

# Cell Signaling by Receptor Tyrosine Kinases

## Review

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A large group of genes in all eukaryotes encode for proteins that function as membrane spanning cell surface receptors. Membrane receptors can be classified into distinct families based upon the ligands they recognize, the biological responses they induce and, more recently, according to their primary structures. A great variety of ligands bind to and regulate the activity of cell surface receptors, including small organic molecules, lipids, carbohydrates, peptides, and proteins. One large family of cell surface receptors is endowed with intrinsic protein tyrosine kinase activity. These receptor tyrosine kinases (RTKs) catalyze transfer of the  $\gamma$  phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter, 1998). RTKs play an important role in the control of most fundamental cellular processes including the cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation. All receptor tyrosine kinases contain an extracellular ligand binding domain that is usually glycosylated. The ligand binding domain is connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic domain contains a conserved protein tyrosine kinase (PTK) core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases (Hunter, 1998; Hubbard et al., 1998). Lymphokines such as erythropoietin and interferon also mediate their responses by tyrosine phosphorylation. However, rather than containing an intrinsic protein tyrosine kinase activity, the relatively short cytoplasmic domains of these receptors interact through noncovalent interactions with members of the Jak family of nonreceptor tyrosine kinases (Darnell et al., 1994; Ihle, 1995). Apart from the lack of covalent linkage to a kinase, the mechanism of activation of these binary receptors largely resembles that of receptor tyrosine kinases (Lemmon and Schlessinger, 1994; Heldin, 1995; Jiang and Hunter, 1999). The purpose of this review is to describe general concepts underlying the mechanism of action of RTKs and the signaling pathways that they regulate, while attempting to shed light on the question of how specificity is defined by the action of RTKs, and how a specific biological response can be generated by the diverse array of signaling pathways activated by all RTKs.

### Paradigms for Receptor Activation

With the exception of the insulin receptor (IR) family of RTKs, all known RTKs (e.g., EGF receptor, PDGF

receptor) are monomers in the cell membrane. Ligand binding induces dimerization of these receptors resulting in autophosphorylation of their cytoplasmic domains (Schlessinger, 1988; Lemmon and Schlessinger, 1994; Jiang and Hunter, 1999). Members of the IR family are disulfide linked dimers of two polypeptide chains forming an  $\alpha_2\beta_2$  heterodimer (Van-Obberghen, 1994). Insulin binding to the extracellular domain of the IR induces a rearrangement in the quaternary heterotetrameric structure that leads to increased autophosphorylation of the cytoplasmic domain. As the active forms of insulin receptor and monomeric RTKs are both dimeric, the signaling mechanisms of the two types of receptor are likely to be very similar (Hubbard et al., 1998).

### Activation by Dimerization

Although all RTKs are activated by dimerization, different ligands employ different strategies for inducing the active dimeric state. Structural studies of growth hormone (GH) in complex with GH receptor (GHR) and erythropoietin (EPO) in complex with EPO receptor (EPOR) show that these cytokines are bivalent, and one ligand binds simultaneously to two receptor molecules to form a 1:2 (ligand:receptor) complex (Kossiakoff and De Vos, 1998; Jiang and Hunter, 1999). Receptor dimerization is further stabilized by additional receptor:receptor interactions.

Several growth factors are homodimers (e.g., VEGF, PDGF) providing the simplest mechanism for ligand-induced receptor dimerization. The VEGF receptors (VEGFR) contain seven immunoglobulin (Ig)-like domains in their extracellular domain, of which only Ig-domains 2 and 3 are required for ligand binding. The crystal structure of VEGF in complex with Ig-like domain 2 of the *flt-1* VEGFR provides a view of ligand-induced receptor dimerization (Wiesmann et al., 1997). The structure shows that one receptor molecule binds at each of the two junctions between VEGF protomers to yield a complex that is close to 2-fold symmetric, and contains two VEGF protomers plus the two Ig-like domains.

The fibroblast growth factor (FGF) family consists of at least 21 related growth factors (Maski and Ornitz, 1998). FGFs are unable to activate FGF receptors (FGFR) without cooperation of the accessory molecule heparin sulfate proteoglycan (HSPG) (Yayon et al., 1991). The crystal structures of FGF in complex with the ligand binding domain of FGFR (consisting of Ig-like domains-2 [D2] and -3 [D3]) provide a molecular view of FGFR dimerization (Plotnikov et al., 1999; Schlessinger et al., 2000) and activation and illustrate the determinants that govern FGF:FGFR specificity (Plotnikov et al., 2000). Each structure shows a 2:2 FGF:FGFR complex, in which FGF interacts extensively with D2, D3, and with the linker that connects these two domains within one receptor (the primary binding site). The dimer is stabilized by a secondary binding site involving interactions between FGF and D2 of the second receptor in the complex, as well as by receptor:receptor interactions. In contrast to the disulfide linked VEGF homodimer, the two FGF molecules in the 2:2 FGF:FGFR complex do not make any contact. Indeed, interactions between FGF and

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FGFR alone are not sufficient for stabilizing FGFR dimers at the cell surface under normal physiological conditions. Heparin or heparan sulfate proteoglycans are essential for stable dimerization of FGF:FGFR complexes (Spivak-Kroizman et al., 1994). It has been shown that heparin binds to a positively charged canyon formed by a cluster of exposed Lys and Arg residues that extends across the D2 domains of the two receptors in the dimer and the adjoining bound FGF molecules (Schlessinger et al., 2000). The full-length FGFR contains an additional Ig-like domain (D1) and a stretch of acidic residues or "acid box" in the linker between D1 and D2. Neither D1 nor the acid box is required for FGF binding to the FGFR. In fact, deletion of D1 and the acid box enhances binding of the receptor to FGF and heparin (Wang et al., 1995). Recent studies we have carried out lead us to propose that D1 and the acid box in full-length FGFR have an autoinhibitory function (Plotnikov et al., 1999). It is thought that the acid box can bind intramolecularly to the heparin binding site in D2, competing with heparin for binding to this site. Similarly, D1 may interact intramolecularly with the ligand binding domain in D2 and D3 and thus interfere with FGF binding to FGFR. This autoinhibition would prevent accidental FGF-independent activation of FGFR by HSPGs that are abundant in the extracellular matrix and on cell surfaces. According to this view, the extracellular domain of FGFR has an autoregulatory function in addition to its roles in ligand recognition and receptor dimerization. A similar mechanism of autoinhibition may apply for other RTKs that contain multiple Ig-like domains in their extracellular domains (e.g., PDGFR, VEGFR). As only 2 out of the 5 Ig-like domains of PDGFR, and just 2 of the 7 Ig-like domains of VEGFR are essential for ligand binding, it is possible that the extra Ig-like domains not involved in ligand binding could play an autoregulatory role in these receptors.

The control of FGFR stimulation by two ligands, FGF and heparin, may provide a mechanism for localized activation of FGFR and vectorial stimulation of cell proliferation or differentiation. The biosynthesis of HSPGs in restricted areas of the extracellular matrix of different tissues may provide a scaffold to which cells expressing FGFR will migrate, and on which these cells will survive, proliferate, or undergo differentiation when supplied with a specific FGF molecule. Indeed, it was demonstrated that FGF8 and FGFR1 are essential for cell migration and mesodermal patterning during gastrulation (Yamaguchi et al., 1994; Sun et al., 1999).

Recent biochemical and structural studies and earlier experiments using monoclonal anti-receptor antibodies have demonstrated that only certain forms of receptor dimers with unique configurations of the extracellular and cytoplasmic domains of both RTKs and cytokine receptors lead to trans-autophosphorylation and PTK stimulation (Lemmon and Schlessinger, 1994; Jiang and Hunter, 1999). Figure 1 depicts a model for how monomeric RTKs (Figure 1A) (e.g., EGFR, VEGFR) or disulfide bridged heterotetrameric RTKs (Figure 1B) (e.g., IR, IGF1R) are activated. It is thought that receptor monomers are in equilibrium with receptor dimers. A limited population of receptor dimers exist with quaternary structures of their extracellular and cytoplasmic domains in configurations that are compatible with trans-

autophosphorylation and stimulation of PTK activity (active dimer). Ligand binding to the extracellular domain stabilizes the formation of active dimers and consequently PTK stimulation. We propose that active dimers exist even in the absence of ligand binding since autophosphorylation of RTKs can be enhanced by inhibitors of protein tyrosine phosphatases or by receptor overexpression even in the absence of ligand binding.

#### ***The Role of Receptor Hetero-Oligomerization***

The EGFR family consists of four RTKs, EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. While EGFR has numerous ligands (e.g., EGF, TGF $\alpha$ , HB-EGF), a ligand for ErbB2 has not been identified. The ligands for ErbB3 and ErbB4, the two other members of this RTK family, are the various isoforms of the neuregulins (NRG). It was demonstrated over a decade ago that EGF-induced stimulation of EGFR leads to activation of ErbB2 by transduction through hetero-oligomerization (King et al., 1988; Stern and Kamps, 1988; Wada et al., 1990). Subsequently, numerous studies have demonstrated that stimulation with EGF or NRG induces a combinatorial hetero-oligomerization of different pairs of members of the EGFR family (Carraway and Cantley, 1994; Lemmon and Schlessinger, 1994; Olayioye et al., 2000). In the absence of a specific ligand for ErbB2, it was proposed that this RTK may function as a heterodimeric partner of the other members of the family, and could provide an additional platform for recruitment of intracellular signaling pathways in response to EGF or NRG stimulation. Moreover, since the sequence of the ErbB3 catalytic domain suggests that this receptor does not have PTK activity, it is thought that ErbB3 may function as a platform to expand the repertoire of intracellular signaling proteins recruited following its trans-phosphorylation by other members of the EGFR family (Carraway and Cantley, 1994).

In the absence of structural information about EGFR, it is difficult to present a clear molecular picture concerning the mechanism of receptor dimerization and hetero-oligomerization. Biophysical studies have suggested that EGF is bivalent toward EGFR and shown that EGF can drive dimerization of the EGFR extracellular domain ending with a stoichiometry of 2:2 EGF:EGFR (Lemmon et al., 1997; Ferguson et al., 2000). It has been proposed that the bivalency of EGF or NRG is the driving force for heterodimerization of ErbB2 with other members of the EGFR family (Tzahar et al., 1997). However, presently there is no evidence for binding of EGF or NRG to the extracellular domain of ErbB2 (Ferguson et al., 2000). The exact mechanism of ligand-dependent dimerization of members of the EGFR family must await the determination of the three-dimensional structures of these complexes. An alternative mechanism is that two receptor homodimers form a heterodimer. Figure 2 shows a potential mechanism for EGF-induced heterotetramer formation between EGFR and ErbB2. According to this scenario EGF-induced homodimers form a tetrameric complex with unoccupied homodimers of ErbB2 by receptor:receptor interactions. The interactions between the two homodimers within the context of a heterotetramer could serve to stabilize the formation of one dimer indirectly by growth factor binding. For example, binding of two monomeric ErbB2 proteins to an EGF-induced homodimer of EGFR may cause homodimeriza-

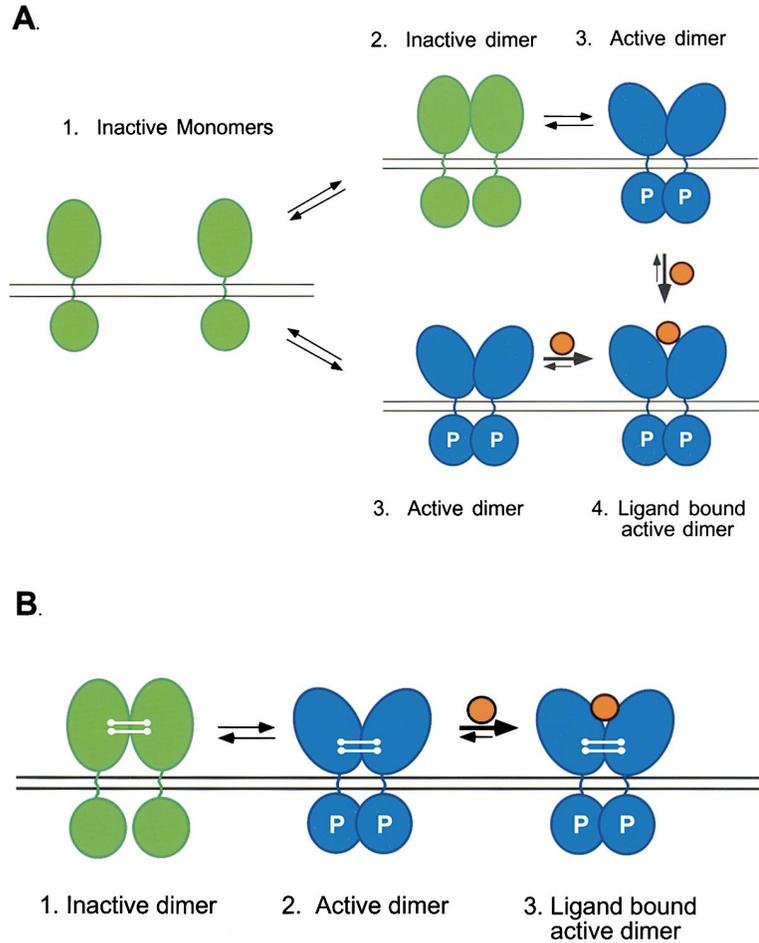


Figure 1. Ligand Binding Stabilizes the Formation of Activated Dimers

(A) Inactive receptor monomers (green) are in equilibrium with inactive (green) or active (blue) receptor dimers. The active receptor dimers exist in a conformation compatible with trans-autophosphorylation and stimulation of PTK activity (blue). Ligand binding stabilizes active dimer formation and hence PTK activation.

(B) Inactive disulfide bridged insulin-receptor (IR) dimers (green) are in equilibrium with active dimers (blue). Insulin binding stabilizes the active dimeric state leading to PTK activation.

tion of the ErbB2 molecules followed by their trans-autophosphorylation and consequent activation (Honegger et al., 1990; Qian et al., 1994; Gamett et al., 1997; Huang et al., 1998). The mechanism of heterotetramer formation between ErbB3 and ErbB4 may be different, since both receptors bind NRG and may undergo NRG-dependent homodimerization (Figure 2). In this case, ErbB3 homodimers may interact with NRG-induced homodimers of ErbB4, which in turn will phosphorylate the cytoplasmic domains of ErbB3 proteins by transphosphorylation. In other words, homodimers of ErbB3 may in fact be preferable substrates of ErbB4 within the context of a heterotetrameric complex.

Structural studies of the catalytic core of several RTKs, together with biochemical and kinetic studies of receptor phosphorylation and activation have provided insights into the mechanism by which RTK dimerization activates enzymatic activity (Hubbard et al., 1994; Mohammadi et al., 1996; Hubbard, 1997). The emerging picture is that receptor oligomerization increases the local concentration of the PTK, leading to more efficient transphosphorylation of tyrosine residues in the activation loop of the catalytic domain (Hubbard et al., 1998). Structural studies have shown that, upon tyrosine phosphorylation, the activation loop adopts an “open” configuration that permits access to ATP and substrates, and enables phosphotransfer from MgATP to tyrosines

on the receptor itself and on cellular proteins involved in signal transmission.

#### Mechanism of Activation of Signaling Proteins

In addition to its central role in the control of protein tyrosine kinase activity, tyrosine autophosphorylation of RTKs is crucial for recruitment and activation of a variety of signaling proteins. Most tyrosine autophosphorylation sites are located in noncatalytic regions of the receptor molecule. These sites function as binding sites for SH2 (Src homology 2) or PTB (phosphotyrosine binding) domains of a variety of signaling proteins. SH2 domain-mediated binding of signaling proteins to tyrosine autophosphorylation sites provides a mechanism for assembly and recruitment of signaling complexes by activated receptor tyrosine kinases. According to this view, every RTK should be considered not only as a receptor with tyrosine kinase activity but also as a platform for the recognition and recruitment of a specific complement of signaling proteins (Pawson and Schlessinger, 1993).

#### Modular Domains of Signaling Proteins

Signaling proteins containing SH2 and PTB domains are modular in nature (Kuriyan and Cowburn, 1997; Pawson and Scott, 1997; Margolis, 1999). Many of these proteins contain intrinsic enzymatic activities and protein modules that bring about interactions with other proteins, with phospholipids, or with nucleic acids. Protein mod-

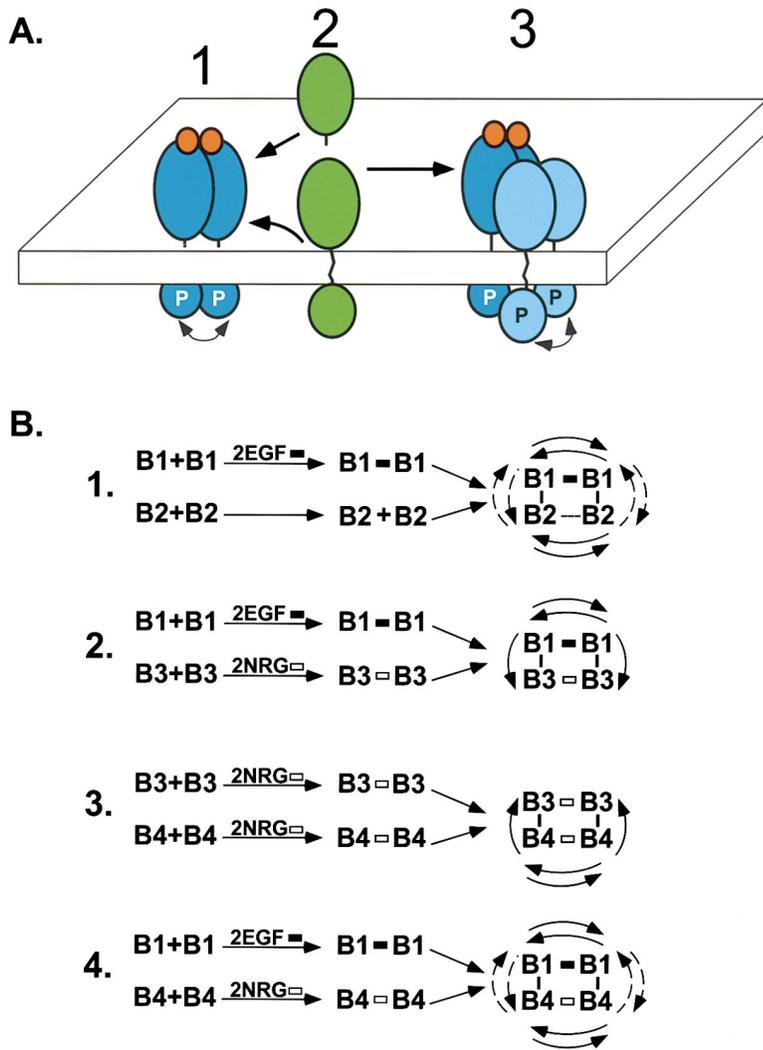


Figure 2. Activation of Members of the EGFR Family by Hetero-Tetramer Formation

(A) EGF binding induces the formation of activated EGFR (blue) homodimers (step 1). Binding of two monomeric ErbB2 (green) proteins (step 2) to an activated EGFR dimer (blue) induces homodimerization of ErbB2 (cyan) molecules followed by autophosphorylation and ErbB2 activation (step 3).

(B) A general scheme for activation of members of the EGFR family by hetero-tetramer formation (EGFR = B1, ErbB2 = B2, ErbB3 = B3, and ErbB4 = B4).

(1) Hetero-tetramer formation between an EGF-induced EGFR homodimer (B1) and two ErbB2 (B2) molecules. Two ErbB2 molecules dimerize by binding to an activated EGFR dimer (as in panel A). Arrows mark autophosphorylation between two EGFRs (B1) or between two ErbB2 molecules (B2). Broken arrows mark potential transphosphorylation between B1 and B2 or between B2 and B1.

(2) Hetero-tetramer formation between an EGF-induced EGFR homodimer (B1) and an NRG-induced ErbB3 homodimer (B3). Arrows mark autophosphorylation of B1 and transphosphorylation of B3 by B1.

(3) Hetero-tetramer formation between an NRG-induced ErbB3 homodimer (B3) and an NRG-induced ErbB4 homodimer (B4). Arrows mark autophosphorylation of B3 by B4.

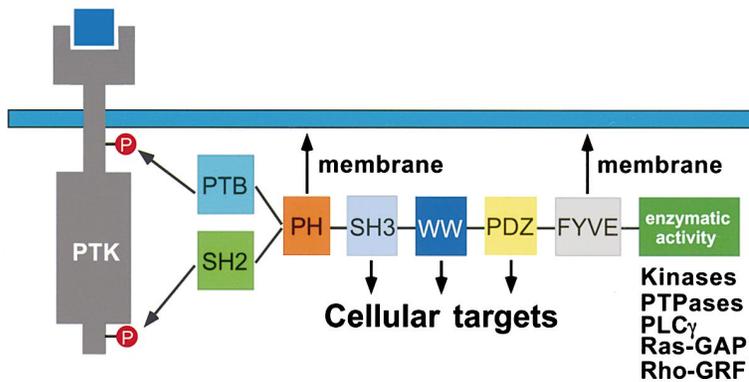
(4) Hetero-tetramer formation between an EGF-induced EGFR homodimer (B1) and an NRG-induced ErbB4 homodimer (B4). Arrows mark autophosphorylation of B1 and B4. Broken arrows mark potential transphosphorylation between B1 and B4 or between B4 and B1.

ules involved in cellular signaling processes range in size from 50 to 120 amino acids. Figure 3 depicts several protein modules that have been shown to be involved in cellular signaling downstream of RTKs and other cell surface receptors. SH2 domains bind specifically to distinct amino acid sequences defined by 1 to 6 residues C-terminal to the pTyr moiety (Songyang et al., 1993), while PTB domains bind to pTyr within context of specific sequences 3 to 5 residues to its N terminus (Margolis, 1999). Certain PTB domains bind to nonphosphorylated peptide sequences, while still others recognize both phosphotyrosine-containing and nonphosphorylated sequences equally well (Margolis, 1999). SH3 domains bind specifically to the proline-rich sequence motif PXXP, while WW domains bind preferentially to another proline-rich motif PXPX (Kuriyan and Cowburn, 1997). Pleckstrin homology (PH) domains comprise a large family of more than a hundred domains. While certain PH domains bind specifically to PtdIns(4,5)P<sub>2</sub>, another subset of PH domains binds preferentially to the products of agonist-induced phosphoinositide-3-kinases (PI-3 kinase) (Ferguson et al., 1995; Lemmon et al., 1995, 1996; Czech, 2000). As only a small subset of PH domains bind specifically to phosphoinositides or

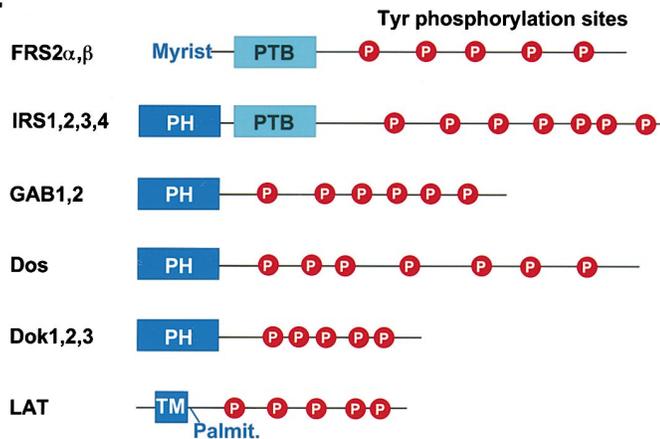
to their soluble head groups, the physiological ligands of the majority of PH domains remain to be identified. However, the weak and nonspecific binding of most PH domains to phosphoinositides may be compensated for by the oligomeric nature of certain PH domain-containing proteins leading to strong membrane association (Lemmon and Ferguson, 2000). Finally, FYVE domains comprise another family of small protein modules that specifically recognize PtdIns-3-P (Fruman et al., 1999), and PDZ domains belong to another large family of independent protein modules that bind specifically to hydrophobic residues at the C termini of their target proteins (Gomperts, 1996).

A large family of SH2 domain-containing proteins possess intrinsic enzymatic activities such as PTK activity (Src kinases), protein tyrosine phosphatase (PTP) activity (Shp2), phospholipase C activity (PLC $\gamma$ ), or Ras-GAP activity among other activities. Another family of proteins contains only SH2 or SH3 domains. These adaptor proteins (e.g., Grb2, Nck, Crk, Shc) utilize their SH2 and SH3 domains to mediate interactions that link different proteins involved in signal transduction. For example, the adaptor protein Grb2 links a variety of surface receptors to the Ras/MAP kinase signaling cascade. Grb2

A.



B.



interacts with activated RTKs via its SH2 domain and recruits the guanine nucleotide releasing factor Sos close to its target protein Ras at the cell membrane (Schlessinger, 1994; Pawson, 1995).

#### Docking Proteins

Agonist-induced membrane recruitment of signaling proteins stimulated by tyrosine phosphorylation is also mediated by a family of docking proteins. Figure 3 depicts a schematic diagram of several docking proteins. All docking proteins contain in their N termini a membrane targeting signal and in their C termini a large region that contains multiple binding sites for the SH2 domains of signaling proteins (Sun et al., 1993; Kouhara et al., 1997). Some docking proteins are associated with the cell membrane by a myristyl anchor (e.g., FRS2), while others have their own transmembrane domain (e.g., LAT) (Zhang et al., 1998a). However, most docking proteins contain a PH domain at their N terminus. Docking proteins such as Gab1 become associated with the cell membrane by binding of its PH domain to PtdIns(3,4,5)P<sub>3</sub> in response to agonist-induced stimulation of PI-3 kinase (Rodrigues et al., 2000). In addition to the membrane targeting signal, most docking proteins contain specific domains such as PTB domains that are responsible for complex formation with a particular set of cell

Figure 3. Protein Modules and Docking Proteins that Participate in Signaling via Receptor Tyrosine Kinases

(A) Protein modules implicated in the control of intracellular signaling pathways. Tyrosine phosphorylated, activated RTKs form a complex with SH2 and PTB domains of signaling proteins. SH2 domains bind to pTyr sites in activated receptors while PTB domains bind to tyrosine phosphorylated and nonphosphorylated regions in RTKs. PH domains bind to different phosphoinositides leading to membrane association. SH3 and WW domains bind to proline-rich sequences in target proteins. PDZ domains bind to hydrophobic residues at the C termini of target proteins. FYVE domains bind specifically to PtdIns(3)P. While adaptor proteins such as Grb2 or Nck contain only SH2 and SH3 domains, other signaling proteins contain additional enzymatic activities such as protein kinases (Src,PKB), PTPase (Shp2) phospholipase C (PLC $\gamma$ ), Ras-GAP or Rho-GRF (Vav).

(B) Docking proteins that function as platforms for recruitment of signaling proteins. All docking proteins contain a membrane targeting region in their N termini. FRS2 is targeted to the membrane by myristoylation, and LAT is targeted to the cell membrane by a transmembrane domains (TM) and by palmytoylation. Most docking proteins are targeted to the cell membrane by their PH domains. Docking proteins contain multiple pTyr phosphorylation sites that function as binding sites for SH2 domains of a variety of signaling proteins.

surface receptors. The PTB domains of IRS1 and IRS2, for example, bind specifically to IR, IGF1-R or IL4-R. The PTB domains of FRS2 $\alpha$  and FRS2 $\beta$  on the other hand, bind preferentially to FGFR or NGFR. It has been shown that docking proteins function as platforms for the recruitment of signaling proteins in response to receptor stimulation. In fact, most of the signaling proteins that are activated in response to insulin or FGF stimulation are recruited via the IRS or FRS families of docking proteins and not by their direct binding to IR or FGFR. It appears that the total amount of signaling proteins that are recruited by a given activated RTK is the sum of the proteins recruited by the receptor directly, and those recruited by docking proteins that are tyrosine phosphorylated by the same receptor.

#### Paradigms for Activation of Effector Proteins

Although many proteins serve as substrates of, and are activated by, RTKs, there appear to be three different general mechanisms for how signaling proteins are activated in response to RTK stimulation. Figure 4 summarizes three effector systems that exemplify these non-mutually exclusive paradigms for activation of effector proteins by RTKs.

**Activation by Membrane Translocation.** PDGF-induced activation of PI-3 kinase leads to generation of the sec-

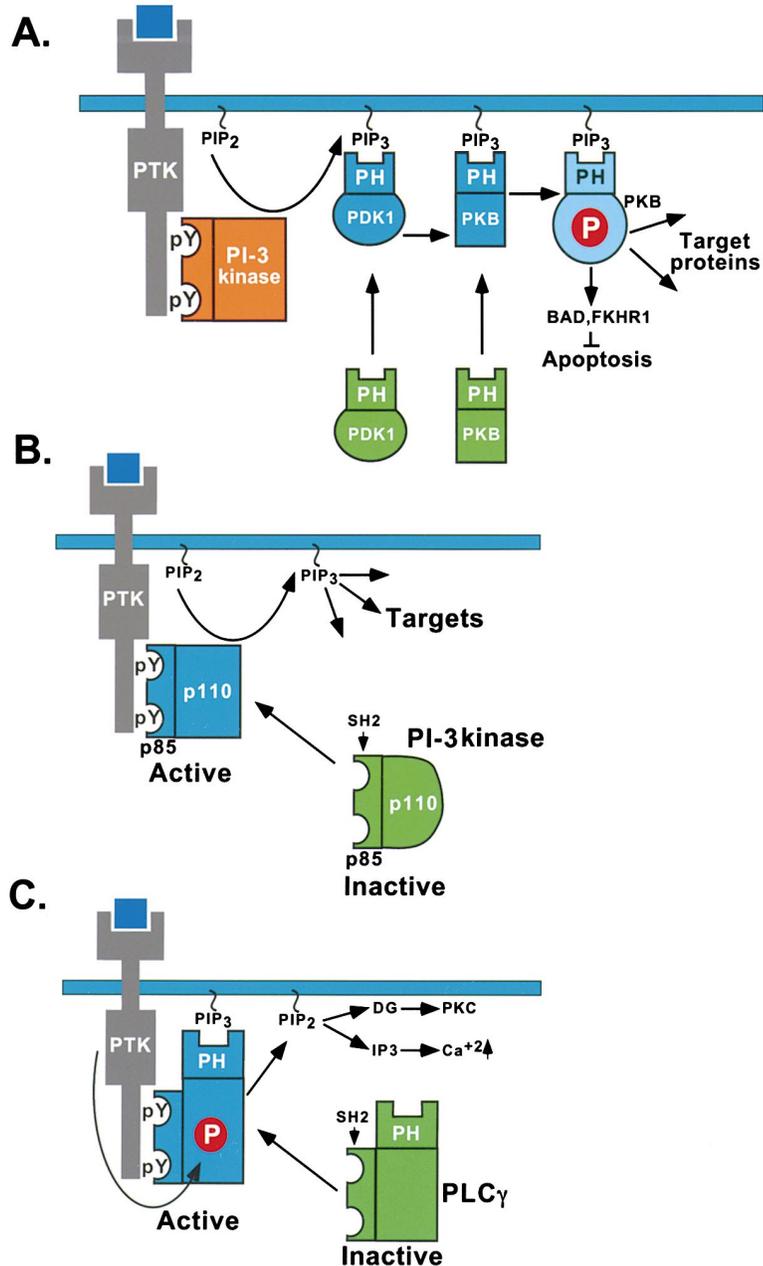


Figure 4. Paradigms for Activation of Signaling Proteins in Response to RTK Activation  
At least two separate molecular events are required for RTK-induced activation of signaling molecules. As many protein targets of RTKs are located at the cell membrane, translocation to the cell membrane is essential for activation of many effector proteins.

(A) Activation of PKB (also known as Akt) by membrane translocation. PtdIns(3,4,5)P<sub>3</sub> generated in response to growth factor stimulation serves as a binding site for the PH domains of PDK1 and PKB. Membrane translocation is accompanied by release of an autoinhibition leading to activation of PDK1 and PKB kinase activities. Full activation of PKB requires phosphorylation by PDK1 (and also by PDK2?). Activated PKB phosphorylates a variety of target proteins that prevent apoptotic death and regulate various metabolic processes.

(B) Activation by a conformational change. Binding of the SH2 domains of p85, the regulatory subunit of PI-3 kinase to pTyr sites on activated receptors releases an autoinhibitory constraint that stimulates the catalytic domain (p110). PI-3 kinase catalyzes the phosphorylation of the 3' positions of the inositol ring of PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> to generate PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, respectively.

(C) Activation by tyrosine phosphorylation. Binding of the SH2 domains of PLC $\gamma$  to pTyr sites in activated receptors facilitates tyrosine phosphorylation of PLC $\gamma$  as well as membrane translocation; a process mediated in part by binding of the PH domain to PI-3 kinase products. Tyrosine phosphorylation is essential for PLC $\gamma$  activation leading to hydrolysis of PtdIns(4,5)P<sub>2</sub> and the generation of the two second messengers Ins(1,4,5)P<sub>3</sub> and diacylglycerol.

ond messengers PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. The generation of these second messengers plays a crucial role in the activation of PDK1 and PKB (also known as AKT), two highly conserved protein kinases that play an important role in stimulation of cell survival, protein synthesis, and metabolic processes (Figure 4A). PDK1 has a PH domain at the C terminus of the protein through which it binds to PtdIns(3,4,5)P<sub>3</sub> leading to membrane translocation (Alessi et al., 1997; Anderson et al., 1998). PKB, which is also recruited to the membrane via its N-terminal PH domain binding to PI-3 kinase products (Franke et al., 1995; Frech et al., 1997), is phosphorylated by PDK1 on Thr308 in its activation loop. It has been proposed that an as yet unidentified protein kinase (hypothetical PDK2) is responsible for PKB phosphorylation on Ser473 leading to complete stimulation of PKB activ-

ity. However, it was recently reported that phosphorylation of Ser473 is mediated by PKB trans-autophosphorylation (Toker and Newton, 2000a, 2000b [this issue of *Cell*]).

**Activation by a Conformational Change.** There is good evidence that SH2 domain-mediated binding of certain signaling proteins to phosphotyrosines on activated receptors induces a conformational change that releases an autoinhibition resulting in stimulation of enzymatic activity. For example, the protein tyrosine kinase activity of Src is activated when its SH2 domain binds to tyrosine autophosphorylation sites on PDGFR (Thomas and Brugge, 1997; Xu et al., 1999). Similarly, binding of p85, the regulatory subunit of PI-3 kinase, to phosphotyrosines in the PDGFR or IRS1 causes conformational changes in p85 that are transmitted to the catalytic sub-

unit p110 leading to enhancement of PI-3 kinase activity (Figure 4B). In addition, by binding to tyrosine phosphorylated PDGFR or IRS1, PI-3 kinase is translocated to the cell membrane where its substrate PtdIns(4,5)P<sub>2</sub> is found.

**Activation by Tyrosine Phosphorylation.** It has been shown that tyrosine phosphorylation of certain target proteins is required for ligand stimulation of their enzymatic activity (Figure 4C). In response to EGF, PDGF, or FGF receptor activation, the SH2 domains of PLC $\gamma$  bind to specific phosphotyrosines in the C-terminal tails of these receptors. Binding of PLC $\gamma$  to the activated receptor facilitates its efficient tyrosine phosphorylation by the RTK. PDGF-induced activation of phospholipase C activity is abrogated in cells expressing PLC $\gamma$  mutated in the tyrosine phosphorylation sites (Kim et al., 1991). Activation of PLC $\gamma$  is also dependent upon agonist-induced generation of PI-3 kinase products. Both tyrosine phosphorylation and membrane translocation of PLC $\gamma$  through binding of its PH domain to PtdIns(3,4,5)P<sub>3</sub> are essential for complete activation of phospholipase-C activity leading to the generation of the two second messengers diacylglycerol and Ins(1,4,5)P<sub>3</sub> (Falasca et al., 1998).

As many of the targets of RTKs are membrane linked, membrane translocation of key signaling components is critical in the process of signal transduction. At least two molecular events must take place before agonist-induced activation of each of the effector proteins described in Figure 4 can occur. PKB activation, for example, requires translocation to the plasma membrane and phosphorylation by PDK1 on a key Thr residue. Furthermore, it was proposed that translocation of PKB to the cell membrane is accompanied by release of an autoinhibition suggesting that a conformational change in PKB may also take place and be required for phosphorylation by PDK1 and for kinase activation (Figure 4A). PDGF-induced activation of PI-3 kinase is mediated by a conformational change in PI-3 kinase induced by p85 binding to pTyr sites on activated PDGFRs (Figure 4B). Stimulation of PLC $\gamma$ , on the other hand, is dependent on both tyrosine phosphorylation and PI-3 kinase activation (Figure 4C). Membrane translocation is essential for PI-3 kinase and PLC $\gamma$  activation, as PtdIns(4,5)P<sub>2</sub>, the substrate of these two enzymes is located in the cell membrane.

### Intracellular Signaling Pathways

The rapid progress in understanding intracellular signaling pathways that took place during the 1990s was largely due to the convergence of information generated by multiple scientific disciplines. Similar proteins were repeatedly identified by applying totally different methodologies. Key components of signaling pathways have been discovered in biochemical studies in which cellular proteins were isolated, cloned, and analyzed. The invertebrate *C. elegans* and *Drosophila* homologs of the same proteins have been found in genetic screens. Moreover, in many cases the same proteins have been identified as products of genes that are mutated in different human diseases such as cancer, severe skeletal disorders, immunodeficiencies, and neurological diseases. A picture is starting to emerge with regard to the different components of several signal transduction pathways and sig-

naling networks that are activated by cell surface receptors (Figure 5). General principles that govern the spatiotemporal information flow from the cell surface to the nucleus, and the modes of communication between the different signaling pathways are becoming unveiled.

### The Ras/MAP Kinase Signaling Cascade

All RTKs and many other cell surface receptors stimulate the exchange of GTP for GDP on the small G protein Ras. Both biochemical and genetic studies have demonstrated that Ras is activated by the guanine nucleotide exchange factor, Sos. The adaptor protein Grb2 plays an important role in this process by forming a complex with Sos via its SH3 domains. The Grb2/Sos complex is recruited to an activated RTK through binding of the Grb2 SH2 domain to specific pTyr sites of the receptor, thus translocating Sos to the plasma membrane where it is close to Ras and can stimulate exchange of GTP for GDP (Schlessinger, 1994; Pawson, 1995; Bar-Sagi and Hall, 2000 [this issue of *Cell*]). Membrane recruitment of Sos can be also accomplished by binding of Grb2/Sos to Shc, another adaptor protein that forms a complex with many receptors through its PTB domain (Margolis, 1999). Alternatively, Grb2/Sos complexes can be recruited to the cell membrane by binding to membrane-linked docking proteins such as IRS1 or FRS2 $\alpha$  which become tyrosine phosphorylated in response to activation of certain RTKs (Sun et al., 1993; Kouhara et al., 1997). There is also evidence that the PH domain of Sos is essential for membrane translocation and for complete activation of Ras. Once in the active GTP-bound state, Ras interacts with several effector proteins such as Raf and PI-3 kinase to stimulate numerous intracellular processes. Activated Raf stimulates MAP-kinase-kinase (MAPKK, MEK) by phosphorylating a key Ser residue in the activation loop. MAPKK then phosphorylates MAPK (ERK) on Thr and Tyr residues at the activation-loop leading to its activation. Activated MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates (e.g., EGFR, Sos). In addition, MAPK is rapidly translocated into the nucleus where it phosphorylates and activates transcription factors (Karin and Hunter, 1995; Hunter, 2000). The signaling cassette composed of MAPKKK, MAPKK, and MAPK is highly conserved in evolution and several MAPK cascades exist in yeast, in invertebrates and vertebrates (Waskiewicz and Cooper, 1995; Madhani and Fink, 1998; Garrington and Johnson, 1999). These highly conserved signaling cascades play an important role in the control of metabolic processes, cell cycle, cell migration, and cell shape as well as in cell proliferation and differentiation (Davis, 2000 [this issue of *Cell*]).

### Phosphoinositol Metabolism and Cell Signaling

Activation of RTKs leads to rapid stimulation of phosphoinositol metabolism and generation of multiple second messengers (Rameh and Cantley, 1999; Czech, 2000). PLC $\gamma$  is rapidly recruited by an activated RTK through the binding of its SH2 domains to pTyr sites in the receptor molecules. Upon activation PLC $\gamma$  hydrolyzes its substrate PtdIns(4,5)P<sub>2</sub> to form two second messengers, diacylglycerol and Ins(1,4,5)P<sub>3</sub>. By binding to specific intracellular receptors, Ins(1,4,5)P<sub>3</sub> stimulates the release of Ca<sup>2+</sup> from intracellular stores. Ca<sup>2+</sup> then binds to calmodulin, which in turn activates a family of Ca<sup>2+</sup>/calmodulin-dependent protein kinases. In addi-

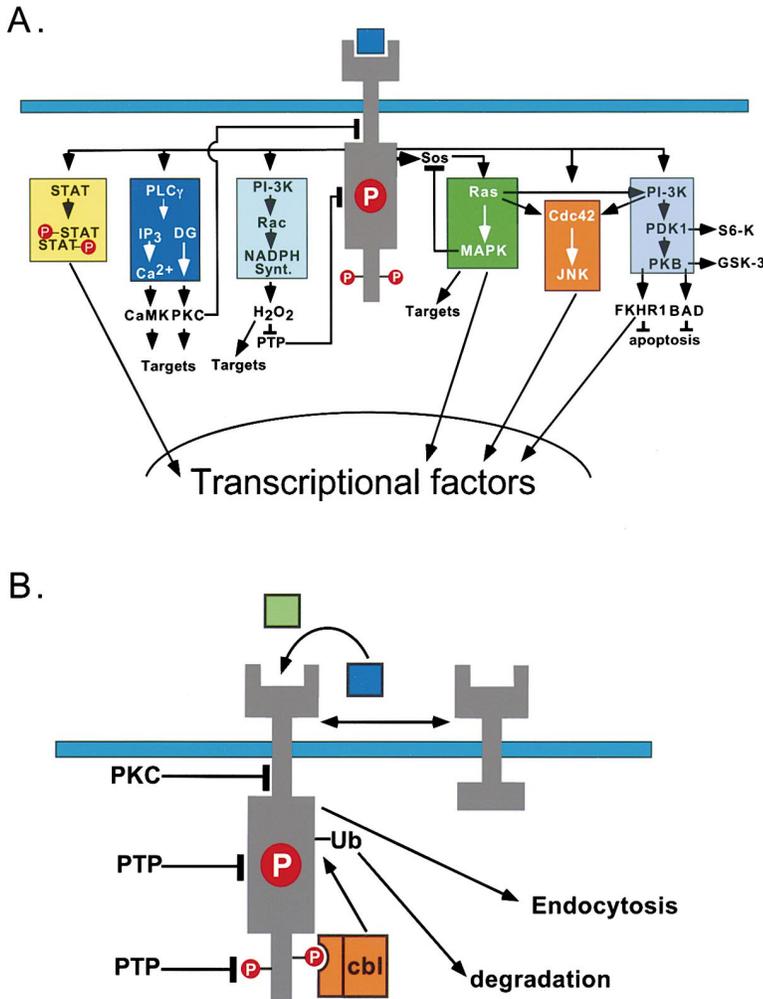


Figure 5. Signaling Pathways Activated by RTKs

(A) Different signaling pathways are presented as distinct signaling cassettes (colored boxes). In several cases the signaling cassettes do not include all the known components of a given pathway. Also shown, examples of stimulatory and inhibitory signals for the different pathways. For example, in addition to activation of the MAP kinase signaling cascade, Ras activates PI-3 kinase and Cdc42. Stimulation of PI-3 kinase leads to activation of PDK1 and PKB, two kinases that regulate various metabolic processes and prevent apoptotic death. In addition, PI-3 kinase activation stimulates generation of hydrogen peroxide which in turn oxidizes and blocks the action of an inhibitory protein tyrosine phosphatase (PTP). The signaling cassettes presented in the figure regulate the activity of multiple cytoplasmic targets. However, the Ras/MAP, STAT, JNK, and PI-3 kinase signaling pathways also regulate the activity of transcriptional factors by phosphorylation and by other mechanisms.

(B) Mechanisms for attenuation and termination of RTK activation. In several cases the activity of RTKs can be negatively regulated by ligand antagonists or by hetero-oligomerization with naturally occurring dominant interfering receptor variants. The PTK activity of EGFR is attenuated by PKC-induced phosphorylation at the juxtamembrane region. Dephosphorylation of key regulatory pTyr residues by protein tyrosine phosphatases (PTP) may inhibit kinase activity or eliminate docking sites. An important mechanism for signal termination is via receptor endocytosis and degradation. The oncogenic protein Cbl binds to pTyr sites in activated RTKs via its SH2-like domain. The RING finger domain of Cbl functions as a ubiquitin-ligase leading to receptor ubiquitination and degradation by the proteasome.

tion, both diacylglycerol and  $Ca^{2+}$  activate members of the PKC family of protein kinases. The second messengers generated by  $PtdIns(4,5)P_2$  hydrolysis stimulate a variety of intracellular responses in addition to phosphorylation and activation of transcriptional factors (Karin and Hunter, 1995; Hunter, 2000).

The phospholipid kinase PI-3 kinase is activated by virtually all RTKs. One group of PI-3 kinases are heterodimers composed of a regulatory subunit p85, which contains two SH2 and one SH3 domain and a catalytic subunit designated p110. Like other SH2 domain-containing proteins, PI-3 kinase forms a complex with pTyr sites on activated receptors or with tyrosine phosphorylated docking proteins such as IRS1 and Gab1. Activated PI-3 kinase phosphorylates  $PtdIns(4)P$  and  $PtdIns(4,5)P_2$  to generate the second messengers  $PtdIns(3,4)P_2$  and  $PtdIns(3,4,5)P_3$ .  $PtdIns(3,4,5)P_3$  mediates membrane translocation of a variety of signaling proteins, such as the non-receptor protein tyrosine kinases Btk and Itk, the Ser/Thr kinases PDK1 and PKB, the Arf exchange factor Grp1, the docking protein Gab1, and  $PLC\gamma_1$ , among many others (Rameh and Cantley, 1999; Czech, 2000). Membrane translocation is medi-

ated through binding of their PH domains to agonist-induced PI-3 kinase products leading to their activation and subsequent stimulation of a variety of cellular responses (Figure 4). One important response is stimulation of cell survival. It has been shown that PI-3 kinase-dependent activation of PKB leads to phosphorylation and inactivation of BAD. Phosphorylation of BAD prevents apoptotic cell death by blocking its complex formation with the apoptotic protein Bcl-2 and Bcl-x1 (Figure 4A) (Datta et al., 1999). Another mechanism for inhibition of apoptosis is via PKB-induced phosphorylation of the transcription factor FKHR1 (Brunet et al., 1999), which in turn suppresses proapoptotic gene expression. Insulin-induced activation of PDK1 leads to phosphorylation and activation of S-6 kinase. Furthermore, glycogen synthase kinase-3 (GSK-3) and phosphofruccokinase, two enzymes that are regulated in response to insulin stimulation, are phosphorylated by PKB. PDK1 and PKB may play a role in the control of protein synthesis, gluconeogenesis, and glycolysis in response to insulin stimulation (Toker and Newton, 2000a).

PI-3 kinase also plays an important role in growth

factor-induced hydrogen peroxide generation. It has been recently shown that PDGF-induced  $H_2O_2$  generation is dependent upon activation of PI-3 kinase and the small G protein Rac (Bae et al., 2000). Earlier studies demonstrated that activation of NADPH synthase, the enzyme complex that catalyzes the production of hydrogen peroxide, is an effector of Rac. Interestingly, EGF-induced generation of  $H_2O_2$  is essential for sustained tyrosine autophosphorylation and activation of EGFR (Bae et al., 1997). Hydrogen peroxide that is generated in response to EGF stimulation oxidizes and inactivates a protein tyrosine phosphatase (PTP) that dephosphorylates activated EGFR (Lee et al., 1998; Bae et al., 2000). Regulation of EGFR kinase activity is not the only role of hydrogen peroxide in response to growth factor stimulation. There is good evidence that  $H_2O_2$  plays an active role in the control of multiple cellular processes.

The activity of effector proteins that are dependent on PI-3 kinase activation can be negatively regulated by PTEN and SHIP, two phosphoinositide-specific phosphatases that dephosphorylate the 3' and 5' positions of the inositol ring of phosphoinositides, respectively (Bolland et al., 1998; Maehama and Dixon, 1998). PTEN is a tumor suppressor protein that is mutated in a variety of human cancers leading to aberrant stimulation of cell survival pathway (Maehama and Dixon, 1998).

#### **Nuclear Translocation of STATs**

All lymphokines induce gene transcription by activating the JAK/STAT signaling pathway (Darnell et al., 1994; Ihle, 1995). The binding of lymphokines to their binary receptor complexes leads to the activation of JAK and subsequent tyrosine phosphorylation of STATs. This is followed by binding of the SH2 domain of STAT to pTyr sites on homotypic or heterotypic STATs enabling formation of STAT homodimers or heterodimers. The dimeric STATs migrate to the nucleus to activate transcription in a target DNA sequence designated the GAS element. In addition to their central role in signaling via lymphokine receptors, there is good evidence that STATs play a role in signaling via RTKs. PDGF, EGF, or FGF stimulation leads to rapid tyrosine phosphorylation and migration of STATs to the nucleus and transcription of target DNA genes. The transcriptional program initiated by STATs is an integral component of the genetic program induced by growth factor stimulation. Moreover, there is good evidence that STAT3 plays a role in PTK-induced oncogenic transformation, as constitutively dimeric forms of STAT3 promote tumor formation.

#### **Mechanism of Signal Attenuation and Termination**

The activity of RTKs must be tightly regulated and properly balanced in order to mediate their normal cellular tasks and their many physiological responses. Indeed, aberrant expression or dysfunction of RTKs is responsible for several diseases and developmental disorders. It is to be expected, therefore, that several mechanisms exist for the attenuation and termination of RTK activity induced by stimulatory ligands.

#### **Antagonistic Ligands**

In *Drosophila*, activation of the EGFR homolog by an EGF-like factor (e.g., Spitz) leads to the expression of a secreted EGF-like protein designated Argos. Genetic and biochemical experiments suggest that Argos binds

to EGFR, competes with Spitz for receptor binding, and inhibits EGFR activity (Figure 5B). It has been proposed that the regulated expression of an EGFR agonist (Spitz), and EGFR antagonist (Argos) is essential for the control of various regulatory networks in which EGFR plays an important role in *Drosophila* development (Casci and Freeman, 1999). No vertebrate homolog of Argos has been identified and the mechanism of its antagonistic activity is not yet understood (Jin et al., 2000).

Another example of an RTK antagonist comes from the family of angiopoietins. Angiopoietins belong to a family of multimeric proteins that regulate mammalian vascularization and angiogenesis. Angiopoietins bind specifically to and activate Tie2, an RTK expressed on the surface of endothelial cells that is implicated in the control of vascularization and angiogenesis. Interestingly, one group of angiopoietins inhibits the biological responses mediated by the Tie2 receptor (Maisonpierre et al., 1997). It is thought that the spatiotemporal expression of the stimulatory and inhibitory angiopoietins is critical for shaping and remodeling the vascular system during development. Moreover, the degree of receptor oligomerization induced by the inhibitory or stimulatory angiopoietins may determine biological outcome.

#### **Hetero-Oligomerization with Receptor Mutants**

In addition to transcripts encoding for full-length RTKs, certain tissues express naturally occurring soluble or membrane-linked receptor variants that are deficient in RTK activity. Expression of an inactive deletion mutant in the same cell may result in dominant negative inhibition of full-length receptor through generation of inactive heterodimers or hetero-oligomers (Jaye et al., 1992). It is thought that one biological role of mutant receptor variants coexpressed in the same cell with full-length receptors is to provide a mechanism for attenuating of the signal generated by ligand stimulation of the full-length receptor (Figure 5B).

#### **Inhibition of RTK Activity**

Activation of protein kinase-C (PKC) by G protein-coupled receptors or by PDGF or phorbol-esters (PMA) results in EGFR phosphorylation on multiple Ser and Thr residues, including Thr654 in the juxtamembrane domain of EGFR. PKC-induced phosphorylation of EGFR results in an inhibition of its PTK activity and in strong inhibition of EGF binding to the extracellular ligand binding domain (Cochet et al., 1984; Davis and Czech, 1985). PKC-mediated phosphorylation of the juxtamembrane domain of EGFR thus appears to provide a negative feedback mechanism for control of receptor activity.

SOCS (suppressor of cytokine signaling) belongs to a family of proteins that function as negative regulators for feedback inhibition in response to cytokine stimulation (Hilton et al., 1998). It has been shown that SOCS proteins inhibit signaling in response to cytokine stimulation by direct binding to the PTK domain of JAK via their SH2 domains. There is now evidence that insulin stimulation induces the expression of SOCS-3 and that SOCS-3 binds directly to the IR suggesting that a similar negative feedback mechanism may take place in signaling via RTKs (Emmanuelli et al., 2000).

#### **Inhibition by Tyrosine Phosphatases**

Protein tyrosine phosphatases (PTP) play an important role in the control of RTK activity and the signaling path-

ways that they regulate. Virtually all RTKs can be activated, even in the absence of ligand binding, by treatment of cells with PTP inhibitors. This experiment demonstrates that the activity of RTKs is continuously being monitored and checked by inhibitory PTPs. The protein tyrosine kinase activity of most RTKs is positively regulated by one or several phosphotyrosine sites in the activation loop. Protein tyrosine phosphatases that dephosphorylate these regulatory p-Tyr residues will inhibit RTK activity and the biological responses mediated by downstream effectors that depend on PTK activity. It was recently demonstrated that targeted gene disruption of PTP1B in mice leads to hyperphosphorylation of IR and IRS1 and sensitization of signaling via the IR *in vitro* and in the mutant mice. These data argue that PTP1B is an important negative regulator of IR (Elchebly et al., 1999).

#### **Receptor Endocytosis and Degradation**

Growth factor stimulation results in rapid endocytosis and degradation of both the receptor and the ligand. Ligand binding induces receptor clustering in coated pits on the cell surface, followed by endocytosis, migration to multivesicular bodies and eventual degradation by lysosomal enzymes. It has been shown that degradation of EGFR is dependent on protein tyrosine kinase activity and that a kinase-negative receptor mutant recycles to the cell surface for reutilization (Ullrich and Schlessinger, 1990). The rapid endocytosis and degradation of activated EGFR and other RTKs attenuates the signal generated at the cell surface in response to growth factor stimulation. Recent studies suggest that the oncogenic protein Cbl plays a role in regulating EGFR and PDGFR degradation. Cbl contains several subdomains, including an SH2-like domain that is responsible for binding to activated RTKs, and a RING finger domain that functions as a ubiquitin ligase. Binding of EGFR or PDGFR to Cbl leads to ubiquitination of the receptor and subsequent degradation by the proteasome (Joazeiro et al., 1999) (Figure 5B). On the other hand, complex formation with activated receptors results in tyrosine phosphorylation of Cbl followed by recruitment to it of signaling proteins such as PI-3 kinase, arguing that Cbl may also function as a docking protein for recruitment of effector proteins.

#### **Coupling with Heterologous Signaling Pathways**

In recent years it has become apparent that RTKs and the signaling pathways they activate are part of a large signaling network that can be regulated by multiple extracellular cues such as cell adhesion, agonists of G protein-coupled receptors, lymphokines or stress signals (Carpenter, 1999). It has also been shown that cell adhesion via integrin receptors leads to activation of several RTKs including the receptors for insulin, EGF, PDGF, and FGF resulting in tyrosine phosphorylation of target proteins and activation of signaling pathways that are normally activated by these receptors. It has been proposed that receptor activation induced by cell adhesion is mediated by coclustering of integrins with RTKs, although the precise mechanism of complex formation between integrins and RTKs is not understood.

RTKs have also been shown to be activated by membrane depolarization, by various stress responses includ-

ing hyperosmotic conditions and ultraviolet radiation, as well as by G protein-coupled receptors (Carpenter, 1999). Agonists of several G protein-coupled receptors (e.g., endothelin, lysophosphatidic acid, angiotensin, and thrombin) have been shown to stimulate the tyrosine phosphorylation of EGFR or PDGFR. It has also been proposed that EGFR and PDGFR, as well as the nonreceptor PTKs, Src and PYK2, are crucial for coupling G protein-coupled receptors stimulation with the Ras/MAP kinase signaling cascade (Luttrell et al., 1999; Hackel et al., 1999). However, it is not yet clear how Gi- and Gq-dependent pathways activate these protein tyrosine kinases. Moreover, MAP kinase stimulation induced by G protein-coupled receptors is normal in fibroblasts deficient in EGFR or in Src kinases.

There is also good evidence for coupling between EGFR signaling and the signaling pathway activated by transforming growth factor- $\beta$  (TGF $\beta$ ) receptors. The TGF $\beta$  family of cytokines mediate their biological responses by binding to and activating a hetero-tetrameric complex composed of receptors with Ser/Thr activity designated TGF $\beta$  receptor-I and -II (Massague et al., 2000 [this issue of *Cell*]). Stimulation of TGF $\beta$  receptors results in the phosphorylation of Smad proteins, followed by their translocation to the cell nucleus and consequent enhancement of transcriptional activity of target genes. EGF exerts an inhibitory response on TGF $\beta$  signaling, by inducing phosphorylation of Smad proteins at specific sites that prevent nuclear translocation and cause an inhibition of transcriptional activity (Kretzschmar et al., 1997; de Caestecker et al., 1998; Zhang et al., 1998b).

It is already apparent that signaling pathways do not function in isolation, and cannot be presented or considered in a simple linear fashion as would be proposed by genetic analyses. A more realistic picture is that signaling pathways are linked together in a large protein network that is subjected to multiple stimulatory and inhibitory inputs, as well as complex feedback mechanisms. Such complexity is essential for mediating the pleiotropic responses of growth factors in development and in the adult animal.

#### **Factors that Determine the Specificity of Signaling Pathways**

A major unanswered question in the field of signal transduction concerns the origin of signal specificity. How are the myriad of extracellular cues transmitted to induce specific biological responses? It is not at all clear how activation of a given RTK at the cell membrane by a specific ligand could utilize the currently known repertoire of intracellular signaling pathways to transduce a unique biological response. Insulin and NGF, for instance, stimulate unique biological responses in their target tissues. Yet, the intracellular signaling pathways that are activated by insulin, NGF, or other growth factors are very similar indeed. In other instances, activation of the same signaling molecules in different cells leads to distinct responses. Why, for example, does stimulation of PI-3 kinase by insulin in muscle cells result in enhancement of metabolic processes, while stimulation of PI-3 kinase by NGF in neuronal cells leads to an antiapoptotic signal? Moreover, what are the factors that determine the biological outcome of a signal gener-

ated by a given receptor tyrosine kinase in different cellular context? Why does stimulation of an RTK (e.g., TrkA, FGFR, Ret) in fibroblasts result in cell proliferation whereas stimulation of the same RTK in neuronal cells results in cell differentiation? Several mechanisms have been proposed for the control of specificity in cell signaling.

#### **Combinatorial Control**

Signal specificity may be defined in part by a combinatorial control. Every RTK recruits and activates a unique set of signaling proteins via its own tyrosine autophosphorylation sites and by means of the tyrosine phosphorylation sites on closely associated docking proteins (e.g., Gab1, FRS2). The combinatorial recruitment of a particular complement of signaling proteins from a common preexisting pool of signaling cassettes is one mechanism for control of signal specificity. This process is further regulated by differential recruitment of stimulatory and inhibitory proteins by the different receptors and downstream effector proteins leading to fine tuning of cellular responses.

#### **The Role of Scaffold Proteins**

It has been shown that scaffolding proteins that bind simultaneously to several proteins are able to insulate key components of signaling pathways from closely related signaling cascades (Whitmarsh and Davis, 1998). In yeast, the scaffolding protein Ste5 has been shown to interact with a pheromone-activated G protein and with components of MAP kinase cascade. Ste5 forms a complex with Ste11, Ste7, and Fus3P leading to insulation of pheromone-induced MAP kinase cascade from closely related signaling pathways. Another example is JIB, a protein that functions as a scaffolding protein in the JNK signaling cascade in mammalian cells (Davis, 2000). There is also evidence that particular members of the MAPK cascade form a complex with a specific upstream activating kinase and downstream effector-kinase to provide insulation from other MAP kinase cascades (Kallunki et al., 1994). It remains to be determined whether RTKs induce specific biological responses by utilizing specific scaffold proteins.

#### **Cellular Compartmentalization**

In recent years it has become apparent that the cellular localization of proteins involved in cell signaling has a profound impact on their biological activity. As many of the targets of RTKs are located at the cell membrane, membrane translocation is required for activation of many cellular processes. Binding of SH2, PTB, or SH3 domains to activated receptors or to membrane-linked docking proteins leads to membrane translocation. In addition, membrane translocation is regulated in part by PH or FYVE domains, two protein modules that bind to different phosphoinositides. It has been shown that binding of proteins containing PDZ domains to their canonical target sequences at the C termini of signaling proteins will induce the assembly of specific sets of signaling proteins in specific regions at the inner face of the cell membrane. Protein assembly at the cell membrane, mediated by multi-PDZ domain containing proteins, may facilitate the phosphorylation of specific substrates by a kinase that is part of the same complex or activation of a GTPase by an exchange factor that is located at the same assembly.

It has been proposed that a variety of proteins that

are involved in cell signaling are concentrated in cholesterol-rich microdomains designated "membrane rafts" (Simons and Ikonen, 1997). It is thought that "membrane rafts" function as sites of assembly of proteins involved in cell signaling including cell surface receptors, GPI-liked proteins, Src kinases, and Ras proteins (Brown and London, 2000). However, it is not clear yet whether membrane rafts exist in the context of living cells (Edidin, 1997) or whether this phenomenon represents an artifact caused by detergent solubilization.

The translocation of STAT proteins from the cell membrane into the nucleus is another example for the role of protein localization in cell signaling (Darnell et al., 1994; Ihle, 1995). Initially, STAT proteins are bound to the cytoplasmic domains of lymphokine receptors in proximity to protein tyrosine kinases of the JAK family. Stimulation of lymphokine receptor or RTKs leads to tyrosine phosphorylation of STAT resulting in homotypic or heterotypic dimerization followed by nuclear translocation and regulation of transcription of target genes.

#### **Signal Duration and Amplitude**

Cellular signaling pathways could be considered as components of intracellular circuits that are generated by protein networks. According to this view, signal transmission and biological outcome should be affected by quantitative considerations such as signal duration and signal strength (Marshall, 1995). For instance, RTKs that induce transient stimulation of MAPK (e.g., EGFR, IR) stimulate PC12 cell proliferation while RTKs that stimulate a sustained and robust MAPK response (e.g., NGFR, FGFR) promote neuronal differentiation of the same cells. In fact, overexpression of IR or EGFR in PC12 cells leads to sustained MAPK response resulting in cell differentiation, although the same receptors give a proliferative response when expressed at lower levels. These experiments shows that biological outcome (proliferation versus differentiation) is determined by quantitative modulation of signal threshold (Marshall, 1995). Signal threshold can be determined by the specific activity of a given RTK, and by the balanced action of the various inhibitory or stimulatory signals that are activated by the RTK. For example, the signal generated by an RTK can be prolonged by generation of hydrogen peroxide that blocks inhibitory protein tyrosine phosphatases or by phosphorylation of docking proteins that promote signal amplification by recruiting of multiple signaling molecules. Signaling pathways are also subjected to multiple negative feedback mechanisms at the level of the receptor itself by inhibitory protein tyrosine phosphatases and by receptor endocytosis and degradation. In addition, the specific activity of key effector proteins can be negatively regulated by inhibitory signals. For example, MAPK responses are inhibited by protein phosphatases that dephosphorylate and inactivate this enzyme. The two phosphoinositide phosphatases PTEN and SHIP dephosphorylate specifically the 3' or 5' phosphate of the PtdIns(3,4,5)P<sub>3</sub> inositol ring, respectively, leading to inhibition of cellular responses mediated by PI-3 kinase products. The balance between the various stimulatory and inhibitory responses will ultimately determine the strength and duration of the signals that are transmitted through the networks of signaling cascades following their initiation at the cell surface in response to RTK stimulation.

### Cellular Context

The biological outcome of signals generated at the cell surface in response to RTK stimulation is strongly dependent on cellular context. The same RTK will induce a totally different response when expressed in different cells or at different stages of differentiation of a particular cell lineage (Sahni et al., 1999). For instance, in early development, FGFR1 plays an important role in control of cell migration, a process crucial for mesodermal patterning and gastrulation. Stimulation of FGFR1 in fibroblasts on the other hand, leads to cell proliferation while stimulation of FGFR1 expressed in neuronal cells induces cell survival and differentiation. The most plausible explanation for these observations is that different cells express cell type-specific effector proteins and transcription factors that mediate the different responses. According to this view, RTKs and their signaling pathways are capable of feeding into multiple processes thus regulating the activity of different effector proteins and transcriptional factors in different cellular environments. A similar input can therefore generate a different output in a different cellular context. In other words, signaling cassettes that are activated by RTKs have evolved in order to relay information from the cell surface to the nucleus and other cellular compartments irrespective of the biological outcome of their activation.

Finally, there is good evidence that critical signaling cascades are regulated by multiple and parallel steps leading to redundancy in signaling pathways. For example, activated EGF receptor recruits the adaptor protein Grb2 directly and indirectly via Shc and Gab1. Therefore, EGFR mutants defective in Grb2 binding are capable of recruiting the adaptor protein Grb2 indirectly resulting in efficient activation of the Ras/MAP kinase signaling cascade. Another example is the redundancy seen in the expression and function of Src kinases (Klinghoffer et al., 1999). While most cells express at least three of the nine known members of the Src family, expression of a single Src kinase is sufficient for mediating an intracellular signal that requires a Src family kinase.

### Conclusions

It has been 20 years since protein tyrosine phosphorylation was discovered (Hunter and Sefton, 1980). The last two decades have seen a rapid progress in the characterization of protein tyrosine kinases, the signaling pathways they activate, and the mechanisms underlying their action and regulation. With the complete determination of the sequences of the genomes of *C. elegans*, *Drosophila*, and *Homo sapiens*, the entire plethora of kinases, phosphatases, and signaling proteins has become accessible to biochemists and geneticists who are interested in deciphering the roles played by RTKs in normal biological processes and in pathological situations.

It is already clear that signaling pathways activated by RTKs are interconnected with other signaling pathways via protein networks that are subjected to multiple positive and negative feedback mechanisms. The frequently applied tool of targeted gene disruption used by geneticists for analyzing signaling pathways is complicated by the existence of redundant signaling pathways and because key components are sometimes

shared by multiple signaling cascades. Consequently, more sophisticated tools should be developed and applied for the analysis of cellular signaling pathways. There is need for new techniques for determination of protein localization (Teruel and Meyer, 2000 [this issue of *Cell*]) and measurement of kinetics of cellular reactions in the context of living cells and even in the live animal. In addition, detailed analyses of gene expression patterns by microarray analysis of genes that are expressed in response to growth factor stimulation (Fambrough et al., 1999) of cells derived from normal or pathological tissues will reveal new links between signaling pathways. Finally, the modern biochemist and geneticists will have to adopt approaches that have been developed by engineers to describe complicated networks (e.g., system analysis) in order to obtain a coherent and realistic perspective on cell signaling (Levchenko et al., 2000; Jordan et al., 2000 [this issue of *Cell*]).

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