FGFR2, also known as bacterial expressed kinase (Bek), KATO-III cell-derived stomach cancer amplified gene (K-sam), Cek3, or TK14 (2,4), FGFR3, also known as Flg2 and Cek2(2,4), andFGFR4 (2,4). Mutations in the FGFR1, FGFR2, and FGFR3 genes are associated with autosomal dominant skeletal disorders, such as Jackson-Weiss syndrome, Crouzon syndrome, PfeifTer syndrome, achondroplasia. hypochondroplasia, and thantophoric dysplasia. However, transgenic mice expressing a dominant negative form of the FGFR1 show a disrupted organization of keratinocytes, indicating a role for FGFR1 in keratinocyte differentiation.

- *Vascular endothelial growth factor receptor family*

The vascular endothelial growth factor receptor (VEGFR) family is highly related to the PDGFR family. The VEGFR family differs from the PDGFR family in that the extracellular domain is composed of seven immunoglobulin repeats (Fig. 1). The catalytic trk domain is interrupted by a large kinase insert region in a similar way as in the PDGFR family. The first member of the VEGFR family that was isolated is Flt-1 (fms-like trk), also known as Quek-1, embryonic receptor kinase 2 (EmRK2) (2,4). Other members of the VEGFR family include fetal liver kinase-1 (Flk-1), also known as KDR (2,4), and Fit-4, also known as Quek-2 (2,4). The VEGFR are exclusively expressed in endothelial cells (2,4). Targeted disruption of the Flt-1 gene suggests an essential role for this protein in the organization of embryonic vasculature, but not for endothelial cell differentiation (16). The Flk-1 gene is essential for yolk-sac blood island formation and embryonic angiogenesis (17).

- *Hepatocyte growth factor receptor family*

The hepatocyte growth factor receptor (HGFR) family are heterodimeric proteins that are formed by proteolytic cleavage of a precursor. The mature receptor consists of an entirely extracellular oc-subunit that is disulfide-linked to the membrane spanning P-subunit (Fig.l) (2,4). The P-subunit contains an uninterrupted trk catalytic domain. Members of the HGFR family include the HGFR, also known as the *c-met* proto-oncogene, the proto-oncogene *c-sea,* and the Ron trk (2,4). Activation of the HGFR is implicated in the mitogenic response of epithelial cells.

Nerve growth factor receptor family

The prototype for the nerve growth factor receptor (NGFR) is

trkA receptor. It is encoded by the cellular counterpart of trk oncogene that has originally been identified as a rearranged human oncogene (18 and Refs therein). The extracellular domain of the NGFR family shows some homology with immunoglobulin and fibronectin type III domains. In addition a leucine-rich repeat is found at the amino terminal part (Fig. 1). Other members of the NGFR family include trkB and trkC (2,4,18). trkB and trkC are both expressed as full length proteins and as C-terminal truncated receptors which have complete extracellular and transmembrane domains, but lack the cytoplasmic catalytic domain. Activation of the trk proteins is implicated in the growth, differentiation and survival of neurons (18). Mice with targeted disruption of trkA or trkC genes survive for a few weeks but have severe defects in the sympathetic nervous system and in muscle afferents, respectively. Mice lacking trkB gene die shortly after birth, apparently because they are unable to feed. In these mice, neurons in the facial, trigeminal, and dorsal root ganglia, which normally express trkB, are not developed (2).

• *Erythropoietin-producing hepatic cell line receptor family*

The erythropoietin-producing hepatic cell line-like (Eph-like) proteins resemble the largest class of Rtrk. The prototype for this family is Eph proteins (2,4). The extracellular domain of the Eph-like proteins are composed of a cysteine-rich domain, and two fibronectin type III domains. The cytosolic trk domain is uninterrupted (Fig.l). Most members of the Eph receptor family, such as Elk (Eph-like kinase), also known as chicken-embryo kinase 6 (Cek6), Eek (Eph- and Elk-related kinase), Cek4, also known as mouse-embryo kinase 4 (Mek4), and human-embryo kinase (Hek), Cek5 (Hek5, Nuk), Sekl (Cek8, Hek8), Sek2, Sek3, Sek4, Eph-homologous kinase (Ehkl, Cek7, Hek7), Ehk2, Ehk3, mouse developmental kinase (Mdk) and brain-specific kinase (Bsk), are almost exclusively expressed in the developing nervous system where they are implicated in axonal guidance (2,4). Eph, epithelial cell kinase (Eck), and hepatoma transmembrane kinase (Htk) show a broader tissue expression. Other Eph family members include Cek9, CeklO (Hek2) and Hekll (2,4).

• *Axl receptor family*

The Axl subfamily of Rtrk possess extracellular domains that are composed of two ammo-terminal immunoglobulin-like domains and two fibronectin type III domains (Fig. 1). This combination is often observed in neural cell adhesion molecules and receptor protein tyrosine phosphatases, and suggests an involvement of the Axl Rtrk in cell adhesion processes.The Axl receptor family contains distinct sequences in the trk domain. The prototype of the Axl family, Axl, also known as Ufo, Ark, was originally identified as a transform-

ing gene from the DNA of patients with chronic myelogenic leukemia (2). Overexpression of Axl in NIH3T3 cells causes neoplastic transformation with the concomitant appearance of a 140 kD Axl tyrosine phosphorylated protein. Other members of the Axl family include c-Eyk, also known as c-Ryk. Rse, also known as Tyro3, Sky, Bit, or Tif, and c-Mer (2). Rse is expressed at high levels in the adult brain, suggesting that Rse may function as a novel neurotrophic receptor. c-Mer is almost exclusively expressed in monocytic lineages.

• *Tie receptor family*

Members of the Tie receptor family are characterized by an exceptional multidomain structure of the extracellular domain, consisting of a cluster of three EGF homology motifs embedded between two immunoglobulin-like loops, which are followed by three fibronectin type III domains (Fig. 1). Members of the Tie family include Tie-1, also known as Tek, and Tie-2 (2). Tie-1 and Tie-2 are specifically expressed in vascular endothelial cells during normal development and pathological conditions such as tumor angiogenesis. The structure of the extracellular domain suggests an involvement of the Tie-proteins in cell-cell adhesion in the vascular endothelium. Mice with a targeted disruption of the Tie-1 gene die immediately after birth as a result of breathing difficulties. Tie-1 ~ embryos fail to establish integrity of vascular endothelial cells, resulting in oedema and localized haemorrhage (19). Mice with a targeted disruption of the Tie-2 gene die at embryonic day 10.5 as a result of malformations in the vascular network (19).

• *Discoidin domain receptor family*

The discoidin domain receptor (DDR) was originally identified as a breast carcinoma phosphoprotein (2). The extracellular domain of DDR, also known as Nep, trkE, Cak, or Ptk-3, has homology to *Dictyostelium dicoideum* lectin dicoidin-I, and contains hydrophilic proline/glycine-rich sequences (Fig.l). Discoidin-I domains show homology with putative phospholipid-binding domains found in other cell adhesion molecules such as the coagulation factors V and VIII and the neuronal recognition protein A5. Discoidin type I domains are also found in Tyro 10, also known as Tkt (2). The catalytic domain of TyrolO exhibits significant similarity to the trk family of neurotrophin receptors.

• *Ror receptor family*

Members of the Ror family (Rorl andRor2) of Rtrk were identified by a polymerase chain reaction-based screen for new members of the trk family. Structural differences in the extracellular domain, which contains a cysteine-rich domain, an immunoglobulin-like domain, and a kringle motif (Fig.l), place the Ror proteins in a separate class of Rtrk (2). The D.

melanogaster homolog of Ror proteins (D-Ror) shows an even greater similarity with the catalytic domain of the trk proteins when compared to the human Ror proteins. Based on its neural-specific expression pattern, it is suggested that D-Ror may encode a neurotrophic receptor that functions during early stages of neural development.

• *Ret receptor family*

The Ret proto-oncogene encodes a Rtrk, in which the extracellular domain contains a sequence homologous to cadherin, a cell adhesion molecule (Fig.l). Germline mutations in the Ret gene are responsible for three different inherited cancer syndromes namely multiple endocrine neoplasia type 2A and type 2B, and familial medullary thyroid carcinoma as well as for Hirschsprung disease (2). Mice with a targeted disruption of Ret gene die shortly after birth. The knock-out mice lack kidneys and enteric neurons pointing to a requirement for Ret in organogenesis and neurogenesis (20).

• *Ryk receptor family*

The Ryk Rtrk, also known as Nyk-r, Vik, Nbtk-1, or Mrk, (2) has a relatively short (-200 amino acids) extracellular domain (Fig.l) and some unusual substitutions at highly conserved residues in their catalytic domain. These substitutions were also found in the *D. melanogaster* counterpart of Ryk, *Drosophila* derailed (Drl) (2). In mice, Ryk is predominantly expressed during hematopoietic development (2). Genetic studies in *D. melanogaster* suggest an involvement for Drl in neuronal pathway recognition (2).

• *Kinase-like gene receptor family*

The kinase-like gene (Klg) protein is a transmembrane protein (2). The extracellular domain contains seven immunoglobulin-like sequences and is homologous to the VEGFR family (Fig. 1). As the catalytic domain lacks the highly conserved DFG motif, it is uncertain whether the Klg protein has intrinsic trk activity

• **Catalytic activity**

• *Ligand-induced dimerization*

Except for the insulin receptor family all Rtrk undergo a transition from a monomeric to a dimeric state upon ligand binding. This dimerization is inititiated through a ligand-induced conformational change of the extracellular domain that stabilizes interactions between adjacent cytoplasmic domains and leads to activation of the catalytic domain by molecular interaction (1,21,22). In the case of the insulin receptor family, that preexists as a dimer of oc-p pairs, ligand binding presum-

ably causes conformational changes in the preformed dimeric receptor that comprise an allosteric transition to yield an active dimer (Fig.2A) (1).

Receptor dimerization can take place between two identical receptor molecules (homodimerization); between different members of the same receptor family (heterodimerization); or between a receptor and an accessory, coreceptor protein (heterodimerization). Ligand binding induces homo- or heterodimerization via two possible mechanisms. It may be mediated by simultaneous binding of a dimeric ligand to two receptor molecules. Examples of dimeric ligands, which thus contain two identical receptor-binding epitopes, include PDGF, CSF-1 and stem cell factor (SCF) (21). In addition to bridging the ligand between both receptors, direct interactions between the receptors involving epitopes located outside the ligand-binding domains may be important for stabilization of the receptor dimer.

A number of other growth factors such as EGF and FGF are monomeric in solution. A monomeric ligand binding can induce conformational changes in the receptor that stabilize its active dimeric form. However, evidence is emerging that both EGF and FGF bind to their cognate receptors in a multivalent manner. In the case of EGF, calorimetric studies indicate that a single EGF molecule can bind simultaneously to two receptor molecules (21). In the case of FGF, plasma membrane heparan sulfate proteoglycans act as coreceptor molecules that bind multiple FGF (23). In this way, FGFR dimerization is induced by multivalent binding of the FGF-heparan complex (Fig.2B) (23).

Ligands for the Eph receptor family are all membrane anchored proteins *via* either glycosylphosphatidylinositol or transmembrane domain (24). These ligands, which are inactive in soluble form, probably facilitate receptor dimerization by their membrane attachment.

• *Homodimerization and heterodimerization*

The occurrence of ligand-induced homo- or heterodimerization provides possible means for increasing the diversity in signaling. In the case of PDGFR family, the two related PDGFR, PDGFRa and PDGFR(5, bind three types of dimeric PDGF ligands. The dimeric PDGF ligands are composed of disulphide linked A- or B-chains in either homodimeric (AA or BB) or heterodimeric (AB) combinations. The A-chain of PDGF binds only to a-receptors, while the B chain binds both a- and p-receptors with high affinity (21,22). In line with this, PDGF AA induces aa-receptor homodimers only, PDGF AB induces aa-receptor homodimers and ap-receptor heterodimers, whereas PDGF BB induces all three combinations of receptors (21,22). It has been observed that each receptor dimer
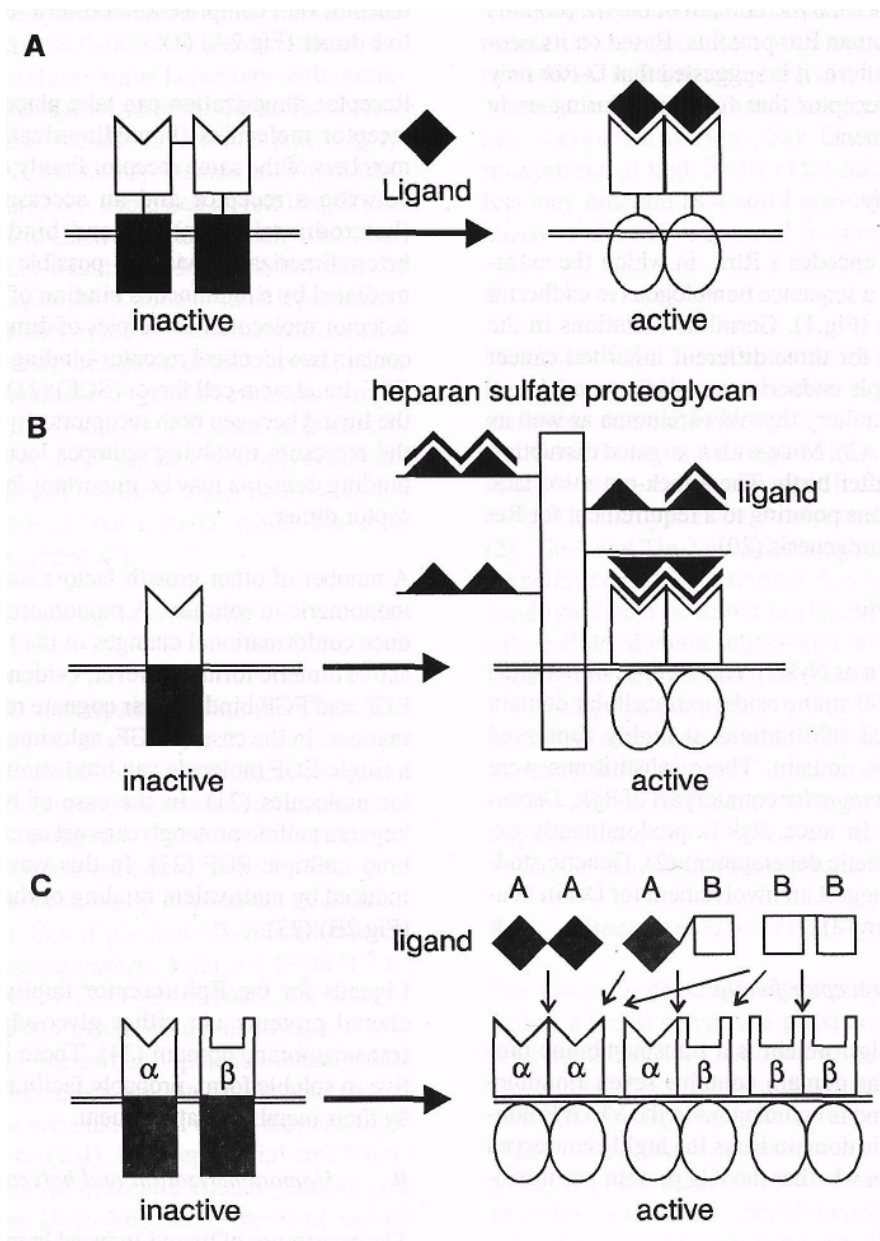
Figure 2. *Models of receptor subclass-specific variations of the mechanism of activation by dimerization. (A) Allosteric activation of disulfide-stabilized receptor dimers. In the insulin receptor family, ligand binding results in conformational changes in the receptor that comprise an allosteric transition to yield an active dimer. (B) Receptor dimerization involving an accessory molecule. In the FGF receptor family, heparan sulfate proteoglycans in the plasma membrane act as accessory molecules that bind multiple FGF molecules. In this -way, FGF receptor dimerization and activation is induced by multivalent binding of the FGF-heparan sulfate proteoglycan complex. (C) Homo- and heterodimerization in the PDGF receptor family. The two related PDGF receptors, PDGFRa and PDGFRfi bind three types of dimeric PDGF ligands. The A-chain of PDGF binds only to a-receptors, -while the B-chain binds both a- and ^-receptors with high affinity. The repertoire of ligand specificity is increased through heterodimerization. As the cytoplasmic domains of the PDGFRaand PDGFRfi contain distinct autophosphorylation sites, specific signals are generated from each receptor dimer formed.*

elicits specific signals with respect to the stimulation of chemotaxis, actin reorganization and mitogenesis (2). Thus, the cell response to PDGF depends on the isoform of PDGF and the type of PDGFR expressed on the target cell (Fig.2C).

Heterodimerization is also observed between members of the EGFR family, EGFR, ErbB2, ErbB3, and ErbB4. Ligands for this receptor family include EOF, transforming growth factor-a, amphiregulin, heparin-binding EGF, betacellulin, and neu differentiation factor (NDF), also known as heregulin (21). So far, it has been reported that EGF binding induces homodimerization of the EGFR (21), and heterodimerization between EGFR/ErbB2, and EGFR/ErbBJ (1,21,22). NDF binding leads to the formation of ErbB3 and ErbB4 homodimers, or ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers (21,22). In analogy to the PDGFR, the formation of homo- or heterodimers in response to ligands for the EGFR family results in the activation of specific signaling pathways leading to different cellular responses. It is interesting to note that ErbB3 lacks certain highly conserved amino acids in the catalytic domain (2), and has no kinase activity. This suggests that ErbB3 acts as a substrate for EGFR and ErbB2 in the heterodimer.

Heterodimeriztion within an existing dimer has been noted between the insulin receptor and the insulin-like growth factor-1 receptor (1).

- *The tyrosine kinase domain*

The trk domain catalyzes the transfer of the y phosphate of a magnesium-adenosine triphosphate complex (Mg-ATP) to a protein substrate. The catalytic domains of serine/threonine kinases and trk are composed of-250-300 amino acids. Within the catalytic domain of distinct trk, sequence identities range from 32 to 95%. Many conserved residues in the catalytic domain of trk are also conserved in the family of serine/threonine kinases (3,25,26).

A 306-residue stretch of the insulin receptor containing the entire catalytic domain has been crystallized (27). The overall structure is similar to the structures obtained from the catalytic domain of the serine/threonine kinases cAMP-dependent protein kinase (PKA) (28), cyclin-dependent kinase 2, extracellular regulated kinase 2, and twitchin kinase. The catalytic domain is composed of two lobes that form a cleft in which Mg-ATP binds. It is assumed that within this cleft the protein substrate and Mg-ATP are brought in close vicinity to each other. The small amino-terminal lobe contains the highly conserved $GlyXGlyXXGlyX(21)Lys^{1030}$ motif and a Glu^{1047} residue which are conserved in all protein kinases. The function of the glycine loop is to anchor the phosphate moiety and, in particular, to help position the y phosphate so that it is poised for transfer. In the structure of PKA, the side chains of Lys^{72}

(Lys1030 in the insmin receptor) and Glu91 (Glu1047) together with the side chain of Asp184 (Asp1150) in the C-terminal lobe form a triad close to the yphosphate of Mg-ATP. Lys72 (Lys1030) is required for ATP-binding by interacting with the a and p phosphates, while Glu91 (Glu1047) and Asp184 (Asp1150) function inthechelation of Mg2+.

The amino-terminal lobe is connected by a single strand to the large C-terminal lobe containing the principal elements of the active site such as the catalytic loop, the activation loop and the peptide-binding site. The relative orientation of the amino- and C-terminal lobe differs between the insulin receptor and PKA. When compared to PKA, the two lobes in the inactive insulin Rtrk domain are rotated away from each other. This orientation holds Lys^{1030}, a residue in the amino-terminal lobe implicated in ATP-binding, at a comparatively large distance from the catalytic residues in the C-terminal lobe.

The catalytic loop in the C-terminal lobe is formed by the highly conserved HRDLAARN (residue 1130-1137) stretch. In the catalytic loop Asp^{1132} (Asp^{166} in PKA) and Asn^{1137} (Asn^{171}) are nearly invariant in both the serine/threonine kinase and trk families. Asp^{1132} is the catalytic base in the phosphotransfer reaction. In the structure of PKA, the side chains of Asn^{171} (Asn^{1137}), Asp^{166} (Asp^{1132}), and Asp^{184} (Asp^{1150}) form a second triad of invariant amino acids implicated in Mg-ATP-binding. In this triad, Asp^{184} (Asp^{1150}) has the potential to shuttle between the two conserved loops, the glycine-rich loop in the small lobe and the catalytic one in the larger lobe. Arg^{1136} (Lys^{168} in PKA) provides charge neutralization.

A marked difference between PKA and the insulin receptor is an extended sequence in the C-terminal lobe, termed activation loop. It contains the three major tyrosine autophosphorylation sites, Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}. The most striking feature of the insulin receptor structure is that one of these residues, Tyr^{1162}, is hydrogen-bonded to the catalytic base, Arg^{1132}, and to Arg^{1136}. The phenolic ring of Tyr^{1162} points to the pyrolidine ring of the conserved Pro^{1172}. Arg^{1136} is also salt-bridged to Asp^{1132} and Asp^{1161}, and has an axial polar interaction with the indole ring of Trp^{1175}. Together, a network of interactions hold Tyr^{1162} in a position that blocks access of ATP to the active site. This structure reveals a novel mechanism of c/5-inhibition in that residues in the activation loop of the unphosphorylated insulin receptor occupy the same space that would otherwise contain ATP. In this way, insertion of $Tyr^{"62}$ represses kinase activity by precluding ATP-binding.

From the ternary structure of the insulin receptor and PKA a model for insulin receptor activation has emerged. In this model, an equilibrium must exist between the m-inhibitory conformation and an "open" conformation, in which Tyr^{1162} is disengaged and Mg-ATP can bind. In this transient "open"

conformation, the kinase can be activated until Tyr[1162] returns to the active site. When insulin binds to the receptor, a conformational change in the heterotetrameric receptor places the phosphorylation sites of one p-chain within the active site of the other p-chain. Intramolecular, /ra/w-phosphorylation occurs when Tyr[1162] is disengaged andMg-ATP can bind. If phosphorylation occurs on one of the tyrosine residues in the active site, the equilibrium shifts to a conformation in which Tyr[1162] is disengaged from the active site and, as a result, the kinase is prone for activation (27). This model points to an essential role for Tyr[1162] in insulin receptor kinase activation. Tyr[1162] is a highly conserved residue in virtually all trk, and it is proposed that phosphorylated Tyr[1162] is salt-bridged to the invariant residue Arg[1131], and probably also Arg[1155], thus stabilizing the non-inhibiting conformation of the active loop.

Once activated, the kinase /raws-phosphorylates tyrosine residues located within the cytoplasmic part of the receptor. The autophosphorylation of the receptor serves as a molecular switch to induce association and activation of cytoplasmic signaling molecules to the activated receptor.

SIGNAL TRAMSDUCTIOH OF RECEPTOR

TYROSINE KIHASES

• **Protein-protein interactions**

Receptor-binding proteins all contain one or more copies of a protein module, termed Src homology 2 (SH2) domain, that directly recognize phosphotyrosine containing sequences, and which are responsible for the interaction of receptor-binding proteins with autophosphorylated receptors (29-31). In addition to SH2 domains, recent studies have identified a second phosphotyrosine-binding domain, termed phosphotyrosine interaction domain (PID) (32). SH2 and PID mediated interactions are important for the activation of signaling pathways initiated by trk. Besides phosphotyrosine binding sequences, the substrates of Rtrk often contain distinct protein modules implicated in the regulation of protein-protein interactions in signal transduction (Fig.3). These domains include SH3 domains (29-31) and pleckstrin homology (PH) domains (33-36).

Src homology 2 domains

SH2 domains have originally been described as regions of homology (~100 amino acids) between the non-Rtrk c-Src, c-Fps, and c-Abl, that lie outside the catalytic domain and are not required for kinase activity (29,30). Subsequently, SH2 domains have also been found in proteins that interact with activated Rtrk, such as phospholipase Cy (PLCy), phosphatidyl-inositol-3'-kinase (PI$_3$,-kinase) and p21[rai] GTPase activating protein (rasGAP) (37), suggesting that SH2 domains may be a common feature of proteins that bind to activated Rtrk.

Studies on the *v-Crk* oncogene of the avian sarcoma virus CT10 subsequently show that the SH2 domain of *v-Crk* interacts with cellular proteins in a tyrosine phosphorylation-dependent manner (38). This implicates SH2 domains in the regulation of protein-protein interactions by recognizing peptide sequences that encompass tyrosine phosphorylation sites (37).

While SH2 domains share the common property of binding phosphotyrosine-containing sequences, binding specificity is determined by only a few amino acids located adjacent to the phosphotyrosine. This has been demonstrated by the differential binding of SH2 domains of the p85a subunit of PI$_3$,-kinase, rasGAP and PLCyto activated growth factor receptors (39,40). Peptide competition and receptor mutagenesis experiments reveal that 3-5 amino acids C-termini to the phosphotyrosine are sufficient to capture the essential elements of sequence-specific recognition (40).

The ternary structure of the SH2 domains of several proteins has clarified the mechanisms of phosphotyrosine and peptide recognition (41). The ternary structures of the Src, Lck, PLCy, and SH-PTP2 SH2 domains complexed with phosphotyrosine containing peptides (42,43,44), and the solution structures of the SH2 domains of the p85a subunit of PI,,-kinase (45) and c-Abl (46) in absence of bound ligands has been resolved. The obtained ternary structures are strikingly similar and share a common secondary structure framework and considerable similarity in ternary folding. In the ternary structure, the amino- and C-terminal ends of the SH2 domains are close together in a globular structure, allowing the SH2 domain to protrude from the rest of the protein, and function independently. ;

The SH2 domain is composed of two a-helices, termed aA and aB, and seven p-strands, termed PA through PG, located in the order appppppapp (47). The spine of the Src SH2 domain is an uninterrupted p-meander that forms two distinct p-sheets. The sheets are connected by a single continuous p-strand, termed PD, in the central p-sheet and PD' in the second. The central three-stranded antiparallel P-sheet (strands PB, pC. PD) divides the SH2 domain into two distinct sides. One side, containing a-helix a A and one face of the central P-sheet, is primarily involved in phosphotyrosine binding. The other side of the SH2 domain, containing a-helix aB, the smaller P-sheet (strands PD', PE, pF), a long loop termed BG, and the other side of the central P-sheet. is implicated in the binding of three peptide residues C-termini to the phosphotyrosine. The peptide recognition site runs along a flat surface perpendicular to the central P-sheet, whereas the phosphotyrosine is contained in a pocket located on this surface.

The interactions in the phosphotyrosine pocket are very similar between the Lck and Src complex (43,44). The side chain of the phosphotyrosine lies in a deep, elongated groove formed
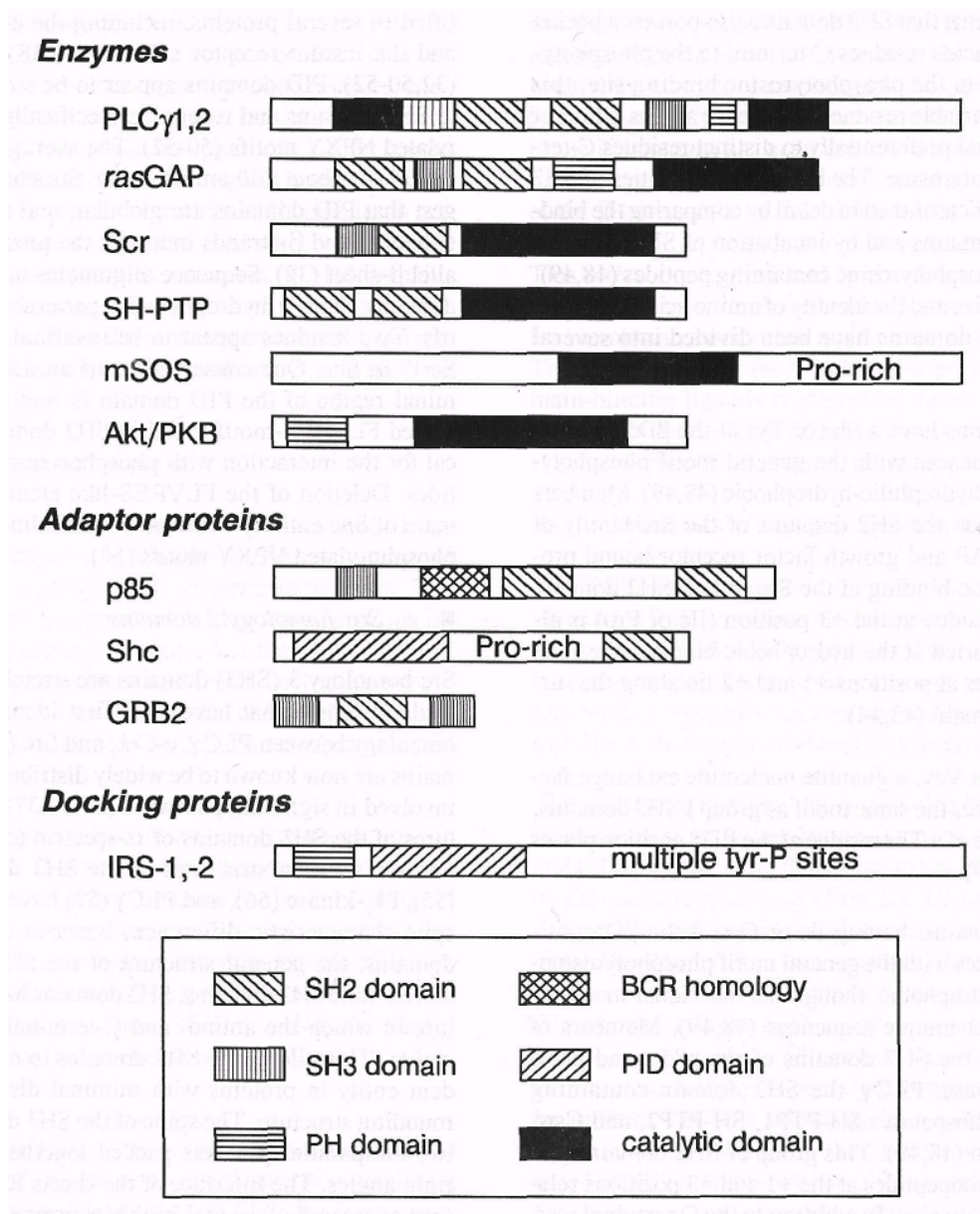
Figure 3. *Modular structure of receptor-binding proteins and other proteins involved in signal transduction. The functions and abbreviations of the distinct domains and of the proteins depicted are outlined in the text.*

by parts of the PB, PC, and PD strands, the aA helix, and the BCloop ("phosphate-binding" loop). Several highly conserved residues in this pocket interact directly with the bound phosphotyrosine. The side chains of HispD4 and LyspD6 and the main-chain atoms of TyrpD5 form a platform beneath the aromatic ring of the phosphotyrosine. The phosphate moiety is held by a network of hydrogen bonds, in which two arginine residues are of particular importance. ArgocA2 donates

four hydrogen bonds to the phosphotyrosine peptide: one to the phosphate oxygen, one amino-aromatic bond to the phosphotyrosine ring, and two to the main chain carbonyl of pY-1. This arginine residue is therefore critical for the conformation of the entire phosphotyrosine residue. The second arginine residue ArgpB5 makes bidentate hydrogen bonds with the phosphate moiety of the phosphotyrosine.

Besides the conserved phosphotyrosine binding pocket, the structural data indicate that SH2 domains also possess a pocket for the 3-6 amino acids residues C-termini to the phosphotyrosine. In contrast to the phosphotyrosine binding site, this pocket is lined by variable residues, and hence allows different SH2 domains to bind preferentially to distinct residues C-termini to the phosphotyrosine. The binding specificities of SH2 domains have been determined in detail by comparing the binding sites of SH2 domains and by incubation of SH2 domains with degenerate phosphotyrosine containing peptides (48,49). Based on these studies and the identity of amino acid pD5 in the SH2 domain, SH2 domains have been divided into several categories (48,49).

Group I SH2 domains have a Phe or Tyr at the PD5 position and recognize sequences with the general motif phosphotyrosine-hydrophilic-hydrophilic-hydrophobic (48,49). Members of this group include the 8112 domains of the Src family of non-Rtrk, of rasGAP and growth factor receptor-bound protein 2 (GRB2). Upon binding of the Src or Lck SH2 domain, the hydrophobic residue at the +3 position (He or Pro) is almost completely buried at the hydrophobic binding site. The hydrophilic residues at positions +1 and +2 lie along the surface of the SH2 domain (43,44).

The SH2 domain in Vav, a guanine nucleotide exchange factor for *ras,* recognizes the same motif as group ISH2 domains, though the presence of a Thr residue at the PD5 position places Vav in group II (49).

Group III SH2 domains, having He or Cys at the PD5 position, select sequences with the general motif phosphotyrosine-hydrophobic-X-hydrophobic, though the individual members of this group select unique sequences (48,49). Members of this group include the SH2 domains of the p85oc and p85 P subunit of PI$_3$,-kinase, PLCy, the SH2 domain containing phosphotyrosine phospatases SH-PTP1, SH-PTP2, and Csw, and the She protein (48,49). This group of SH2 domains primarily binds phosphopeptides at the +1 and +3 positions relative to the phosphotyrosine. In addition to the C-terminal residues, evidence is emerging that also residues amino-termini to the phosphotyrosine may be involved in sequence specific binding of some SH2 domains.

To date, SH2 domains have been identified in many proteins, that include enzymes, adaptors, structural proteins, and transcription factors. The binding of activated Rtrk to SH2 domain-containing proteins provides a mechanism for recruiting substrates and targets to activated Rtrk dimers.

Phosphotyrosine interaction domains

In addition to SH2 domains, a distinct PID, also known as

She and IRS-1 NPXY-binding domain (SAIN), has been identified in several proteins, including the adapter protein She, and the insulin receptor substrates (ERS), IRS-1 and IRS-2 (32,50-52). PID domains appear to be structurally unrelated to SH2 domains and recognize specifically tyrosine phosphorylated NPXY motifs (50-52). The average length of the PID domain is about 160 amino acids. Structure predictions suggest that PK) domains are globular, and the arrangement of a-helices and p-strands indicates the presence of an antiparallel p-sheet (32). Sequence alignments have shown that PID domains share a hydrophobicity pattern and conserved motifs. Two residues appear to be invariant, notably Gly[38] and Ser[151] in She. One conserved motif located in the ammo-terminal region of the PID domain is reminiscent of the conserved FLVRES-motif found in SH2 domains which is critical for the interaction with phosphotyrosine-containing peptides. Deletion of the FLVRES-like element in the Pro domain of She entirely eliminates the binding of She to tyrosine phosphorylated NPXY motifs (50).

• *Src homology 3 domains*

Src homology 3 (SH3) domains are stretches of 55-70 amino acids in length that have been first identified as regions of homology between PLCy, *v-Crk,* and Src (29,30,37). SH3 domains are now known to be widely distributed among proteins involved in signaling pathways (29,30,37). The crystal structures of the SH3 domains of oc-spectrin (53) and c-Fyn (54), and the solution structures of the SH3 domains from c-Src (55), PI$_3$,-kinase (56), and PLCy (57) have been resolved. Despite characteristic differences between the individual SH3 domains, the general structure of the SH3 domains is conserved. Like SH2 domains, SIC domains have a globular structure in which the amino- and C-terminal ends are close together. This allows the SH3 domains to exist as an independent entity in proteins with minimal disruption of the surrounding structure. The spine of the SH3 domain is formed by two antiparallel p-sheets packed together at approximately right angles. The interface of the sheets forms a hydrophobic core composed of several highly conserved residues. The hydrophobic pocket may serve as a binding site for target proteins.

A number of SH3 domain-binding proteins have been identified, and subsequent sequence comparisons have led to the recognition that a short proline-rich peptide motif of approximately 10 amino acids binds to SH3 domains (58). Screening of combinational libraries of proline-rich peptides identify two classes of peptides as ligands for Src and PL,-kinase SH3 domains (59): class I ligands have a consensus sequence RXLPPZP (Z=L for Src SH3; Z=R for PI$_3$,-kinase SH3), and class II ligands contain a XPPLPXR motif. Structural studies of SH3 domains complexed with proline-rich ligands indicate

that the bound ligands adopt a left-handed polyproline type II helix (59). In this helix, two prolines interact directly with the surface of the SH3 domain. Other prolines contribute to the formation of the helix, whereas the non-proline residues, consisting of combinations of arginine and leucine, interact extensively with the SH3 domain and apparently confer ligand specificity. Remarkably, structures of ligand-bound SH3 domains reveal that SH3 domains bind class I ligands in an orientation that is opposite to that observed for bound class II ligands (59). This indicates that activation of SH3 domain-mediated pathways does not only depend on the subset of sequences bound by the SH3 domain, but also on the correct orientation required for the assembly of an active signalling complex.

• *Pleckstrin homology domains*

The pleckstrin homology (PH) domain is a sequence of approximately 120 amino acids homologous to two regions of pleckstrin which is the major PKC substrate in platelets. This region of homology is frequently found in many proteins involved in signaling pathways (33-36). More than 70 PH domain containing proteins have been identified, including serine/threonine kinases, trk, sevmreal isoforms of PLC, GAP, nucleotide exchange factors, adaptors, and cytoskeletal proteins (33-

36)

PH domains are implicated in intramolecular interactions in signaling pathways. This notion has emerged from the finding that a naturally occurring mutation in the PH domain of Bruton's tyrosine kinase (Btrk) is sufficient to cause the human genetic disease agammaglobulinaemia. Moreover, *in vivo* functional analysis of the *D. melanogaster* guanine nucleotide exchange factor son of sevenless (SOS) revealed that the PH domain is essential for the development of the *D. melanogaster* eye.

The crystal structure for the PH domain of dynamin and the solution structure of the PH domains of spectrin, pleckstrin and dynamin has been determined (60,61). The basic structure of the molecule is an antiparallel p-sheet consisting of seven p-strands, with a long a-helix at the C-terminus. The p-sheet has a strong bend, that is stabilized by the a-helix packing. Opposite this helix are three loops that vary among most PH domains. The overall structure of the PH domain is electrostatically polarized, with the three variable loops forming a positively charged surface that may serve as a ligand binding site. This positively charged surface includes the position of mutation in the Btrk PH domain.

The structure of the PH domain shows similarity to the structure of lipid-binding proteins. A characteristic shared by all PH domain-containing proteins is the functional importance

of membrane localization (33-36,60). In line with a putative membrane targeting role for PH domains, fusion proteins containing PH domains of several proteins bind specifically to the negatively charged phospholipid phosphatidyl-inositol-4,5-biphosphate (62). In these experiments, the ammo-terminus of the PH domain has been identified as the important site of lipid interaction. In addition to lipid binding, the PH domain of the p-adrenergic receptor kinase-1 has been demonstrated to bind to the $G_{p\gamma}$ subunits of heterotrimeric G proteins (60). This interaction is mediated by the C-terminus of the PH domain (60). Both PH domain-binding ligands are neccessary for membrane association and activation of the kinase (60). The requirement for the simultaneous presence of two PH domain-binding ligands represents a novel mechanism for effecting membrane localization of a protein and may have relevance to other PH domain-containing proteins.

• **Substrates of receptor tyrosine kinases**

The binding of cytoplasmic signaling molecules to autophosphorylated Rtrk provides a mechanism for recruiting substrates and targets to activated Rtrk dimers. The receptor-binding proteins all contain one or more copies of SH2 or PDD domain that directly recognizes the phosphotyrosine-containing binding sites in the autophosphorylated receptor.

The specificity in signaling is provided by sequence specific recognition of phosphotyrosine-containing sequences by SH2 and PID domains, though appears to be ultimately determined by the catalytic specificity of the trk. Using a degenerate peptide library, with peptides with the sequence Met-Ala-XXXX-Tyr-XXX-Ala-Lys-Lys as a substrate, the substrate specificity of trk has been investigated (63). Most trk select peptides with Glu or Asp residues at the -4 to -2 positions relative of the Tyr. Rtrk also prefer a Glu at the -1 position, while the non-Rtrk preferentially phosphorylate tyrosine residues preceded by an lie or Val. The selectivity for residues C-termini to the Tyr is strikingly similar to the phosphopeptide motifs recognized by SH2 domains. All trk show a selection for hydrophobic residues at the +3 position. Rtrk phosphorylate a general motif Tyr-hydrophobic-X-hydrophobic, a sequence generally recognized by group III SH2 domains as found in the p85 subunit of PI_3-kinase, PLCy, and SH-PTP2 (48,49). In contrast, non-Rtrk show a preference for Tyr-hydrophilic-hydrophilic-hydrophobic, a sequence recognized by group ISH2 domains (48,49).

As described earlier, SH2 and Pro domains are found in many proteins. Based on their structural properties, the receptor-binding proteins have been classified into distinct classes (29). The first class of receptor-binding proteins is formed by proteins with known enzymatic activity. The binding of the enzymes to the receptor can facilitate efficient tyrosine phospho-

rylation leading to activation of the enzyme, or may induce allosteric activation of the bound enzyme. Members of this class include PLCy, *rasGAP,* SH2 domain containing protein tyrosine phosphatases, and non-Rtrk. The second class of receptor-binding proteins, termed adaptors, are devoid of any enzymatic activity. These proteins are often composed of SH2 and SH3 domains, and function as intermediates between Rtrk and downstream signaling molecules. Members of the adaptors family include the p85 subunit of PI_3,-kinase, GRB2, Crk, Nek, and She. Docking proteins represent the third class of receptor-binding proteins. They have no enzymatic activity, but act as an Rtrk surrogate by providing multiple SH2 domain binding sites following tyrosine phosphorylation by an activated Rtrk. Members of this class include IRS-1 and IRS-

The recruitment of SH2 or PID domain-containing proteins to activated Rtrk or to a tyrosine phosphorylated docking protein represents the first step in the activation of signaling cascades. The best characterized signaling pathways in fibroblasts initiated by activated Rtrk are described below.

- **Activation of the p21"" signaling pathway**

The p2 1 "" genes are the cellular counterparts of the transforming genes of the Harvey and Kirsten strains of rat sarcoma virus, and of the transforming gene of a neuroblastoma cell line. The products of the p21™ genes are small GTP-binding proteins of approximately 21 kD localized at the inner side of the plasma membrane. By analogy to other GTP-binding proteins, p2 1 "" cycles between an inactive GDP-bound state and an active GTP-bound state (Fig. 4) (64-66). In the oncogenic forms of p21^{TO} which have been detected in a variety of human tumors, point mutations at positions 12, 13 or 61 lock the protein in a constitutively activated GTP-bound state (67).

Genetic evidence from *D. melanogaster* and *C. elegans,* microinjection studies with neutralizing antibodies and overexpression of p21^{ras} proteins in fibroblasts have placed p21"* in signal transduction downstream of Rtrk (2). A wide variety of growth factors increase the intracellular levels of p21""-GTP (2). Moreover, cells expressing oncogenic forms of trk such as *v-Src, v-Abl, oiv-ErbB2* also exhibit increased levels ofp21^{ral}-GTP (2).

The p2 1 "* guanine nucleotide content can be regulated at two levels. First, at the level of exchange of GDP for GTP by guanine nucleotide exchange factors (GNEF) (Fig.4). Second, at the level of hydrolysis of p21""-GTP, a process catalyzed by GTPase activating proteins (GAP) (64-66,68) (Fig.4). Evidence has been obtained that growth factors enhance the exchange rate of GDP-bound to p21^{ray} by exogeneous guanine nucleotides, indicating an involvement of GNEF in the formation

of p21™-GTP (69,70). Based on homology with the catalytic domain of *S. cerevisiae* and *D. melanogaster* GNEF for *ras* (cdc25 and SOS protein, respectively), several putative mammalian GNEF have been identified, including mSOS (71). Despite the cloning of GNEF, the mechanism by which activated trk couple to p21"" remains unclear, though the identification of the GRB2 protein contributes to the elucidation of the activation mechanism.

GRB2 was originally identified by screening a protein expression library with the autophosphorylated C-terminal tail of the EGFR and by screening a cDNA library with degenerate oligonucleotides recognizing SH2 domains (72). GRB2 encodes a 23 kD protein that is devoid of any enzymatic function and consists almost entirely of two SH3domains separated by one SH2 domain. Genetic counterparts for GRB2 have been isolated from *C. elegans* (Sem-5; sex muscle abnormal) and *D. melanogaster* (Drk; downstream of receptor kinases) (73-75).

GRB2 associates through its SH2 domain to tyrosine phosphorylated phosphotyrosine-hydrophilic-hydrophilic-hydrophobic sequences with a strong preference for Asn at the +2 position (49). Many activated Rtrkbind GRB2 directly, though in some cases the binding of GRB2 requires an adaptor protein, such as She, SH-PTP2, RPTPoc, IRS-1 or IRS-2 (2). An involvement of GRB2 in the regulation of p21"" comes from the observation that overexpression of GRB2 enhances the activation of p21$^{"K}$ by EGF (76) and the activation of ERK2 by insulin (77). Moreover, coinjection of GRB2 together with P21$^{™l}$ leads to the induction of DNA synthesis in quiescent fibroblasts, while injection of GRB2 or p21^{rai} alone has no effect (72). Also genetic evidence from *C. elegans* places GRB2 in a pathway upstream of p21"". GRB2 is the mammalian homologue of the *C. elegans* Sem-5 (73). In *C. elegans,* the induction of vulval differentiation is regulated by the Let-23 trk and the Let-60 *ras* protein. Mutation of the Sem-5 allele abrogates vulval development, and experimental evidence places Sem-5 downstream of Let-23 and upstream of Let-60 (73).

Genetic analysis of the *D. melanogaster* Drk protein suggests that Drk function is essential for signaling of the sevenless Rtrk that mediates proper specification of the R7 photoreceptors in the *D. melanogaster* eye (74,75). In these studies, the biological activity of Drk correlates with the binding of the Drk SHE domain to the activated sevenless receptor and concomitant localization to the plasma membrane. A major breakthrough in the investigation of the mechanism for linking trk to p21^{rai} is the finding that Drk SH3 domains bind *in vitro* to proline-rich sequences in the C-terminal tail of SOS (74,75). SOS is a *D. melanogaster* guanine nucleotide exchange factor which is, like *ras* and Drk, required for sevenless signaling (71).
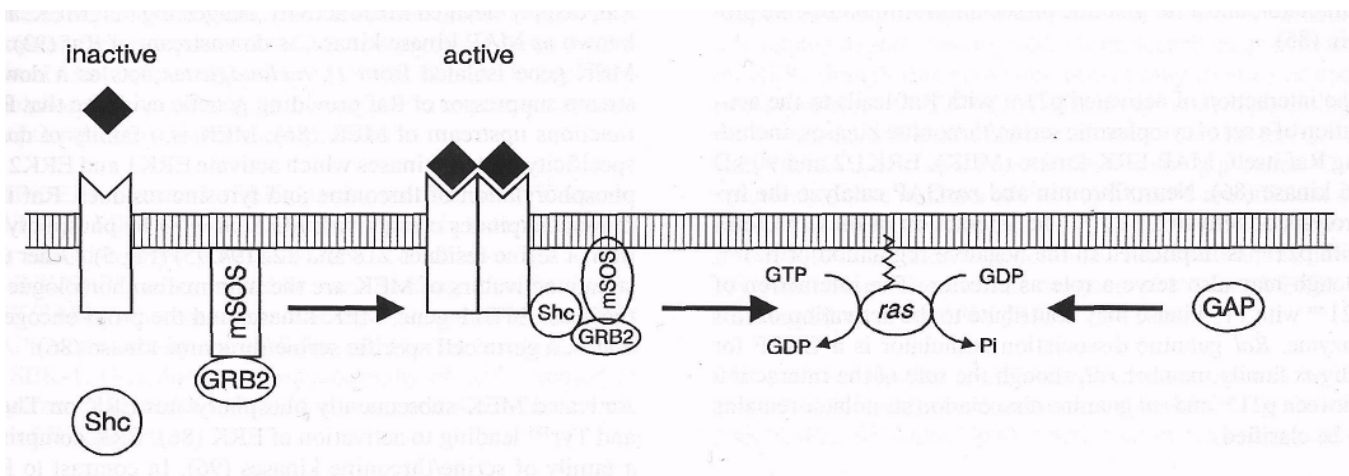
Figure 4. Regulation of p21^{ras} guanine nucleotide content by receptor tyrosine kinases. Upon ligand induced activation of receptor tyrosine kinase, She can bind to phosphotyrosine residues within the activated receptor. The activated receptor tyrosine kinase induces tyrosine phosphorylation of She which is followed by the association of GRB2. Alternatively, GRB2 can bind directly to an activated receptor tyrosine kinase. The SH3 domain of GRB2 is bound to the C-terminal tail of mSOS. The interaction of GRB2/mSOS with phosphotyrosine residues probably induces a conformational change that relieves the inhibitory constraint of the C-terminal tail of mSOS. In this way, active mSOS can promote the exchange of GDP for GTP on p21^{ras}. p21^{ras}-GTP is inactivated by hydrolysis of GDP to GTP which is catalyzed by GTPase activating proteins (GAP).

Subsequently, it has been demonstrated that GRB2 binds to the mammalian homolog of SOS (mSOS) and that this complex exists constitutively (78-82). From this result, it has been proposed that the binding of the GRB2/mSOS complex to an activated Rtrk translocates mSOS to the vicinity of membrane-bound p21"". As a result of this translocation, the exchange of GDP for GTP on $p2l^{TM}$ may be stimulated (Fig.4).

In vivo structure-function analysis of SOS reveals that the amino-terminus of SOS is essential for its function in *D. melanogaster*. The amino-terminal region contains a PH domain which is implicated in membrane binding. In line with this membrane recruitment model, modified mSOS proteins that are constitutively located at the plasma membrane activate the $p2l''''$ signaling pathway without external stimuli. These membrane-targeted mSOS derivatives are even more active upon deletion of the C-terminal region, the binding site for the GRB2 SH3 domains. This suggests that a secondary activation mechanism may rely on a conformational change induced by binding of GRB2/mSOS to tyrosine phosphorylated proteins that relieves the inhibitory effect of the C-terminal domain of mSOS (Fig. 4).

Besides interacting with activated Rtrk, the 8112 domain of GRB2 also binds to tyrosine phosphorylated Src-homology

collagen (She) (83). The primary structure of She contains an amino-terminal PID domain and a C-terminal SH2 domain separated by a glycine/proline-rich motif with regions of homology to the a,-chain of collagen (50,51,83). She has been implicated in p21"* activation, since its overexpression transforms cells and induces p21 ""-dependent neurite outgrowth (83,84). She interacts with several activated Rtrk through its PID and/or SH2 domain (2), and as a result She becomes tyrosine phosphorylated. Tyrosine phosphorylated She associates with GRB2/mSOS (84) (Fig.4). This interaction is presumably mediated by the binding of the GRB2 SH2 domain to a tyrosine phosphorylated YVNV motif within the She protein. In this way, the interaction of tyrosine phosphorylated She with GRB2/mSOS provides an alternative mechanism for the activation of the p21"^K signaling pathway by Rtrk.

Effectors of p21™

Activated p21^{OT} exerts its effects by interacting with one or more cellular effector molecules. Mutations in p21™ which abrogate its biological activity without reducing GTP-binding have delineated a domain in p21™ that associates with effector molecules, termed effector domain, to a region between amino acids 35-40. Among the proteins that physically interact with the effector domain in a GTP-dependent manner are

neurofibromin, Raf , PL,-kinase, a *ral* guanine dissociation stimulator, and a *ral* guanine dissociation stimulator-like protein (85).

The interaction of activated P21"* with Raf leads to the activation of a set of cytoplasmic serine/threonine kinases, including Raf itself, MAP-ERK-kinase (MEK), ERK1/2 and 90 kD S6 kinase (86). Neurofibromin and rasGAP catalyze the hydrolysis of activated p21"*. The interaction of these proteins with pll"" is implicated in the negative regulation of p21[rai], though may also serve a role as effector. The interaction of p21"* with PI$_3$-kinase may contribute to the activation of this enzyme. *Ral* guanine dissociation stimulator is a GNEF for the *ras* family member *ral,* though the role of the interaction between p21 "* and *ral* guanine dissociation stimulator remains to be clarified.

• *Activation of the Raf/MEK/ERK pathway*

As described above, GTP-bound p21"" interacts with the amino-terminal domain of the serine/threonine kinase Raf (87-89). However, p21[ras]-GTP does not stimulate the kinase activity of Raf *in vitro* (88). Furthermore, purified active Raf is not associated with p21[rar] and thus appears to be locked into the active state through a covalent modification, or through high-affinity interaction with an unknown regulator (90). This raises the possibility that p21"" functions as a regulated, membrane-bound anchor to recruit Raf to the plasma membrane. Indeed, modified Raf proteins that associate with the plasma membrane are constitutively active, and under these circumstances independent of *pll*™ (91,92).

However, several lines of evidence point to an involvement of additional factors in the activation of Raf. First, growth factors can further activate membrane-bound Raf *via* a mechanism independent of P21"" (91). This suggests that other kinases are required for full activation and phosphorylation of Raf. Second, once deposited to the plasma membrane, Raf becomes associated with structural elements as it cannot be released from the plasma membrane with detergent (92). These components are expected to contain the missing link that lock Raf in its kinase-active state.

A putative group of proteins that may contribute to Raf activation are members of the family of 14-3-3 proteins. The 14-3-3 P and 14-3-3 £ isoforms interact with both the amino-terminal and the kinase domain of Raf, though without interfering with the binding of Raf to p21™-GTP (90). 14-3-3 proteins associate with Raf in mammalian cells and accompany Raf to the plasma membrane during the process of activation. Moreover, both 14-3-3 (3 and 14-3-3 £ activated Raf *in vitro,* thus suggesting that 14-3-3 proteins may participate in or be required for activation of Raf.

Cells transformed with v-Raf, or the oncogenic mutants of c-Raf, display elevated MEK activity, suggesting that MEK, also known as MAP kinase kinase, is downstream of Raf (93). A MEK gene isolated from *D. melanogaster* acts as a down-stream suppressor of Raf providing genetic evidence that Raf functions upstream of MEK (86). MEK is a family of dual-specificity protein kinases which activate ERK1 and ERK2 by phosphorylation of threonine and tyrosine residues. Raf immunoprecipitates can activate MEK *in vitro* by phosphorylation of serine residues 218 and 222 (94,95) (Fig.5). Other upstream activators of MEK are the mammalian homologue of the yeast STE11 gene, MEK kinase, and the proto-oncogene *c-mos,* a germ cell specific serine/threonine kinase (86).

Activated MEK subsequently phosphorylates ERK on Thr[183] and Tyr[185] leading to activation of ERK (86). ERK comprises a family of serine/threonine kinases (96). In contrast to Raf and MEK, ERK appears to have multiple substrates, including phospholipase A2, 90 kD S6 kinase, the transcription factors *myc, fun,* Elk-1, and PHAS-I (96). ERK also phosphorylates mSOS which may represent a negative feedback of the p21™VERK pathway (97).

• *Activation of the JNK/SAPK and other MAPK pathways*

In addition to the p21 ""/Raf/MEK/ERK pathway, several MAP kinase (MAPK)-related pathways have been characterized (Fig.5). These pathways are regulated by a three-component protein kinase cascade consisting of a serine/threonine protein kinase (MAPKKK), which phosphorylates and activates a dual-specificity kinase (MAPKK) that in turn activates another serine/threonine kinase (MAPK). The MAPK serve to link signals from the plasma membrane to the cytosol and the nucleus, and are implicated in the regulation of cell shape, osmotic integrity, and pheromone responses in yeast, stress responses in mammalian cells, and cytokine signaling (96). In mammalian cells, at least three MAPK-related families exist, including the *jun* N-terminal/stress-activated protein kinase (JNK/SAPK), /bs-regulating kinase (FRK), andMAPKAPkinase-2 reactivating kinase (RK/MPK2/p38) (96). These subgroups are distinguished by the sequence of the tripeptide dual specificity phosphorylation motif that is required for activation: Thr-Glu-Tyr (ERK), Thr-Pro-Tyr (JNK/SAPK), andThr-Gly-Tyr (RK/MPK2/p38).

The JNK/SAPK family comprises several isoforms, the human 46 kD JNK1 and 54 kD JNK2 protein, and the rat p54a, p54p, and p54y SAPKs (98). JNK/SAPK are potently activated in response to cellular stress, ultraviolet radiation, incubation with protein synthesis inhibitors, genotoxic agents, interleukin-1 and tumor necrosis factor-a (TNF-a), and to a lesser extent by oncogenic p21[OT], EOF andNGF. The activation of JNK/SAPK by EOF and by NGF depends on p21"", whereas JNK/SAPK ac-

tivation by TNF-a is p21^TO-independent (96,98). JNK/SAPK binds to the ammo-terminal transactivation domain of the transcription factors *c-jun* and ATF-2, and phosphorylates serines 63 and 63 *of c-jun* and threonines 69 and 71 of ATF-2 (96,98). The phosphorylation of ATF-2 and *c-jun* is implicated in transcriptional activation of these proteins.

SAPK/ERK kinase-1 (SEK-1), also known as JNK kinase (JNKK or MKK4), a dual-specificity protein kinase structurally related to MEK, phosphorylates the Thr ^{183}-Pro-Tyr^{185} motif of JNK/SAPK *in vitro* and *in vitro* leading to activation of JNK/SAPK, whereas an inactive SEK-1 mutant blocks JNK/SAPK activation by extracellular stimuli (96,98). In addition to SEK-1, Q-sepharose chromatography of cells exposed to hyperosmolar media indicate the existence of another activator of JNK/SAPK.

SEK-1 on its turn is activated through phosphorylation by MEKK (96,98). MEKK, a serine/threonine kinase structurally related to Raf, has originally been identified as activator of MEK, though this activation occurs only *in vitro* or upon overexpression of MEKK (86). When expressed at physiological levels, no MEK activation can been seen. In analogy to Raf, MEKK is activated by small GTP-binding proteins. Constitutively active mutants of Racl and Cdc42 are efficient activators of the kinase cascade leading to JNK/SAPK activation (99,100). Studies with dominant negative mutants identify Racl as an intermediate between P21"* and MEKK in Rtrk signaling toward JNK/SAPK (99,100).

The pathway leading to RK/MPK2/p38 activation is highly related to the pathway regulating JNK/SAPK (Fig.5). Like JNK/SAPK, RK/MPK2/p38 is activated in response to cellu-
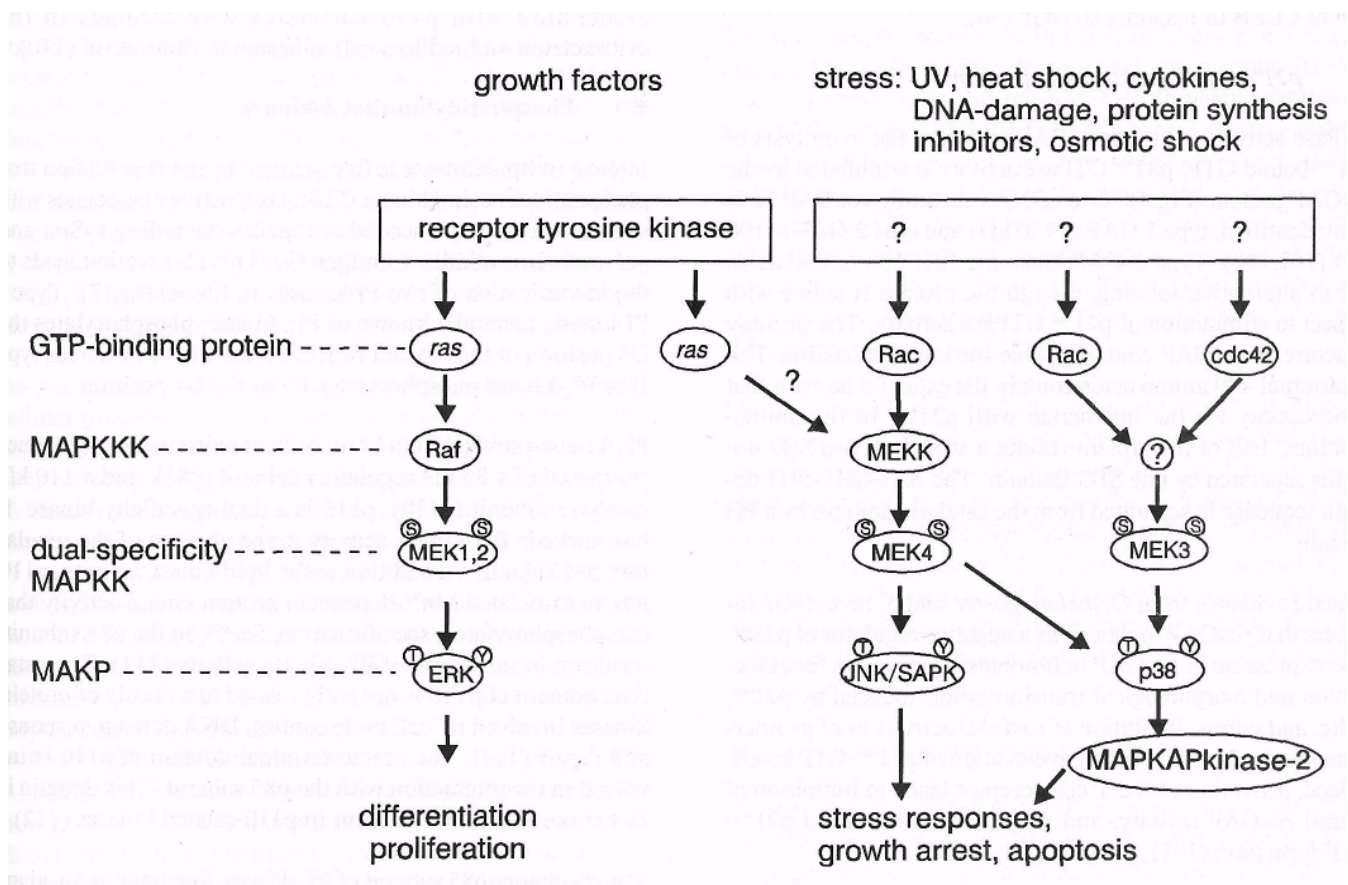

Figure 5. *MAP kinase pathways in mammalian cells. MAP kinase pathways in mammalian cells contain a central core of GTP-binding protein, a serine/threonine kinase (MAPKKK), a dual-specificity kinase (MAPKK) and a serine/threonine kinase (MAPK). The activation of the ERK pathway is implicated in proliferation and differentiation, while JNK/SAPK and p38 are involved in the regulation of stress responses and apoptosis.*

lar stress, such as heat shock or osmotic stress, and by interleukin-1 (98). The activation of RK/MPK2/p38 leads to activation of MAPK-activated protein kinase-2 (MAPKAP kinase-2) which subsequently phosphorylates small heat shock proteins (98). Activation of RK/MPK2/p38 is mediated by phosphorylation of the Thr^{180}-Gly-Tyr^{182} motif. RK/MPK2/p38 is specifically activated by the dual-specificity kinase MKK3 (98). Also SEK-1 activates RK/MPK2/p38 *in vitro,* though coexpression of SEK-1 and its activator MEKK does not lead to a marked activation of RK/MPK2/p38 (98). This implicates a novel uncharacterized MEKK related protein kinase in the regulation of the RK/MPK2/p38 kinase cascade. Upstream of the MEKK-related protein kinase lies the small GIF-binding proteins Racl and Cdc42 (99,100).

Other putative p21 ""-dependent MAPK pathways include FRK (96), which phosphorylates the transactivation domain of Fos, and an unidentified kinase responsible for the phosphorylation of CREB in response to NGF (96).

- **p21^ras GTPase activating proteins**

GTPase activating proteins (GAP) catalyze the hydrolysis of p21""-bound GTP. p21™* GTPase activity is stimulated by the *rasGAP* protein (Fig.4). Two cDNAs encoding rasGAP have been identified, type 1 GAP (-120 kD) and type 2 GAP (-100 kD) (101,102). Type 2 GAP lacks the first 180 amino acids due to alternative splicing, though the protein is active with respect to stimulation of p21^ras" GTPase activity. The primary structure of rasGAP contains some intriguing domains. The C-terminal 400 amino acids contain the catalytic domain that is neccessary for the interaction with p21^raj. In the amino-terminus, half of the protein resides a stretch of two 8112 domains separated by one SH3 domain. The SH2-SH3-SH2 domain sequence is separated from the catalytic domain by a PH domain.

Genetic evidence from *D. melanogaster* and *S. cerevisiae* indicates that rasGAP functions as a negative regulator of p21 ^r<*. Overexpression of *rasGAP* in fibroblasts suppresses focus formation and morphological transformation induced by p21™, v-Src. and v-Fms. Inhibition of rasGAP activity or of its interaction with p21"* may result in elevation of p21^m-GTP levels. Indeed, activation of the T cell receptor leads to inhibition of overall rasGAP activity, and may thus contribute to p21^r<"-GTP formation (103).

The SH2 domains of rasGAP are implicated in the interaction of rasGAP with activated Rtrk, including PDGFR, EGFR, insulin receptor, and HGFR (2). Upon this association, rasGAP becomes tyrosine phosphorylated and associates with two other phosphoproteins, p62 and p!90 (104). Cells expressing mutant PDGFR that lack rasGAP binding site, display normal p21ras-GTP formation, suggesting that this interaction is not involved in the process (40).

Although rasGAP has been purified as a negative regulator for p21"*, rasGAP may also contribute positively to signaling. Expression of the SH2-SH3-SH2 stretch of rasGAP inhibits opening of K* channels in atrial cell membrane, and induces the expression of *c-fos* promoter-driven luciferase gene (105,106). The effector functions of rasGAP may involve the formation of a complex between rasGAP and p62 and pi90 (104). p62 shows homology with a putative hnRNP protein (GRP33), and recombinant p62 binds to RNA and DNA *in vitro,* suggesting a role for p62 in RNA and DNA metabolism (107). p!90 that binds to the SH3 domain of rasGAP has a region homologous n-chimerin and rho-GAP (108). It has been shown that p 190 has GTPase activity toward Rho, a small GTP-binding protein implicated in actin stress fiber formation (109), and that expression of the rasGAP SH2-SH3-SH2 stretch and association with pi90 correlates with changes in the cytoskeleton and reduces cell adhesion to fibronectin (110).

- **Phosphatidylinositol 3-kinase**

Interest in lipid kinases is first sparked by the observation that phosphatidylinositol kinase (Pl-kinase) activity associates with several retrovirally encoded oncogenes, including v-Src, and polyomavirus middle T antigen (2). This observation leads to the identification of two Pl-kinases in fibroblasts (2). Type I Pl-kinase, generally known as PI_3,-kinase, phosphorylates the D3 position in the inositol ring of PI and PI(4)P, whereas type II or PL_4-kinase phosphorylates PI on the D4 position.

PI_3,-kinase purified from brain or liver exists as a heterodimer composed of a 85 kD regulatory subunit (p85), and a 110 kD catalytic subunit (pi 10). pi 10 is a dual-specificity kinase. It has intrinsic PI_3,-kinase activity in the absence of the regulatory p85 subunit. In addition to the lipid kinase activity, pi 10 has an associated Mn^{2+}-dependent protein kinase activity that can phosphorylate a specific serine, Ser^{608}, in the p85 subunit, resulting in inhibition of PI_3,-kinase activity (111). The catalytic domain of pi 10 is distantly related to a family of protein kinases involved in cell cycle control, DNA damage response and repair (112). The amino-terminal domain of pi 10 is involved in the interaction with the p85 subunit. This domain is less conserved or even absent in pi 10-related kinases (112).

The regulatory p85 subunit of PI_3,-kinase functions as an adaptor linking tyrosine phosphorylated Rtrk to PI_v-kinase activation (113). The primary sequences of p85 a and p85 [3 isoforms reveal a multidomain structure containing a number of non-catalytic domains. At the amino-terminus of the protein lies a SH3 domain followed by a region with significant sequence homology to the product of the breakpoint cluster region (BCR)

gene. This region of BCR has GTPase-activating activity toward the small GTP-binding protein Rac (113). The C-terminal half of the molecule is dominated by two SH2 domains (113). The region between the SH2 domains contains the binding site for pi 10 subunit (113). Ser^{608} which is phosphorylated by pi 10 subunit is located in this region and is implicated in the regulation of PI$_3$,-kinase activity (111). In addition, the p85 subunit contains two binding sites for SH3 domains (2). Binding of SH3 domains of non-Rtrk to purified PI$_3$,-kinase leads to activation of the enzyme (2). The SH2 domains of the p85 subunit mediate the binding of PI$_3$,-kinase to phosphotyrosine residues in activated Rtrk or in so-called adaptor molecules, such as IRS-1 and IRS-2 (2). The p85 SH2 domains recognize a tyrosine phosphorylated YXXM motif (48), and both SH2 domains are required for stable binding. The association between Rtrk and p85 subunit is stimulated by numerous growth factors, and results in allosteric activation of theholoenzyme.

The involvement of active PI$_3$,-kinase and 3'-phosphoinositide metabolites in cellular signaling is studied with receptor mutants that lack PI$_3$,-kinase binding sites, with mutant p85 proteins that lack the pi 10 binding site, and using the specific inhibitors of PI,, -kinase activity. From these studies, evidence has been obtained that PI$_3$,-kinase may lie on the pathway leading to cell proliferation (40,114,115). Furthermore, activation of PI,,-kinase is apparently required for insulin, IGF-1, HGF, and PDGF (2). This observation fits with a role for PI$_3$,-kinase in Rac activation, a protein regulating membrane ruffling (113).

Despite evidence for an involvement of PI$_3$,-kinase in several cellular processes, relatively little is known about the mechanisms that transmit the signal beyond this pointRecently, a putative target of 3'-phosphoinositides has been characterized. Evidence indicates that the 60 kD serine/threonine kinase Akt, also known as PKB or Rac, lies on a pathway downstream of PI,,-kinase (116): (/') activation of Akt by growth factors is inhibited by wortmannin, a specific inhibitor of PI,, -kinases, (ii) PDGFR mutants that fail to activate PI$_3$,-kinase also fail to activate Akt/PKB, (Hi) a dominant negative mutant of PI,,-kinase inhibits PDGF activation of Akt/PKB, and (iv) phosphatidylinositol-3-monophosphate (PtdIns3P), but not Ptdlns or PtdIns(4,5)P2, can activate Akt/PKB in vitro. Activation of Akt/PKB is inhibited by mutations in the amino-terminal PH domain (116). As this domain is essential for protein homodimerization, it is tempting to speculate that binding of 3'-phosphoinositides to the PH domain can promote dimerization of Akt/PKB and so activate it, possibly by inducing protein autophosphorylation.

Among the potential targets of Akt/PKB is the 70 kD S6 kinase. p70 S6 kinase is activated by constitutively active Akt/PKB in vivo (116). In addition, growth factor induced activation of p70 S6 kinase is inhibited by PI$_3$,-kinase inhibitors (117,118). However, PDGFR mutants that fail to activate PI$_3$,-kinase and Akt/PKB still activate p70 S6 kinase (117,118), suggesting that several pathways contribute to the activation of this enzyme (118).

• **Phospholipase Cy**

Activation of phospholipase Cy (PLCy) induces the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP$_2$) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). DAG is the physiological activator of PKC, whereas IP, mobilizes Ca^{2+} from intracellular stores, thereby affecting Ca^w-dependent intracellular processes.

The primary structure of PLCy, which is similar in its two known isoforms (PLCyl and PLC y2), contains two central SH2 domains and a SH3 domain C-terminus to the central SH2 domain. This SH2-SH2-SH3 structure is flanked by a split PH domain. The SH2 domains of PLCy direct the interaction with activated Rtrk. Both SH2 domains belong to group III that bind phosphotyrosine-hydrophobic-X-hydrophobic sequences, though the amino- and the C-terminal SH2 domain display different binding specificities (48). The amino-terminal SH2 domain preferentially binds to the sequence phosphotyrosine-Ile/Leu/Val-acidic (Glu/Asp)-Ile/Leu/Val, while the C-terminal SH2 domain binds the sequence phosphotyrosine-Val/Ile-hydrophobic (Ile/Leu)-Pro/Ile/Val. The SH3 domain localizes PLCy to the cytoskeleton (119). The significance of the split PH domain remains to be clarified.

Most but not all activated Rtrk bind PLCyl (2). Binding of PLCyl to the activated Rtrk apparently facilitates the tyrosine phosphorylation of PLCyl. Both binding and tyrosine phosphorylation of PLCyl are essential for its activation. Studies with mutant FGFR and PDGFR that lack PLCyl binding sites indicate that activation of PLCyl per se is not required for the induction of DNA synthesis (2). However, restoration of Tyr^{1021}, the PLCyl binding site in PDGFR, partially restores DNA synthesis (114). This indicates that activation of PLCy itself can induce a mitogenic response.

• **Protein tyrosine phosphatases**

Protein tyrosine phosphatases (FTP) represent a diverse family of enzymes that catalyze the dephosphorylation of phosphotyrosine residues in activated Rtrk and their substrates. PTP can be divided into two groups. One group comprises receptor-like PTP, that contain a putative extracellular ligand binding domain, a transmembrane domain and a highly conserved cytoplasmic catalytic domain (120,121). The other group is formed by a large expanding family of cytoplasmic PTP (120,121). Within the family of cytoplasmic PTP, two PTP

have been characterized that contain SH2 domains, termed SH-PTP1 and SH-PTP2 (120,121).

The primary structure of SH-PTP1, also known as FTP 1C, SHP, and HCP, indicates the presence of two amino-terminal SH2 domains and a C-terminal catalytic domain (122). The expression of SH-PTP1 is limited to lymphocytes. The amino-terminal SH2 domain of SH-PTP1 recognizes a broad selection of phosphotyrosine-hydrophobic-X-hydrophobic sequences with a slight preference for Phe at the +1 and +3 position relative to the phosphotyrosine (49). The optimal binding motif for the C-terminal SH2 domain has not been determined yet. The amino-terminal SH2 domain of SH-PTP1 associates *in vitro* with tyrosine phosphorylated EOF and SCF receptors (122). Expression of SH-PTP1 in 293 cells induced a partial or complete dephosphorylation of the EGFR, ErbB2, overexpressed PDGFRoc and PDGFRp, and the P subunits and unprocessed precursors of the insulin and IGF-1 receptor (123). No direct interaction between CSF-1 receptor and SH-PTP1 could be demonstrated (2). Despite this, SH-PTP1 has been identified as a tyrosine phosphorylated protein in macrophages incubated with CSF-1 (2). *In vivo,* SH-PTP1 is probably involved in attenuation of the mitogenic response in macrophages, as macrophages isolated from mice homozygous for the motheaten locus, which encodes the SH-PTP1 gene, show CSF-1 independent proliferation (2).

SH-PTP2, also known as PTP1D, PTP2C, Syp, or SH-PTP3, has a primary structure similar to SH-PTP 1 (123). The amino-terminal SH2 domain recognizes phosphotyrosine-hydrophobic-X-hydrophobic sequences with a small preference for He or Val at the +1 position, and a Val, lie, Leu, or Pro at the +3 position (48). In response to growth factor stimulation, SH-PTP2 associates to activated growth factor receptors through its SH2 domains, becomes tyrosine phosphorylated and thus leading to increased FTP activity (2,123). For full activation of the enzyme, the interaction of both SH2 domains with phosphoproteins is required. In contrast to what is found for SH-PTP 1, SH-PTP2 does not dephosphorylate the cytoplasmic domains of EFGR, ErbB2, PDGFRoc, PDGFRP, insulin and IGF-1 receptors (123). The main tyrosine phosphorylation site in SH-PTP2 has been mapped to Tyr^{542} (124). This tyrosine lies in the consensus binding motif (YTNI) for the SH2 domain of GRB2, which by association with mSOS couples Rtrk to the activation of $p21^{rsM}$. In response to PDGF stimulation, tyrosine phosphorylated SH-PTP2 acts as an adapter between PDGFR and GRB2-mSOS complex (125), suggesting that SH-PTP2 maybe an upstream component in the activation of p21"". Indeed, restoration of the SH-PTP2 binding site in a PDGFR mutant lacking all the tyrosine autophosphorylation sites, is sufficient for full activation of $p21^{ras}$ hi response to PDGF (114), whereas expression of a dominant negative mutant of SH-PTP2 attenuates insulin-induced activation of $p21^{rat}$/ERK pathway (126,127).

- **Non-receptor tyrosine kinases**

- *Src-like tyrosine kinases*

The prototype of Src-like trk family, c-Src, is the cellular homologue of the transforming protein of Rous sarcoma virus (v-Src). Members of this family of non-Rtrk include c-Src itself, c-Yes, Fyn, Lyn, and Lck. All Src-like trk contain a Gly residue at position 2 which is required for myristylation and membrane attachment. The first 70-80 amino acids of the amino-terminus are unique among the members of Src-like trk. This stretch is followed by a SH3 domain and a SH2 domain. The C-terminal half of the protein contains the catalytic domain and a regulatory tyrosine phosphorylation site (128).

The trk activity of c-Src is increased by dephosphorylation of the C-terminal tyrosine residue, Tyr^{527} (128). Mutation of this tyrosine increases trk activity and transforming potential of Src. The kinase activity of c-Src is negatively regulated by phosphorylation of Tyr^{527}, presumably by C-terminal Src kinase (Csk) (129). The negative regulation of Src by phosphorylation involves the binding of Src SH2 domain to the phosphorylated Tyr^{527}, thus leading to steric hinderence of the catalytic domain (128).

The activity of Src and Src-related kinases is increased after stimulation of fibroblasts with PDGF or CSF-1, and during mitosis (130). So far, several substrates for Src have been characterized, including paxillin, focal adhesion kinase, pi 10, p!30, PI_3,-kinase, and src-associated in mitosis 68 kD (Sam68) (2,129).

- *Focal adhesion kinase*

Focal adhesion kinase ($pp!25^{FAK}$) is a cytosolic trk which is predominantly localized to focal adhesions (131,132). Its structure reveals a central catalytic domain flanked by large amino- and C-terminal sequences that are devoid of SH2 and SH3 domains (131,132). The amino-terminal non-catalytic sequence binds to the cytosolic part of the p-integrin subunit (133). The C-terminal stretch of $pp!25^{FAK}$ contains the focal adhesion targeting sequence (FAT) which is neccessary for its localization to focal adhesions. The C-terminus also contains binding sites for two other focal adhesion-associated proteins, talin and paxillin (133).

$pp!25^{FAK}$ is tyrosine phosphorylated in response to cellular adhesion and subsequent integrin clustering and after stimulation of cells with polypeptide growth factors and mitogens (133). Tyrosine phosphorylation of tyrosine 397 of $pp!25^{FAK}$ facilitates the SH2 domain directed binding of c-Src and Fyn, resulting in activation of these enzymes (134). Activation of c-Src is implicated in the phosphorylation of other tyrosine

residues within pp!25[FAK], thus enhancing the enzymatic activity of pp!25[FAK] (133). In addition, activation of pp!25[FAK] and c-Src induces the tyrosine phosphorylation of focal adhesion-associated proteins like paxillin, p!30 and tensin (133). The tyrosine phosphorylation of these proteins is implicated in the formation of focal adhesions (133-135).

INSULIN RECEPTOR SIGNALING

• The insulin receptor

The insulin receptor is a member of Rtrk family. While most Rtrk elicit a mitogenic response, the insulin receptor also induces a metabolic response like stimulation of glucose uptake and glycogen synthesis. Upon insulin binding, the insulin receptor undergoes tyrosine phosphorylation starting at Tyr^{1138} and one of the two vicinal Tyr^{1162} and Tyr^{1163}. The phosphorylation of the three major tyrosine autophosphorylation sites increases the phosphotransferase activity of trk. In addition to phosphorylation of the triple tyrosines in the kinase domain, minor tyrosine phosphorylation occurs at position 972 in the juxtamembrane domain, and at positions 1328 and 1344 in the C-terminal tail (136-138).

Following receptor autophosphorylation and concurrent receptor activation, several substrate proteins become tyrosine phosphorylated. These proteins include IRS-1, IRS-2 and She (52, 139,140). The tyrosine phosphorylation of these proteins contributes to the activation of postreceptor signaling pathways that lead to the various cellular responses mediated by insulin.

• Postreceptor signaling

Insulin receptor is among the Rtrk that stimulate the formation of p21 [ras]-GTP (141). This involves a GNEF that enhances the rate of guanine nucleotide binding on p21"* (70). Several studies point to an involvement of She in this process (142-144). She binds directly *via* its PID domain to the activated insulin receptor, thus facilitating tyrosine phosphorylation of She (50,140). This tyrosine phosphorylation is followed by the binding of She to GRB2/mSOS (143). The formation of a multiprotein complex between the activated insulin receptor, She, GRB2, and mSOS brings mSOS in the vicinity of membrane-bound *p2l[ras]*. As a result, the exchange of GDP for GTP on p21[OT] is stimulated. The activation of p2r[<K] is followed by activation of Raf, MEK and ERK as described earlier in this review.

Tyrosine phosphorylation of TRS-1 and IRS-2 is an unique response of the insulin receptor family, although some members of the cytokine receptor family also induce this response (52). IRS-1 and IRS-2 are cytoplasmic proteins, which contain at least 22 potential tyrosine phosphorylation sites and

two domains involved in protein-protein interactions, e.g. PH and PID domains (52,139). The tyrosine phosphorylation of IRS-1 and presumably IRS-2 directs the binding of several SH2 domain-containing proteins, including the p85 subunit of PI_3,-kinase, SH-PTP2, GRB2, and Nek (9). The binding of p85 subunit to tyrosine phosphorylated TRS-1 reflects the main step in insulin-Induced activation of PI_3,-kinase (9). Regarding GRB2, several lines of evidence have indicated that p21[TM]-GTP formation can proceed independently of TRS-1/GRB2 complex formation (142-144).

The requirement of p21 [TM] and PI_3, -kinase for insulin-stimulated glucose uptake and glycogen synthesis has been investigated. Activation of the p21 ""/ERK pathway alone appears not to be sufficient to stimulate glucose transport in 3T3L1 adipocytes (145). The involvement of this pathway has been subsequently exluded by expression of a dominant negative mutant of p21[ras], p2i"z,(ASNi7) jhi[s p]2i^ [mu]tant abrogates the signaling pathway mediated by endogeneous p21""-GTP (146), but has no effect on insulin-stimulated glucose uptake and glycogen synthesis (147). In contrast, insulin-mediated glucose uptake and glycogen synthesis is completely abolished by wortmannin, an inhibitor of PI_3,-kinase (115,147). This suggests that PI_3,-kinase, or another wortmannin-sensitive enzyme, is an essential intermediate in insulin-induced metabolic signaling.

Insulin receptor activation also promotes the tyrosine dephosphorylation of pp!25[FAK] and paxillin (148,149). The dephosphorylation of pp!25[FAK] requires phosphotyrosine residue 1158 in the activated insulin receptor and is accomplished by activation of PTP ID (149,150). pp!25[FAK] dephosphorylation by insulin is accompanied by a reduction in the amount of cytosolic stress fibres, though the physiological relevance of this event remains to be identified (150).

CONCLUSIONS

• In the last decade, considerable progress has been made towards the understanding of Rtrk signaling pathways. Ligand binding induces dimerization, activation and autophosphorylation of the Rtrk. The autophosphorylated tyrosine residues form binding sites for proteins containing SH2 or PID domains. The recruitement of these proteins to the receptor represents the first step in the activation of postreceptor signaling pathways. The specificity in signaling is probably achieved by identity of the proteins that bind to a specific Rtrk as well as tissue-specific expression of substrates.

Although a number of early postreceptor signaling pathways has been elucidated by now, their contribution to cellular responses remains to be clarified. In case of the insulin receptor, the elucidation of signaling pathways that are involved in the regulation of glucose uptake and glycogen synthesis may con-

tribute to the identification of candidate genes involved in the pathogenesis of NIDDM.

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