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MINI-REVIEW

Structure and Function of Tyrosine Kinase Receptors

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Abstract

Over the past ten years, several growth factor receptors have been shown to be ligand-regulated tyrosine kinases. Tyrosine kinase activity is essential for signal transmission, suggesting that phosphorylation cascades may play an important role. Considerable effort has gone into understanding the structure and function of tyrosine kinase receptors in order to define their mechanisms of signal transmission. However, the protein substrates of the receptor kinases have proven to be difficult to isolate and clone. This review focuses on the receptors for insulin, epidermal growth factor, and platelet-derived growth factor. They are all tyrosine kinases, but emerging evidence suggests that they utilize multiple separate signal transduction pathways. Work carried out during the next several years should yield considerable insight into the complexity of the components which interact with these tyrosine kinase receptors to regulate cellular growth and metabolism.

Key Words: Insulin receptor; EGF receptor; PDGF receptor; tyrosine kinase; tyrosine phosphorylation; signal transduction.

Introduction

Phosphorylation of proteins plays an important role in the control of cellular metabolism, the cell cycle, and growth. Protein phosphorylation is catalyzed by kinases which transfer the gamma-phosphate from ATP to the hydroxyl group of specific serine, threonine, or tyrosine residues in target proteins. A regulatory role for protein phosphorylation cascades was appreciated several years ago by Krebs *et al.* (1959). They demonstrated that glycogen phosphorylase is activated by serine phosphorylation catalyzed by phosphorylase

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kinase (Krebs *et al.*, 1959). A decade later, the *c*AMP-dependent protein kinase was identified, which is activated when the level of cellular *c*AMP increases during hormonal stimulation of the adenylate cyclase system (Walsh *et al.*, 1968). The finding that phosphorylase kinase was activated by the *c*AMP-dependent protein kinase provided the first evidence that phosphorylation cascades initiated through catacholamine stimulation and mediated intracellularly by second messengers play an important role in the regulation of cellular metabolism.

In 1979, $pp60^{v\text{-}src}$, the transforming gene product of the Rous sarcoma virus, was shown to be a protein kinase (Erikson *et al.*, 1979). The presence of phosphotyrosine in $pp60^{v\text{-}src}$ was subsequently established by Hunter and Sefton (1980). Tyrosyl kinases are Ca^{2+} and *c*AMP-independent, and are regulated through direct phosphorylation or through associations with other proteins such as growth factors. Since 1980, over 100 protein kinases were identified, nearly half of which are protein tyrosine kinases (Hunter, 1987). Although the amount of phosphotyrosine in cells is much smaller than the amount of phosphoserine or phosphothreonine, the growing number of tyrosine kinases identified as oncogenes or found to be regulated by growth factors indicates that this family of enzymes plays an important role in the control of cellular growth and metabolism.

Soon after the discovery of phosphotyrosine in $pp60^{v\text{-}src}$, the epidermal growth factor (EGF) receptor was shown to be an EGF-stimulated tyrosyl kinase (Carpenter *et al.*, 1979; Cohen *et al.*, 1980). This finding suggested that tyrosyl phosphorylation may play a central role in the mechanism of signal transduction by growth factor receptors. These observations stimulated similar studies with other polypeptide hormones and growth factors, and lead to the observation that the receptors for insulin (Kasuga *et al.*, 1982a,b), insulin-like growth factor-1 (IGF-1) (Jacobs *et al.*, 1983; Rubin *et al.*, 1983), platelet-derived growth factor (PDGF) (Nishimura *et al.*, 1982; Frackelton *et al.*, 1983; Ek and Heldin, 1984), and macrophage colony-stimulating factor-1 (CSF-1) (Rettenmier *et al.*, 1985) are tyrosine-specific protein kinases. These receptors were identified as tyrosyl kinases because, in each case, the specific ligand stimulated tyrosine autophosphorylation of a membrane protein that was known to be its receptor. Many other tyrosyl kinases were subsequently identified by molecular cloning. The approach was very successful because of a high degree of DNA and amino acid sequence homology between the catalytic regions of all protein tyrosine kinases (Hunter and Cooper, 1986; Hanks *et al.*, 1990). Low stringency screening of *c*DNA libraries yielded many related receptor molecules. For example, additional growth factor receptors have been cloned for which specific ligands are not yet characterized, which resemble the PDGF and EGF receptors (Yarden and Ullrich, 1988) and the insulin (Shier and Watt, 1989) and IGF-1

receptors (Yee *et al.*, 1989). Although more kinases will be identified during the next decade, our challenge is to clarify the molecular links between the tyrosine kinases and the cellular response.

Biosynthesis of the Insulin Receptor: An Example of a Tyrosine Kinase Receptor

The *c*DNA for the insulin proreceptor has been cloned from human (Ullrich *et al.*, 1985; Ebina *et al.*, 1985), *drosophila* (Petruzzelli *et al.*, 1986; Nishida *et al.*, 1986), and mouse (Sibley *et al.*, 1989), and all the molecules are very similar. The coding portion of the human *c*DNA is a little over 4 kb long and predicts a receptor consisting of 1346 to 1358 amino acids depending on the tissue source. The 5′-end of the *c*DNA begins with a signal sequence, which is followed by the coding sequence for the α-subunit which in turn is followed by the sequence for the β-subunit. The α- and β-subunit segments are separated by four basic amino acid residues that represent the site for cleavage to the individual subunits. The predicted α-subunit sequence includes several sites for N-linked glycosylation, and a cysteine-rich domain thought to be involved in insulin binding; it has no apparent transmembrane domain. The β-subunit, on the other hand, has an extracellular segment also containing sites for N-linked glycosylation, a single transmembrane segment of 23 amino acids, and an intracellular domain with regions homologous to the larger family of TKRs.

In human cells, there appears to be a single insulin receptor tyrosine kinase gene per haploid genome that is located on the short arm of chromosome 19. Genomic Southern analysis and cloning of portions of the 5′ and 3′ regions of the receptor gene indicate that it is at least 150 kb long containing 22 exons (Fig. 1) (Seino *et al.*, 1989). Exon 11 is variably expressed so that

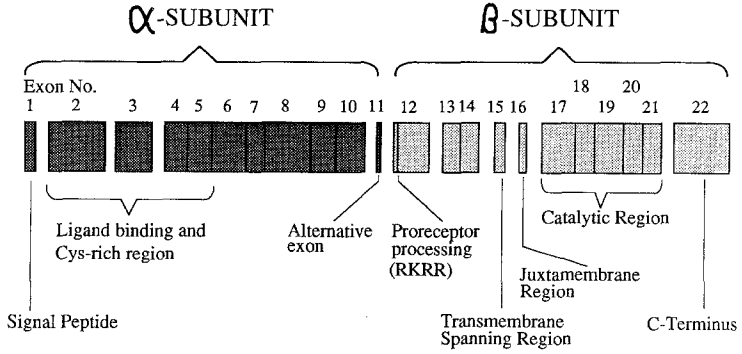

Fig. 1. The exon structure of the human insulin receptor gene.

the α-subunit either contains or lacks a 12 amino acid C-terminal extension. In all cells studied, there are multiple species of insulin receptor *m*RNA (Ullrich *et al.*, 1985). During *in vitro* translation of receptor *m*RNA or when cells are biosynthetically labeled in the presence of inhibitors of glycosylation, the proreceptor can be identified and has a molecular weight of about 160,000. In normal intact cells, the proreceptor is rapidly glycosylated to give species of 180–210 kD, which are then disulfide linked, cleaved, and further glycosylated to give the mature receptor (White and Kahn, 1986).

On the cell surface, the insulin receptor is a heterotetrameric glycoprotein consisting of two α-subunits of M_r = 135,000 and two β-subunits of M_r = 95,000 linked by disulfide bonds to give a β-α-α-β structure (Fig. 2) (White and Kahn, 1986). The half-life of the native receptor is 8–12 h in the cells studied so far (Kasuga *et al.*, 1981). Biochemical studies confirm that the α-subunit is entirely extracellular and contains the insulin binding site (Hedo and Simpson, 1984). This binding site can be affinity labeled using [^{125}I]insulin and bifunctional cross-linking agents or affinity insulin analogues. Although there are two α-subunits per receptor, there is still a debate as to whether the holoreceptor contains one or two insulin binding sites in its native form (Pang and Shafer, 1984). The β-subunits are transmembrane proteins and involved in intracellular signaling, the details of which are described below.

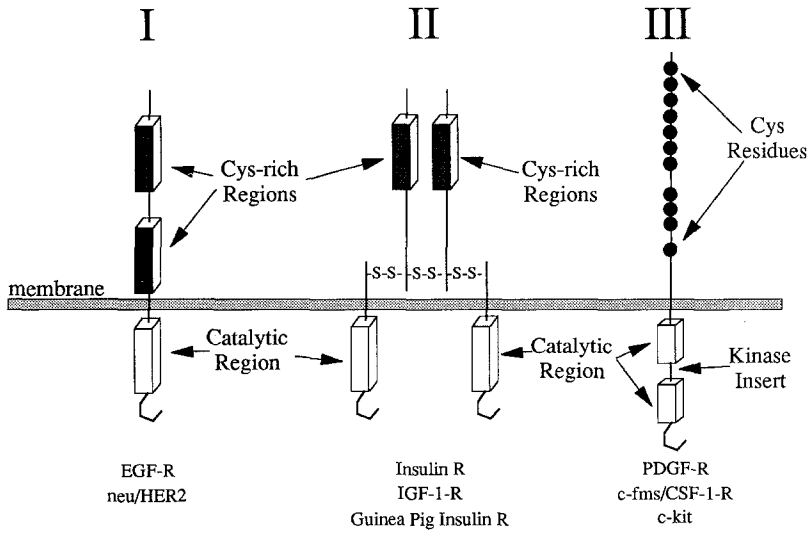

Fig. 2. A schematic diagram of the three classes of tyrosine kinase receptors.

An Overview of the Structural Features of Protein Tyrosine Kinase Receptors

Introduction

Tyrosine kinase receptors (TKRs) have a similar overall structure consisting of an extracellular domain for ligand recognition, a single transmembrane domain, and a cytoplasmic domain that generates regulatory signals. Based on conserved amino acid sequences and the overall molecular structure, three subclasses of TKRs have been proposed by Ullrich *et al.* (Fig. 2). Members of *class I* are monomeric transmembrane proteins in which the extracellular and intracellular domains are on the same molecule. The extracellular domain contains two cysteine-rich repeat regions. The EGFr is an example of this class of TKR. The *class II* TKR (Fig. 2) is a heterotetrameric enzyme in which two α-subunits and two β-subunits are linked covalently by intermolecular disulfide bonds. The α-subunits contain a single cysteine-rich repeat region stablizing the ligand binding domain. The insulin receptor is an example of this class of TKR. The *class III* TKR (Fig. 2) is similar to class I in that it is monomeric, but the cysteine residues are distributed over the entire extracellular domain, and the intracellular domain contains an insert of about 120 amino acids located in the middle of the conserved kinase domain.

The Extracellular Domain

The external domain of TKRs contains the ligand binding site. This region is apparently stabilized by a large number of disulfide bonds which probably form a rigid secondary structure necessary to establish a highly specific binding pocket in the extracellular environment. Undoubtedly, the ligand binding specificity of the TKRs is established by the conformation of the total amino acid sequence, which is stabilized by the disulfide bond network. The structural rather than functional role of the disulfide bond network is emphasized by the fact that the cysteine residues are located in identical positions in the insulin and IGF-I receptors, whereas these receptors selectively bind with high-affinity insulin or IGF-1, respectively.

The Transmembrane Domain

The extracellular and the intracellular domains of TKRs are linked by a single α-helical region of about 24 amino acids (Fig. 2). This transmembrane domain is not conserved between TKRs. Even closely related TKRs such as the insulin and IGF-1 receptor contain distinct transmembrane

domains. It is possible that the transmembrane domain plays only a structural role linking the external and internal domains of TKRs, where only its length, hydrophobicity, and stability in an α-helix is important. This may be the case for certain TKRs such as the EGF receptor where mutations in this region have no effect on signal transmission (Kashles *et al.*, 1988). The role of the transmembrane region of the insulin receptor has not yet been explored.

The Cytoplasmic Domain

The cytoplasmic domain of TKRs respond to ligand binding by undergoing conformational changes and autophosphorylation on tyrosyl residues. Several important structural features are known about this domain (Fig. 2). It contains the highest degree of homology between the members of the TKR family. All protein kinases contain a consensus amino acid sequence encoding an ATP binding domain. The distinctive amino acid sequence Gly-Xxx-Gly-Xxx-Xxx-Gly followed by an Ala-Xxx-Lys residue 14 to 22 amino acids downstream is a perfect indicator of a protein kinase (Hanks *et al.*, 1990; Smith and Smith, 1990). The lysyl residue is absolutely required for kinase activity and substitution with any other amino acid invariably blocks kinase activity and signal transduction by all TKRs (Hunter and Cooper, 1986; Chou *et al.*, 1987). The ATP binding domain is located about 50 amino acid residues downstream from the transmembrane domain.

A second region, distinctive for TKRs, is found about 140 amino acids beyond the ATP binding site (White and Kahn, 1986). This region is flanked by the amino acid sequences Asp-Phe-Gly at the N-terminal side, and Ala-Pro-Glu at the C-terminal side. The tyrosyl residues in this region which are surrounded by acidic amino acids are often major sites of ligand-stimulated autophosphorylation. Most tyrosyl kinases contain one tyrosyl residue, and in most cases its role in activation of the kinase is unclear (Hanks *et al.*, 1990). For the insulin receptor, three tyrosyl phosphorylation sites are found here, Tyr_{1146}, Tyr_{1150}, and Tyr_{1151}, according to the numbering of Ullrich (Ullrich *et al.*, 1985). Insulin-stimulated autophosphorylation of these tyrosyl residues activates the insulin receptor kinase (Rosen *et al.*, 1983; White *et al.*, 1988b); for the insulin receptor, this region is referred to as the regulatory region. In contrast, the conserved tyrosyl residue in this region of the EGF receptor is not a major autophosphorylation site.

In type III TKRs, the catalytic domain is interrupted by a sequence of about 100 amino acids. This was first appreciated with the PDGF receptor and later found in other closely related molecules (Yarden and Ullrich, 1988). In the PDGF receptor, this insert contains an autophosphorylation site that may be required for associations with other proteins necessary for signal transduction, such as phosphatidylinositol 3-kinase (Kazlauskas and Cooper,

1989, 1990) and GAP (Hall, 1990). However, the kinase insert region is not necessary for interaction with all putative signal-transducing molecules, as phospholipase C-gamma and c-raf do not require this region (Hall, 1990).

A third characteristic of all TKR catalytic domains is the presence of a C-terminal tail that diverges between TKRs, contains autophosphorylation sites, but in many cases is not necessary for catalytic activity. The C-terminal region of the EGF receptor may be essential for specificity of signal transmission (Khazaie *et al.*, 1988). For the insulin receptor, both proteolytic removal of the C-terminus and truncation of the *c*DNA yield receptor molecules that are still catalytically and biologically active (Goren *et al.*, 1987; McClain *et al.*, 1988). The C-terminal tail of the insulin receptor (Ullrich *et al.*, 1985), the IGF-1 receptor (Ullrich *et al.*, 1986), and the guinea pig insulin receptor (Shier and Watt, 1989) are strikingly different, suggesting, on the one hand, that the C-terminus plays an important role in the specificity of signal transmission for this class of TKRs, and, on the other hand, that it may have diverged because it does not play an important role.

The Regulation of Tyrosine Kinase Activity by Ligand Binding

The catalytic activity of TKRs is regulated by ligand binding. However, the mechanism by which the intracellular kinase is activated by external ligand binding is unknown. The two domains are only connected by a short α-helix, and interactions between the domains are restricted by a lipid bilayer. Activation of TKRs may involve dimerization of adjacent receptor monomers, a mechanism which has been studied most extensively for the EGF receptor (Schlessinger, 1988). According to this model, the EGF receptor forms dimers during EGF binding which bring adjacent catalytic regions close together; the conformational changes resulting from this contact presumably activate the kinase. However, other changes are implicated as well. For example, a chimeric receptor molecule composed of the intracellular and transmembrane domains of the EGF receptor and the extracellular domain of the insulin receptor is fully activated by insulin binding (Riedel *et al.*, 1986). Thus the extracellular domain of the insulin receptor and the EGF receptor may use a common mechanism of signal transmission across the plasma membrane even though the structures of the native receptors are quite different. The main difference is that the EGF receptor is monomeric whereas the insulin receptor is dimeric (Fig. 2). Thus the chimeric insulin : EGF receptor exists as a covalent dimer before insulin binding, suggesting that some other conformational change must also be involved in signal transmission besides bringing two catalytic domains into close proximity.

Activation of some TKRs may be thought of as a release of the catalytic domains from an inhibitory conformation. This is implicated for the insulin receptor by several observations employing both biochemical and genetic experiments. Before insulin binding the kinase activity of the β-subunit is undetectable; however, a low concentration of trypsin mimics the effect of insulin and activates the tyrosyl kinase, presumably by removing most the α-subunit (Shoelson *et al.*, 1988). Thus, the external domain of the insulin receptor apparently inhibits the kinase before ligand binding, whereas the kinase is released from this inhibition during insulin binding or after removal of this domain. Removal of the α-subunit also breaks the covalent links holding two β-subunit catalytic regions together, suggesting that dimer formation may not be one of the primary events necessary for insulin receptor activation. In contrast, removal of the EGF receptor binding domain does not fully activate this tyrosyl kinase. Thus, it appears that EGF binding causes conformational changes which release the catalytic domain from an inhibitory constraint and promote dimerization, which fully activates the kinase (Wedegaertner and Gill, 1989). Thus, a general mechanism to explain the activation of TKRs by ligand binding is not completely established.

The Regulation of Tyrosine Kinase Activity by Ligand-Stimulated Tyrosyl Autophosphorylation

The Insulin Receptor

It is generally thought that autophosphorylation of TKRs is necessary for regulation and signal transduction. But only for the insulin receptor has the link between autophosphorylation and tyrosine kinase activity been unambiguously established. Tyrosyl autophosphorylation in the β-subunit of the insulin receptor occurs through a cascade of intramolecular reactions (White *et al.*, 1988b; White and Kahn, 1989). As a result, at least five tyrosine residues are phosphorylated. Three of these residues are located in a cluster including Tyr_{1146}, Tyr_{1150}, and Tyr_{1151} (Fig. 3); the other residues are located in the C-terminal tail of the β-subunit, but their phosphorylation does not appear to play a role in activating the kinase (Goren *et al.*, 1987). The simultaneous phosphorylation of Tyr_{1146}, Tyr_{1150}, and Tyr_{1151}, that is, tris-phosphorylation, appears to be necessary to activate the tyrosyl kinase. The role of tris-phosphorylation of the regulatory region was established initially by interrupting the autophosphorylation cascade with antiphosphotyrosine antibodies, followed by elution of the partially phosphorylated receptor and assays of the kinase activity (White *et al.*, 1988b). Activation of the receptor by tris-phosphorylation was recently confirmed by kinetic arguments

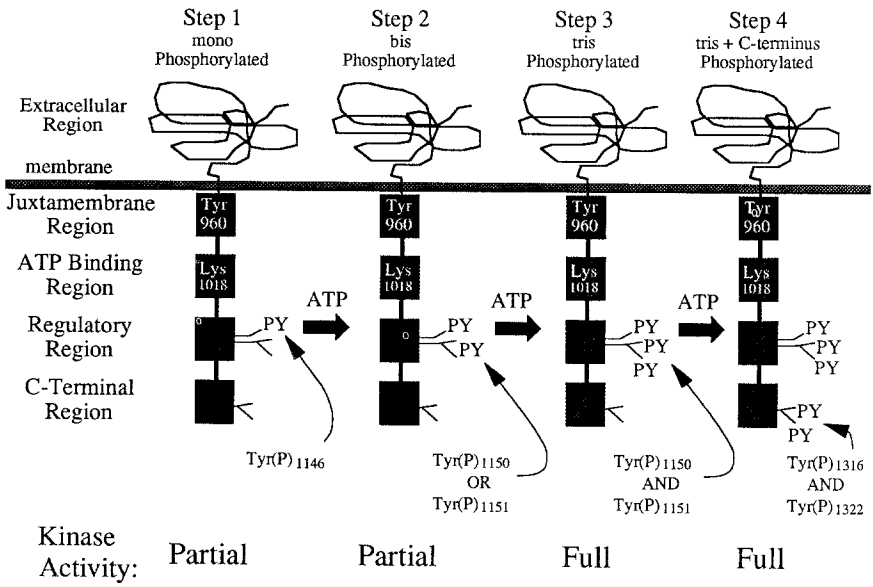

Fig. 3. A schematic diagram of the cascade of autophosphorylation of the insulin-stimulated receptor β-subunit. For simplicity only the β-subunit is shown here.

(Flores-Riveros *et al.*, 1989). The region containing these three tyrosine residues is called the regulatory region of the β-subunit as the phosphorylation of these sites plays a major role in kinase activation.

Based on kinetic studies and other analysis, Tyr_{1146} appears to be one of the major autophosphorylation sites in the insulin receptor. Functional analysis of a mutant receptor in which Tyr_{1146} was replaced with phenylalanine (IR_{F1146}) provides an interesting example of the complicated relation between autophosphorylation, receptor function, and biological activity. Autophosphorylation of the partially purified IR_{F1146} was reduced 60 to 70% when compared to the wild-type IR, but was still stimulated by insulin. The reduction in autophosphorylation is consistent with Tyr_{1146} playing a central role in the autophosphorylation cascade, perhaps by facilitating the phosphorylation cascade at the other sites; however, it is not obligatory as insulin can still stimulate autophosphorylation of some of the residual sites (Fig. 4). The phosphotransferase activity of the dephosphorylated form of both the wild-type IR and IR_{F1146} toward exogenous substrates is stimulated 3- to 4-fold by insulin. However, only the wild-type IR which undergoes autophosphorylation normally was activated 20-fold by autophosphorylation (Wilden *et al.*, 1990). In Chinese hamster ovary (CHO) cells, insulin binding to IR_{F1146} is normal, whereas autophosphorylation is reduced 80% compared to cells expressing the wild-type IR. An endogenous substrate of the insulin

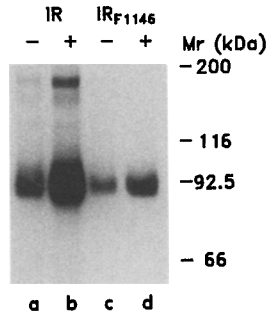

Fig. 4. *In vitro* autophosphorylation of IR and IR_{F1146}. WGA-purified IR (a and b) and IR_{F1146} (c and d) were adjusted to equal insulin binding and incubated for 30 min at 23°C in either the absence (a and c) or presence (b and d) of 100 nM insulin. The receptors were autophosphorylated and analyzed by reducing SDS-PAGE and autoradiography. [^{32}P]Phosphate incorporated into the β-subunit was quantified. Cerenkov counting of the excised β-subunit band: (a) β-subunit = 504 cpm; (b) β-subunit = 1146 cpm; (c) β-subunit = 70 cpm; (d) β-subunit = 151 cpm.

receptor, pp185, is not detected during insulin stimulation of CHO/IR_{F1146} cells, which is consistent with the model that tris-phosphorylation is necessary to fully activate the kinase of the receptor. These data suggest that activation of the IR tyrosyl kinase can be resolved into two components: the first is dependent on insulin binding and the second is dependent on the subsequent insulin-stimulated autophosphorylation cascade.

Although the tyrosyl kinase activity of the IR appears to be absolutely necessary for biological activity, the exact role of the individual autophosphorylation sites during receptor signaling is more complicated. The IR_{F1146} does not internalize insulin rapidly or stimulate DNA synthesis in the presence of insulin. In contrast, both the IR and IR_{F1146} stimulate glycogen synthesis equally in CHO cells. Thus, at least two signal transduction pathways diverging from the insulin receptor (IR) are implicated in the mechanism of insulin action. Each of these signals appears to require a distinct pattern of autophosphorylation, and may have a different requirement for kinase activity. As pp185 is not phosphorylated in the IR_{F1146} cells, it is possible that this protein is involved in the regulation of thymidine incorporation and the regulation of receptor internalization, but not the stimulation of glycogen synthase. The data is consistent with the possibility that internalization of the IR is necessary to mediate thymidine incorporation. Interestingly, opposite biological results have been obtained when Tyr_{1150} and Tyr_{1151} are substituted with phenylalanine, leaving Tyr_{1146} intact (Debant *et al.*, 1988). In this mutant, insulin stimulation of glucose uptake and the conversion of glucose into glycogen is defective, but the stimulation of thymidine incorporation into DNA is normal. These opposite results suggest that the relation between autophosphorylation and biological activity is more complicated than simply

the activation of the tyrosyl kinase measured during *in vitro* assays. Whatever the explanation is, it appears that autophosphorylation of the regulatory region plays an important role in selecting the signal transduction pathways that are activated during insulin binding.

The EGF Receptor

The EGF receptor also undergoes tyrosyl autophosphorylation during EGF binding. Activation of the EGF receptor kinase by autophosphorylation is controversial, with some reports finding it (Bertics and Gill, 1985; Shoelson *et al.*, 1989) and others not (Downward *et al.*, 1985). Unlike the insulin receptor, alterations of individual autophosphorylation sites do not have a major effect on kinase activity (Honegger *et al.*, 1988a). However, the major autophosphorylation sites of the EGF receptor are located in the C-terminal portion of the molecule including Tyr_{1068}, Tyr_{1148}, Tyr_{1173}, and Tyr_{1086}; Tyr_{845} of the EGF receptor, which corresponds to Tyr_{1150} in the regulatory region of the insulin receptor, has not been shown to be phosphorylated (Honegger *et al.*, 1988a; Margolis *et al.*, 1989a). Removal of the C-terminus may increase the biological sensitivity of the EGF receptor, suggesting that the C-terminal phosphorylation sites may play a regulatory role through competitive inhibition rather than through activation (Honegger *et al.*, 1988b). Thus, it is not surprising that mutations of Tyr_{1068}, Tyr_{1148}, and Tyr_{1173} have no effect on activation of the EGF receptor kinase. Owing to kinetic similarities between the insulin receptor and the EGF receptor demonstrated by Shoelson *et al.* (1989), it is possible that the phosphorylation of Tyr_{845} will eventually be demonstrated to play a role in the activation of the EGF receptor.

Heterologous Regulation of Protein Tyrosine Kinase Receptors

Receptor tyrosyl kinases are ordinarily activated through binding of the cognate ligand at the extracellular face of the plasma membrane, and their kinase activity may be sustained and enhanced through autophosphorylation. However, other ligands also appear to play roles in the activation or inhibition of TKRs. Heterologous regulation of TKRs may involve the formation of hybrid receptor molecules which are capable of responding to different ligands, or TKRs may be activated or inactivated by phosphorylation catalyzed by a distinct kinase that is activated through a different messenger system. The inhibition of the insulin (Takayama *et al.*, 1988) and EGF (Glenney, 1985) receptor kinase by phorbol ester-stimulated serine or threonine phosphorylation is an example of the latter mechanism.

IGF-1 and insulin share certain biological effects, which may be partially explained by cross-reactivity between insulin and IGF-1 to their receptors. Recently another mechanism to account for cross-reactivity has been proposed, suggesting the formation of insulin/IGF-1 receptor hybrid tetramers (Moxham *et al.*, 1989; Yee *et al.*, 1989). The insulin and IGF-1 receptors are class II TKRs composed of closely related by distinct subunits (Fig. 2). Based on immunochemical and structural analysis, hybrid receptors have been isolated from *in vitro* preparations and cells which are composed of one insulin receptor $\alpha\beta$ heterodimer and an IGF-1 receptor $\alpha\beta$ heterodimer. This hybrid receptor in NIH-3T3 and HepG2 cells behaves like an IGF-1 receptor based on its relative potency for autophosphorylation (Moxham *et al.*, 1989). However, in this case, IGF-1 stimulates the autophosphorylation of one insulin receptor β-subunit and one IGF-1 receptor β-subunit. The activation of two distinct β-subunit by a single ligand could explain the overlap in the biological responses triggered by IGF-1 and insulin receptors (Moxham *et al.*, 1989).

Indirect heterologous regulation of TKRs can be mediated through serine, threonine, or tyrosine phosphorylation. Phosphorylation of the EGF receptor at threonine residues inactivates the tyrosine kinase. Thr_{654}, located just after the transmembrane domain, is a major site of phosphorylation by the protein kinase C (Davis and Czech, 1986). Phosphorylation of this residue inhibits high-affinity EGF binding and inhibits EGF-stimulated tyrosyl autophosphorylation (Glenney, 1985). The addition of a negative charge near the inner face of the plasma membrane may inhibit dimerization of occupied EGF receptors, preventing activation of the kinase, but the actual mechanism of this inhibition is unknown. Thus agents which stimulate the protein kinase C may inactivate the EGF receptor through a phosphorylation cascade.

A possible mechanism for feedback inhibition of the EGF receptor involves activation of phospholipase C and the protein kinase C by the EGF receptor itself. It is well known that EGF binding stimulates phosphatidylinositol bis-phosphate hydrolysis and the release of two key second messengers, inositol tris-phosphate and diacylglycerol (Moolenaar *et al.*, 1988). The diacylglycerol activates the protein kinase C which is capable of phosphorylating the EGF receptor at Thr_{654}. A key step in this mechanism is the activation of phospholipase C. This activation step may occur through the direct tyrosyl phosphorylation of the PLC-gamma isozyme (Wahl *et al.*, 1989; Margolis *et al.*, 1989b; Meisenhelder *et al.*, 1989). Tyrosyl phosphorylation of PLC-gamma in EGF stimulated cells has been demonstrated by several laboratories; however, activation of PLC-gamma by phosphorylation is difficult to establish. Nevertheless, the regulatory mechanism outlined in Fig. 5 may play an important role in the regulation of the EGF receptor signal transmission, and variations of this paradigm may eventually be found for other TKRs as well.

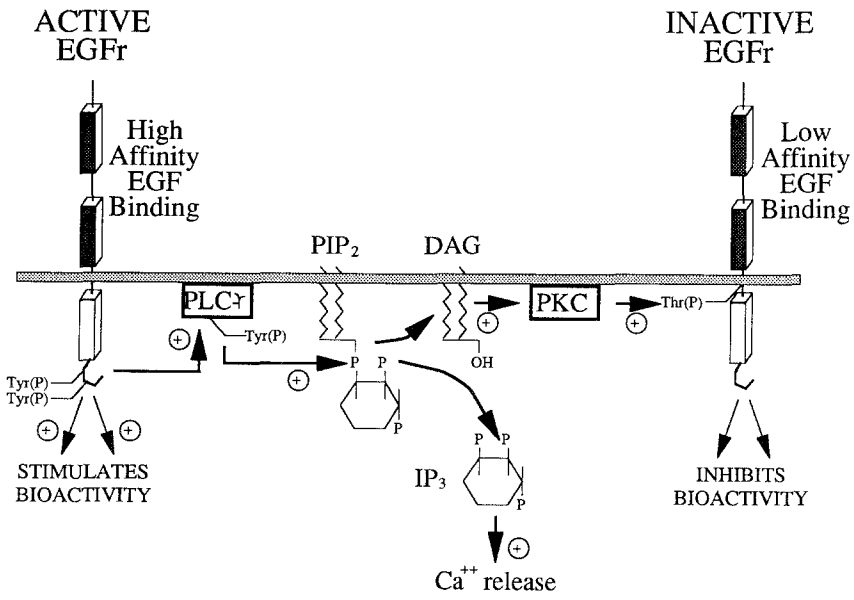

Fig. 5. A hypothetical mechanism showing feedback inhibition of high-affinity (dark line between EGF and the receptor) EGF binding and the activity of the EGF receptor by threonine phosphorylation catalyzed by the protein kinase C. Abbreviations used are: PLC-gamma, phospholipase C gamma subtype; PIP_2, phosphatidylinositol bis-phosphate; DAG, diacylglycerol; PKC protein kinase C.

Details of this mechanism are currently evolving, so the complexity of the final scheme and the exact molecular events involved are unknown.

Signal Transmission

Introduction

One of the most important problems facing us is to elucidate the mechanisms of signal transmission used by the TKRs. Ligand-activated TKRs stimulated many cellular responses including nutrient uptake, protein synthesis, cell cycle progression, DNA synthesis, and cellular replication, but all TKRs do not elicit the same set of responses. EGF increases Na^+/H^+ antiport, intracellular Ca^{2+} concentration, phospholipase C activity, and amino acid and glucose transport systems, but insulin only stimulates the transport systems. Thus specificity must come between the coupling of the receptor to intracellular enzymes; some progress has been made recently with several TKRs. In the previous section, the molecular link between the EGF

receptor and the PLC-gamma was discussed. In this section, the interaction between TKRs and other cellular proteins will be considered.

The Requirement for Kinase Activity and Substrate Phosphorylation

The requirement of kinase activity for signal transduction has been clearly demonstrated by using kinase-deficient mutant receptors. The mutant molecules are obtained by substituting the lysyl residue in the consensus sequence of the ATP binding site of the receptors for EGF, insulin, and PDGF. Any other amino acid in this position completely eliminates both autophosphorylation and substrate phosphorylation, and prevents signal transduction (Moolenaar *et al.*, 1988). For example, substitution of Lys_{1018} in the β-subunit of the insulin receptor with alanine (IR_{A1018}) destroys the ability of insulin to stimulate glycogen synthase, thymidine incorporation, and all other biological responses tested in transfected CHO or NIH-3T3 cells. In fact, expression of this mutant receptor molecule inactivates the endogenous insulin receptor, presumably through the formation of mixed tetramers ($\beta\alpha\alpha\beta_{A1018}$).

The requirement for substrate phosphorylation is more controversial, because the target proteins of the TKRs have not been identified. A role for

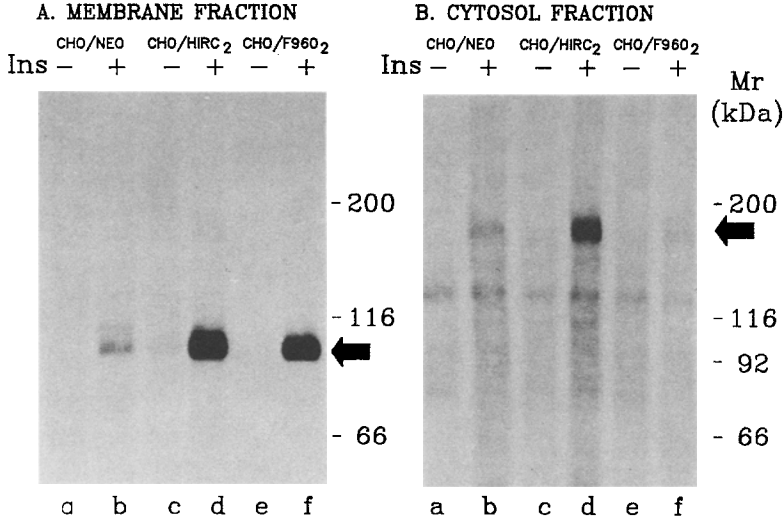

Fig. 6. Immunoprecipitation of phosphotyrosine-containing proteins from [^{32}P]phosphate-labeled CHO cells. Control CHO/neo cells or the CHO/$HIRC_2$ and CHO/$F960_2$ cells were labeled for 2 h with [^{32}P]orthophosphate. The cells were incubated without (−) or with 100 nM insulin (+) for 1 min and fractionated into membrane (A) and cytosolic (B) components. The phosphotyrosine-containing proteins were immunoprecipitated with the αPY, reduced with DTT and separated by SDS-PAGE. The insulin receptor β-subunit is found only in the membrane (A), and the pp185 is found mainly in the cytosol (B).

substrate phosphorylation is implicated in the mechanism of insulin action (White *et al.*, 1988a). A mutant insulin receptor (IR_{F960}) was prepared in which point mutations were introduced into the juxtamembrane region of the insulin receptor purposely at Tyr_{960} (changed to Phe_{960}), and accidentally at Ser_{962} (changed to Thr_{962}). The mutations have no effect on insulin-stimulated autophosphorylation as Tyr_{960} is not an autophosphorylation site (Fig. 6A), but it prevents biological activity, suggesting that autophosphorylation alone is insufficient for signal transmission (White *et al.*, 1988a). The wild-type insulin receptor phosphorylates at least one endogenous protein during insulin stimulation that can be readily detected with anti-phosphotyrosine antibodies; the protein is cytosolic and migrates around 180 kDa under reduced conditions during SDS-PAGE (Fig. 6B). However, the mutant IR_{F960} did not mediate the tyrosyl phosphorylation of pp185. Therefore, β-subunit autophosphorylation was not sufficient for the insulin response, but substrate phosphorylation appears to be needed. It is unlikely that pp185 mediates all of the responses to insulin; rather, the detection of pp185 should be viewed as a marker for a fully functional insulin receptor kinase that can initiate the phosphorylation cascades.

Activation of Serine Kinases by Tyrosyl Phosphorylation

Insulin stimulates metabolic pathways leading to the synthesis of DNA, protein, glycogen, and fat. In cases where some of the mechanistic details are known, protein phosphorylation or dephosphorylation appears to play an important role (Czech *et al.*, 1988). The molecular link between the tyrosine kinase messenger systems and serine/threonine phosphorylation and dephosphorylation is unclear. One hypothesis states that the signal is propagated through a phosphorylation cascade initiated by activation of the receptor tyrosine kinase. Several reports are emerging which suggest that some serine protein kinases are activated by tyrosyl phosphorylation. Three recent examples include the MAP-II (microtubual-associated protein) kinase (Ray and Sturgill, 1988), KIK (Kemptide insulin-stimulated kinase) (Klarlund *et al.*, 1990), and the raf-I kinase (Morrison *et al.*, 1989).

The MAP-II kinase is assayed by its ability to phosphorylate microtubual-associated proteins (MAPs). Insulin stimulates its activity and slightly increases its Tyr(P) content; perhaps activation occurs directly through tyrosine phosphorylation by the insulin receptor (Fig. 7). The MAP-II kinase is structurally similar and possibly identical to pp42, a widely studied tyrosyl phosphorylated protein identified in cells stimulated by EGF, PDGF, IGF-II, thrombin, or phorbol 12-myristate 13-acetate, and also in some oncogene-transformed cells (Rossomando *et al.*, 1989). Thus MAP-II/pp42 kinase may be a common target of all TKRs, suggesting that intermediate

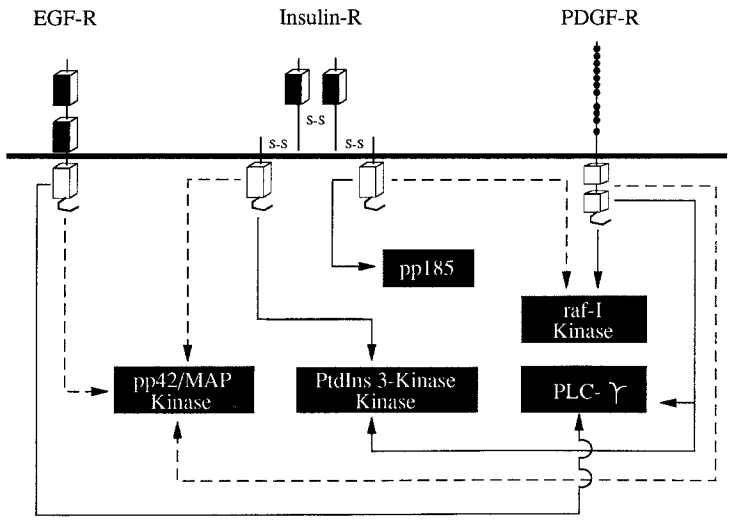

Fig. 7. Possible proteins involved in interactions between the receptors for EGF, insulin, and PDGF. The solid lines indicate proteins which are thought to be activated through tyrosine phosphorylation by the activated TKR. The dashed lines indicate proteins that are activated indirectly by the TKR. Details of this mechanism are currently evolving, so the complexity of the final scheme and the importance of each protein to cellular growth and metabolism is unknown.

steps may exist linking each receptor to this common protein. Interestingly, the activated MAP-II kinase phosphorylates and activates the S6 kinase purified from frog oocytes (34); the phosphorylated S6 kinase may play a role in stimulating protein synthesis. This phosphorylation cascade, beginning at the TKRs, may be involved in the regulation of protein synthesis.

The PDGF and insulin receptors have been shown recently to activate raf-1, a serine/threonine kinase implicated in signal transduction mediated by membrane-bound oncogene products and growth factor receptors (Morrison *et al.*, 1988). However, the exact steps which activates raf-1 appear to be different for each receptor. Raf-1 and the PDGF receptor associate non-covalently after tyrosyl phosphorylation of the PDGF receptor (Morrison *et al.*, 1989). Activation of raf-1 during PDGF stimulation was attributed to tyrosyl phosphorylation which occurs in the complex (Morrison *et al.*, 1989). Thus a direct molecular link may exist between the PDGF receptor and this serine kinase. In contrast, activation of the raf-1 kinase by the insulin receptor appears to occur through serine phosphorylation (Kovacina *et al.*, 1990; Blackshear *et al.*, 1990). Moreover, a stable association between raf-1 and the insulin receptor has not been demonstrated. These observations suggest that the activation of raf-1 by insulin probably occurs through multiple intermediate steps (Fig. 7).

The Relation Between the PDGF Receptor and a Phosphatidyl Inositol-3-kinase

Another signaling system may involve the activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) by TKRs. Although PtdIns kinases have been characterized for many years, only recently has a PtdIns 3-kinase been identified which phosphorylates the hydroxyl group at the D3-position of the inositol ring of phosphatidylinositol. The PtdIns 3-kinase was first shown to associate specifically with the middle-T/pp60$^{c\text{-}src}$ complex or to pp60$^{v\text{-}src}$ (Kaplan *et al.*, 1986; Whitman *et al.*, 1988; Cohen *et al.*, 1990a). Later, it was found to associate with the PDGF receptor following PDGF stimulation of cells (Whitman *et al.*, 1987; Kaplan *et al.*, 1987; Auger *et al.*, 1989), and most recently with the insulin receptor (Endemann *et al.*, 1990; Ruderman *et al.*, 1990) and CSF-I receptor (Varticovski *et al.*, 1989). The PtdIns 3-kinase separates from the tyrosyl kinase in deoxycholate, suggesting that it is not an intrinsic activity of the tyrosyl kinase (Whitman *et al.*, 1987). In the case of the PDGF receptor, an 85-kDa Tyr(P)-containing protein is thought to be the PtdIns 3-kinase (Kaplan *et al.*, 1987; Cohen *et al.*, 1990a,b). However, it is not established whether the PtdIns 3-kinase is activated by tyrosyl phosphorylation or through noncovalent associations with tyrosine kinases (Whitman *et al.*, 1987; Kaplan *et al.*, 1987). The role of the PDGF and insulin-stimulated PtdIns 3-kinase remains to be determined, but it could play an important role in the generation of a second messenger for signaling.

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