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# Signaling—2000 and Beyond

Review

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*Ships that pass in the night and speak each other in passing*
*Only a signal shown and distant voice in the darkness*
—Henry Wadsworth Longfellow, "The Theologian's Tale" (1863)

## The Past

When the history of signal transduction is written in 2100, what will be remembered of the exciting advances that occurred in the latter half of the twentieth century? Everyone will surely have their own list of landmark findings that have contributed to our current picture of signal transduction. Undoubtedly there are seminal earlier events, but from my own perspective this era began with the discovery in the mid-1950s that phosphorylation can reversibly alter the activity of an enzyme through the combined action of a protein kinase and a protein phosphatase (Krebs and Beavo, 1979). At much the same time the hormones adrenalin and glucagon were found to increase the level of intracellular 3′5′ cyclic AMP, and the concept of the second messenger was born. About 10 years later the cAMP-dependent protein-serine kinase (PKA) was isolated as a target for cAMP, and through its pleiotropic substrate specificity PKA was shown to be responsible for many of the effects of cAMP. With these findings the field of signal transduction was born.

Since these early days, progress in understanding mechanisms of signal transduction has been astonishingly rapid. In the past 40 years many important themes and principles of signal transduction have emerged. The highly conserved nature of eukaryotic signaling pathways has been revealed, and defects in signaling have been found as the underlying basis of cancer and other human diseases. Progress has benefited tremendously from structural and genetic analysis, the development of analytical techniques and reagents, and the availability of pharmacological modulators of signaling. What are some of the major milestones?

### *G Protein–Coupled Receptors*

The identification of G proteins and their role in activating membrane bound adenylyl cyclase to synthesize cAMP in response to hormonal stimulation in the mid-1970s was another great step forward in understanding transmembrane signal transduction (Gilman, 1987). The study of G proteins revealed the principle that hydrolysis of protein-bound GTP could act as a signaling switch, and also brought us very near to the membrane receptor itself. The cloning of hormone receptors that are coupled to adenylyl cyclase as well as several neurotransmitter and drug receptors revealed that they all had a close relationship to rhodopsin, the seven transmembrane domain G protein–coupled light receptor. This suggested that serpentine G protein–coupled receptors would be a large family, and, indeed, with the recent addition of odorant receptors, this has become by far the largest receptor family, numbering over a thousand. Analysis with purified components showed that the liganded receptor interacts directly with the heterotrimeric G protein leading to the exchange of GTP for GDP bound to the $\alpha$ subunit, which thereupon dissociates from the $\beta/\gamma$ subunits, allowing both the $\alpha$.GTP and the $\beta/\gamma$ complexes to signal to downstream effectors, such as adenylyl cyclase, again by direct interaction. Sustained signaling in response to a stimulus is generally undesirable, and therefore mechanisms for signal termination are required. The study of G proteins revealed that signaling is turned off when the $\alpha$ subunit hydrolyzes the bound GTP, either spontaneously or upon interaction with a GTPase activating protein (GAP), permitting the $\beta/\gamma$ complex to rebind.

### *Transmembrane Signaling by Phosphorylation*

During the 1980s and 1990s several other distinct principles of transmembrane signaling have been uncovered. The discovery of a new class of protein kinases associated with the polyomavirus, v-Src and v-Abl viral transforming proteins that phosphorylate tyrosine immediately suggested that tyrosine phosphorylation plays a role in growth control (Hunter and Cooper, 1985). Tyrosine phosphorylation was quickly revealed as a major mechanism of transmembrane signaling. The demonstration that EGF stimulated tyrosine phosphorylation EGF receptor in membrane preparations led to the identification of a large family of ligand-stimulated receptor protein-tyrosine kinases (PTKs). Receptor PTKs are all type I transmembrane proteins with a cytoplasmic domain that has intrinsic catalytic activity that is activated upon ligand binding. This established that intracellular protein phosphorylation can be used as a direct means of transmembrane signal transduction. Another important step forward in understanding how signals are transmitted across the membrane came with the discovery that receptors lacking intrinsic catalytic activity can be coupled to nonreceptor PTKs via noncovalent association with the cytoplasmic domain of a receptor subunit, thus forming "binary" receptors (Neet and Hunter, 1996). For instance, cytokine receptors use members of the JAK PTK family. The discovery of transmembrane protein-serine kinases, first in plants and then as receptors for TGF$\beta$ family cytokines in vertebrates, provided another example of the use of ligand-induced intracellular protein phosphorylation as means of transmembrane signal transduction.

The fact that receptor PTKs proved to be type I transmembrane proteins raised the conundrum of how a theoretically flexible protein with a single transmembrane domain could propagate a signal across the membrane in response to ligand binding. The seminal finding was that activation of receptor PTK cytoplasmic catalytic domains requires ligand-induced dimerization (Schlessinger, 1988). This juxtaposes the two catalytic domains allowing mutual transphosphorylation of residues in the activation loop of the catalytic domain, leading to enzymatic activation, and autophosphorylation of tyrosines

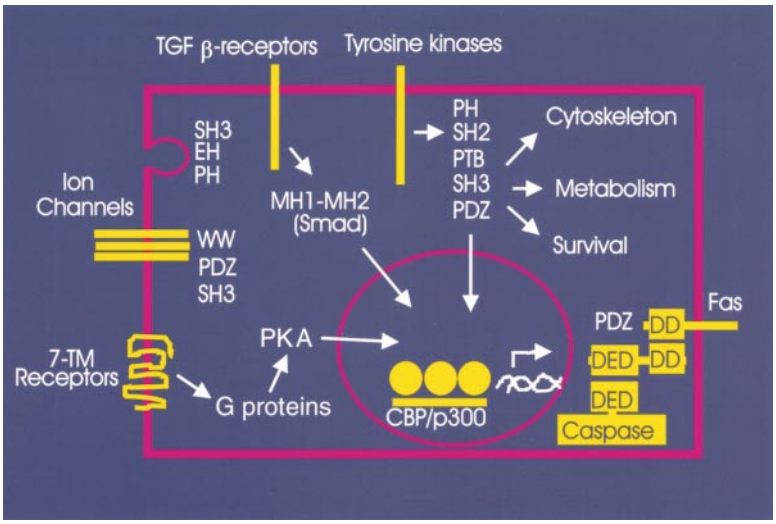


Figure 1. Protein Modules and Signal Transduction

This figure of a cell, showing how modular protein and lipid interaction domains are used in a variety of cell signaling pathways, has been modified from a concept provided by Tony Pawson.

outside the catalytic domain, which are the key to downstream signaling. Receptor protein-serine kinases are also activated by ligand-induced dimerization. Subsequently, ligand activation of many types of surface receptor, including those that do not activate protein kinases directly, has been found to involve oligomerization.

***Protein Signaling Modules***

The discovery of the SH2 domain as a means of recognizing specific phosphorylated tyrosines in activated receptor PTKs and receptor PTK targets was a breakthrough in understanding how activated PTKs propagate signals, since it revealed how the association of two proteins could be induced by phosphorylation thus propagating a signal (Pawson and Gish, 1992). Indeed, this discovery illustrated a totally new function for protein phosphorylation, namely the regulation of protein–protein association. Other types of phosphotyrosine (P.Tyr)-binding domain have subsequently been found (e.g., PTB domains), but SH2 domains are the most prevalent type of P.Tyr-binding domain involved in signaling downstream of activated receptor PTKs. SH2 domains bind in a sequence-specific fashion, recognizing one or more residues in positions 1–6 C-terminal to the P.Tyr. PTB domains recognize residues up to 5 away on the N-terminal side of P.Tyr, but only a subset of PTB domains (e.g., the Shc and IRS-1 PTBs) bind to their target proteins in a phosphorylation-dependent manner (van der Geer and Pawson, 1995).

SH2 and PTB domain interactions are used as a means of recruiting target proteins to activated PTKs, thus permitting their phosphorylation, and also for translocation to the plasma membrane, where many effector proteins activated by receptor PTKs, such as phospholipase C$\gamma$ and PI-3′ kinase, have their substrates (Schlessinger, 1994). SH2/PTB domains are present not only in proteins with intrinsic enzymatic activity that can be regulated by tyrosine phosphorylation, such as phospholipase C$\gamma$, but also in so-called adaptor proteins, such as Grb2, that bind to and thereby bring effector enzymes to the plasma membrane. Receptor-induced recruitment of proteins to the plasma membrane as a mechanism of activating signaling pathways has emerged as a major theme in signaling.

With the identification of the SH2 domain came the realization that most protein–protein interaction domains involved in signal transduction are modular in nature (Figure 1). The study of signaling initiated by receptor PTKs and other protein kinases has uncovered a plethora of different protein interaction domains, ranging in size from 40 to 150 residues, used for inducible or constitutive interactions involved in signaling (Pawson, 1995). Examples are SH2, PTB, SH3, WW, FHA, SAM, LIM, PX, EH, EVH1, and PDZ domains. Like the SH2 domain, most of these domains recognize short linear sequences from 4 to 10 amino acids in length, in some cases requiring phosphorylation of a specific Ser/Thr or Tyr within the recognition sequence, thus providing inducible association. These domains fold independently and their N and C termini protrude from one side of the domain with the interaction surface on the opposite side, allowing them to be assembled almost like beads on a string (Kuriyan and Cowburn, 1997).

Another important step forward was the discovery that domains involved in membrane signaling can recognize molecules other than proteins. For instance, pleckstrin homology domains (PHD), found in many signaling proteins, recognize specific phospholipids and thus allow inducible membrane association dependent on the formation of lipid second messengers (Ferguson et al., 1995). For example, the PHDs of the PDK1 and Akt/PKB protein-serine kinases bind $PIP_3$ and this promotes their membrane recruitment in response to PI-3′ kinase activation and leads to activation of Akt/PKB by PDK1. $Ca^{2+}$ elevation can regulate membrane association of proteins via $Ca^{2+}$-dependent interaction of C2/CalB domains with phospholipids.

Recently, phosphoserine (P.Ser)-binding domains have also been identified, and these too play roles in signal propagation, by facilitating phosphorylation-dependent protein–protein interactions (Yaffe and Cantley, 1999). The first example was the phosphorylation-dependent binding of the CBP coactivator protein to the CREB transcription factor when phosphorylated at Ser133. Subsequently, other families of P.Ser/P.Thr-binding domains have been found.

### *Intracellular Signaling Pathways*

Following the identification of the transmembrane receptors, and their proximal targets, intracellular signaling pathways were the next to come on stage. The emergence of a large family of small monomeric G proteins, heralded by Ras, was an important step in understanding transmembrane signaling. Ras is anchored via C-terminal lipid modifications to the inner face of the plasma membrane, and, like heterotrimeric G proteins, is activated by GTP-GDP exchange catalyzed by GTP exchange factors. Ras.GTP levels are increased upon activation of many types of receptor. A key to understanding how Ras is activated was the finding that the Cdc25-related Sos protein has Ras.GTP exchange factor activity, and that the Grb2 SH2/SH3 adaptor protein is constitutively associated with Sos via its SH3 domains (Schlessinger, 1993). The Grb2/Sos complex is recruited to an activated receptor PTK upon binding of the Grb2 SH2 domain to an autophosphorylated tyrosine in the appropriate sequence context, thus bringing the Sos catalytic domain into proximity with Ras at the plasma membrane and stimulating GTP exchange. Once activated, Ras.GTP interacts with a series of effector proteins, including the Raf protein-serine kinase and PI-3′ kinase, which initiate downstream signaling (Wittinghofer and Nassar, 1996). Analysis of Ras function also led to the discovery of the first GAP, which stimulates GTP hydrolysis and thus acts to terminate Ras signaling (McCormick, 1989). Other small G proteins also function as signaling switches that are activated by receptor-induced GTP exchange. For instance, the Rho family small G proteins, Rho, Cdc42, and Rac, are all activated via receptor signaling pathways, and, like Ras, induce divers signaling pathways, which lead to gene expression and, perhaps more importantly, cause dramatic rearrangements in the actin cytoskeleton.

Ras was the first signaling protein whose function was shown to be conserved from yeast to vertebrates, and this presaged the identification of many highly conserved signaling pathways throughout the eukaryotic kingdom. A prime example is the receptor PTK-Ras-MAP kinase pathway, where genetic and biochemical analysis has revealed that this pathway exists in essentially identical form in species ranging from nematodes to vertebrates, with most of the components being functionally interchangeable between organisms.

### *Transcriptional Regulation by Surface Receptors via Transcytoplasmic Signaling*

Upon binding ligand, many receptors invoke gene expression responses. Indeed, the discovery that growth factors induce de novo expression of a specific set of genes independent of new protein synthesis led to the search for transcytoplasmic signaling pathways that regulate transcription (Karin and Hunter, 1995). In the past 10 years, there has been spectacular progress in understanding how plasma membrane signals are transmitted to the nucleus. The first and simplest transcytoplasmic nuclear signaling mechanism to be identified was the PKA/CREB system (Montminy et al., 1990). In this pathway, cAMP, elevated in response to receptor stimulation, activates the $R_2C_2$ PKA holoenzyme localized in the cytoplasm by binding to the regulatory (R) subunits, thus releasing the catalytic (C) subunit which translocates to the nucleus. An important step in understanding the cAMP pathway was the identification of the CREB transcription factor, which binds to cAMP response elements in inducible genes, and the demonstration that it is a nuclear target for PKA. Once the C subunit enters the nucleus it phosphorylates CREB at Ser133, triggering binding of the CBP/p300 coactivator and transcription of cAMP-responsive genes. This finding reinforced the notion that transcytoplasmic signaling pathways could use transcription factor phosphorylation as a regulatory mechanism. Many transcription factors are now known to be directly regulated by phosphorylation, through positive or negative control of nuclear import or export, DNA binding, or transactivation activity (Karin and Hunter, 1995).

### *MAP Kinase Pathways*

Two separate lines of inquiry, namely analysis of protein-serine kinases activated by receptor PTKs and the study of transcription factor phosphorylation, converged to establish that the MAP kinase pathway is a major mechanism for controlling transcription in eukaryotes (Seger and Krebs, 1995). MAP kinase was originally discovered as an insulin-activated protein-serine kinase, and biochemical studies, reinforced by genetic analysis of the pheromone response in budding yeast, showed that this pathway consists of a cascade of three protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK) (Waskiewicz and Cooper, 1995). These protein kinases are activated in series, such that the MAPKKK phosphorylates the MAPKK at serines in its activation loop, which is thereby activated and phosphorylates the MAPK at a threonine and tyrosine in its activation loop, leading to its activation. Every eukaryotic organism has multiple MAPK pathways, which are largely separate from one another. The elucidation of the modular MAPK cascade with its three consecutive protein kinases had immediate implications for understanding signal amplification and switching, and most importantly for nuclear signaling, because the terminal MAPK, once activated, can migrate into the nucleus, and there phosphorylate and activate transcription factors.

### *Nuclear Translocation of Transcription Factors*

Receptor-induced nuclear translocation of a latent cytoplasmic transcription factor into the nucleus emerged as a different transcytoplasmic signaling principle. The NF-κB activation pathway provided the first example of a cytoplasmically sequestered transcription factor (Karin and Hunter, 1995). In this case, NF-κB is held in the cytoplasm through binding to an inhibitor protein, IκB, which masks the nuclear localization signal in the NF-κB heterodimer. Activating stimuli induce phosphorylation of IκB, at specific sites, which targets it for ubiquitin-mediated degradation, thus releasing active NF-κB to migrate into the nucleus. The simplest pathway of this sort is the JAK/STAT system, in which ligand binding to a cytokine receptor activates associated JAK family PTKs, which first phosphorylate receptor subunits and then specific STAT transcription factors, whereupon the STATs dimerize, migrate to the nucleus and activate transcription (Karin and Hunter, 1995). Other examples are activation of the Wnt/Frizzled pathway, which results in translocation of β-catenin/LEF1 transcription factor

complexes into the nucleus, and activation of TGFβ family receptors, which leads to phosphorylation of receptor-specific Smad transcription factors, which then assemble with the common Smad4 subunit, translocate into the nucleus, and induce transcription of target genes.

Signaling via Notch family receptors utilizes the novel principle of ligand-induced proteolytic cleavage releasing the Notch cytoplasmic domain, which acts as a protein second messenger that migrates to the nucleus and regulates gene expression by binding into and converting the suppressor-of-hairless transcriptional repressor into a transcriptional activator (Artavanis-Tsakonas et al., 1999).

***Nuclear Receptors***

The finding that lipid-soluble ligands, such as retinoic acid, estrogen, and other hormones, can traverse the plasma membrane without utilizing surface receptors and induce cellular responses by binding to and activating members of a family of zinc finger transcription factors revealed yet another nuclear signaling mechanism (Mangelsdorf et al., 1995). These so-called nuclear receptors prove to be a huge family that transduce transcriptional responses to an astonishing variety of chemicals. In principle, the nuclear receptors represent the simplest possible mechanism for nuclear signaling, although analysis of how ligand binding activates receptor-mediated transcription of target genes reveals a surprisingly complex machinery in which the unliganded receptor heterodimer acts as repressor as a result of its association with histone deacetylases, and ligand binding converts the receptor into an activator by triggering dissociation of histone deacetylases and recruitment of histone acetylases.

***Phospholipid- and Ion-Based Signaling***

The discovery in the 1960s of the phosphatidylinositol (PI) cycle, which results in stimulus-induced turnover of $PIP_2$, was the harbinger of the finding of a series of phospholipid-derived second messengers, including diacylglycerol (DAG), $IP_3$, and $PI_{3,4,5}P_3$, and more recently $IP_4$ and $IP_6$ (Liscovitch and Cantley, 1994). The identification of protein kinase C as a DAG-regulated enzyme provided another example of a second messenger-regulated protein kinase. Phospholipase action on $PIP_2$ not only generates DAG but also $IP_3$, which acts as a second messenger to release $Ca^{2+}$ from intracellular stores, thus triggering a program of $Ca^{2+}$-activated events. The unexpected discovery of PI-3′ kinases and the family of 3′ phosphoinositides they generate led to the elucidation of a new phospholipid-based signaling system in which proteins are recruited via $PIP_3$-binding PH domains to the membrane where they are activated (e.g., the Akt/PKB protein-serine kinase) (Kapeller and Cantley, 1994).

The regulated entry and exit of ions across the plasma membrane, through ligand-gated, voltage-sensitive and stretch-activated ion channels and via ion pumps, has important signaling functions, particularly in impulse propagation in the nervous system and in muscle contractility. Receptor-induced changes in cytoplasmic and nuclear $Ca^{2+}$ levels as a result of release of membrane-bound $Ca^{2+}$ stores through $IP_3$ or entry of extracellular $Ca^{2+}$ through plasma membrane ion channels constitute an important signaling mechanism (Berridge and Irvine, 1989). The discovery of calmodulin as a major $Ca^{2+}$-sensing protein in the cells, and identification of protein targets for $Ca^{2+}$/calmodulin complexes, including a family of $Ca^{2+}$/calmodulin activated protein kinases, has provided us with an explanation for how $Ca^{2+}$ release is translated into molecular consequences. Moreover, the development of cell-permeant $Ca^{2+}$ sensor fluorophores has afforded us with the most detailed spatiotemporal picture of signaling events occurring in the cell. The intricate and dynamic real-time patterns of local and global $Ca^{2+}$ release and resorption and the propagation of $Ca^{2+}$ waves and oscillations are surely the harbinger of how other signaling systems work at the subcellular level.

***New Second Messengers***

With cAMP, cGMP, and $IP_3$ the concept of the second messenger was well established. However, the discovery that NO is the endothelial cell–derived relaxing factor for vascular smooth muscle and that NO activates soluble cytoplasmic guanylyl cyclase to elevate cGMP revealed, somewhat unexpectedly, that a gas could act as a second messenger (Murad, 1994). This finding rekindled interest in cGMP-mediated signaling, which is mainly effected by the cGMP-dependent protein kinase, although there are other targets for cGMP.

***Signaling in Prokaryotes and Plants***

Although much of the emphasis in signal transduction has been on eukaryotes, prokaryotes should not be neglected in this litany of progress. For instance, an important new signaling principle was established with the discovery of the two component histidyl-aspartyl phosphorelay systems, comprised of a sensor "histidine" protein kinase that phosphorylates a response regulator on an aspartate residue (Stock et al., 1990). These systems allow prokaryotes to respond to a wide variety of extracellular stimuli. However, somewhat surprisingly, two component systems are rare in eukaryotes, and for the most part seem to have been superseded by conventional protein kinases.

Likewise, although animals and fungi have received the lion's share of attention, plants have also taught us new principles in signal transduction. For instance, the first receptor protein-serine kinases were found in plants, and one type of leucine-rich receptor kinase may be directly regulated by plant steroid family ligands. Moreover, ethylene, an important plant hormone, was the first gas shown to induce cellular responses, and significant progress has recently been made in understanding how ethylene signals.

## The Future

What does the future hold? There are many areas in signaling where it is safe to predict that rapid progress will be made, but because of its preeminence as a mechanism of signal transduction, most attention will be paid to protein phosphorylation and its role in intracellular signaling.

***Modular Protein Interaction Domains in Signaling***

A central theme in receptor-initiated signaling is the use of modular protein–protein interaction domains, either for inducible or constitutive interactions (Pawson, 1995). Well over a hundred putative protein domains have been defined by comparative sequence analysis (http://smart.

EMBL-Heidelberg.de). Although the function of many of these domains is known, many remain uncharacterized and a majority of them could prove to be new protein–protein interaction domains utilized in signaling pathways. Partners for these domains can be identified by two-hybrid screens and affinity methods combined with the use of degenerate peptide libraries to establish a binding consensus sequence. Several phospholipid interaction domains, including PH and FYVE domains, are already known that provide inducible membrane association in response to generation of lipid second messengers (e.g., $PIP_3$) or stabilize membrane association of signaling proteins via binding to constitutive membrane phospholipids. Given the importance of membrane recruitment in signaling, additional lipid recognition domains seem certain to be discovered. Identification of nonprotein targets for such domains should be aided by affinity chromatography combined with mass spectrometric analysis.

The finding that short linear motifs were sufficient for recognition by protein interaction domains came as something of a surprise, given the complex nature of the subunit interaction surfaces found in multisubunit proteins. However, the fact that recognition only requires such short sequences combined with the functional independence of these domains presumably facilitated the rapid genesis of new protein–protein interactions during evolution. Indeed, how easily these domains could be duplicated and used to evolve new signaling proteins is illustrated by the experimental portability of these domains. For example, replacement of the C terminus of the Sos Ras activator with the Grb2 SH2 domain yields a fusion protein that largely rescues the defects in $Grb2^{-/-}$ ES cell differentiation (Cheng et al., 1998). The ability to make chimeric signaling proteins of this sort to test hypotheses about specific signaling connections will be increasingly useful as an analytical tool.

### *Phosphorylation-Dependent Protein–Protein Interaction*

In just 10 years it has become apparent how widely used sequence-dependent P.Tyr recognition is for tyrosine phosphorylation-mediated signaling. To add to the well-established SH2 and PTB domains, novel P.Tyr-binding domains have recently been identified in c-Cbl, IRS-2, and Gab1. Interestingly, structural analysis indicates that the P.Tyr-binding domain in c-Cbl is a variant SH2 domain (Meng et al., 1999), whereas the Gab1 P.Tyr-binding domain appears to have a novel structure. Additional P.Tyr-binding domains are likely to be identified, although they probably will not be large families. A distinct type of P.Tyr-binding domain is exemplified by the "anti-phosphatases", which are proteins structurally related to the PTP or dual-specificity phosphatase families, but which lack one or more of the residues essential for catalytic activities, and therefore can bind but not hydrolyze P.Tyr-containing proteins (Hunter, 1998a).

Although it has only recently been appreciated that P.Ser/Thr can also be recognized by modular binding domains, three families of sequence-specific P.Ser/Thr-binding domains are already known (Yaffe and Cantley, 1999). 14-3-3 proteins, which recognize RSXpSXP and RXY/FXpSXP motifs, bind many protein kinases, and as a result of their dimeric nature can potentially assemble signaling complexes. FHA domains bind pTXXD motifs in proteins, such as Rad9p involved in DNA damage responses. One group of WW domains bind P.Ser/P.Thr.Pro motifs in mitotic phosphoproteins and in the RNA polymerase II large subunit CTD. A subset of WD40 domains and leucine-rich repeats can also recognize P.Ser. Given the great potential and specificity of phosphorylation-induced protein interaction as a signal transfer mechanism, it is a good bet that additional P.Ser/P.Thr-binding domains will be discovered.

### *Induced Protein Proximity in Signaling*

Another overriding principle that has emerged from analysis of receptor-activated signaling pathways is that signaling can be initiated and propagated using the principle of induced protein proximity (Austin et al., 1994). As described above, ligand binding to receptor PTKs initiates signaling by causing dimerization, which facilitates transphosphorylation. Recruitment of proteins with P.Tyr-binding domains to activated membrane-localized receptors where they can be phosphorylated or act on membrane targets is another example. This mechanism elevates the local concentration of proteins at the membrane thereby increasing the number of productive signaling complexes. SH2 domain binding also greatly increases the affinity of these proteins as substrates for phosphorylation by activated receptor PTKs. A dramatic illustration of the importance of protein proximity has been afforded by the use of artificial ligand-binding domains fused to signaling proteins that can then be induced to oligomerize by membrane-permeant dimeric chemical ligands. In several cases, this has been found to trigger the activation of signaling pathways just as efficiently as extracellular ligands (Spencer et al., 1993).

Induced protein proximity is used to activate signaling pathways by many other receptor systems, particularly the TNF, IL-1, Toll, and death receptors, where initial signaling events are critically dependent on oligomeric protein–protein interactions. The subset of TNF family receptors that promote cell death use homotypic interactions between death domains, death effector domains and CARD domains to trigger an intracellular signaling cascade that involves sequential proteolytic activation of a series of proenzymes in the caspase protease family. In death receptor-induced caspase activation, protein proximity is used to accentuate the low basal catalytic activity of a procaspase leading to efficient autoprocessing in the context of an oligomer (Salvesen and Dixit, 1999).

A new concept in protein proximity has emerged from the study of signaling by receptor PTKs. Although it is well established that ligand-induced dimerization is required for activation of receptor PTKs, recent evidence suggests that dimerization per se may not be sufficient for activation, and that there is an additional requirement for the relative orientation of the two dimer subunits to be such that the catalytic domains are correctly juxtaposed (Jiang and Hunter, 1999). In fact, there is good evidence in some systems that receptor dimers can exist in the absence of ligand, and that ligand-induced conformational switches in preformed receptors are critical for activation of the catalytic domains. In this context, an understanding of the way in which

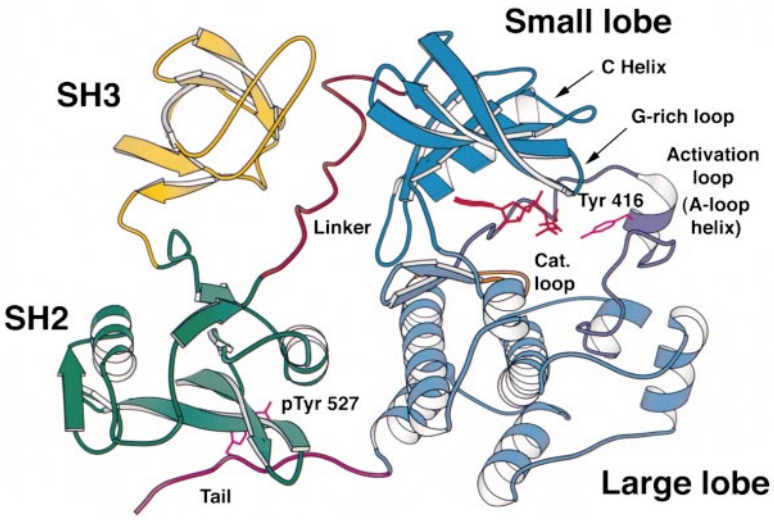


Figure 2. Ribbon Diagram Showing the Structure of the c-Src Protein-Tyrosine Kinase in Its Inactive State Bound to AMP-PNP

This figure is reproduced with permission from Xu et al. (1999).

multimeric ligands are presented to receptors will be increasingly important.

***Structural Analysis of Signaling Proteins***

Many of the most important advances in understanding signaling pathways initiated by tyrosine phosphorylation have come through the determination of the three-dimensional structures of PTKs and PTPs, their immediate targets, including modular domains such as SH2, SH3, PTB and PH domains, and downstream components, including protein-serine kinases and phosphatases.

Structural analysis of signaling proteins has already revealed new and unexpected principles of regulation. For instance, the structures of several protein kinases have shown how the "activation" loop acts as a negative regulator of activity, by blocking ATP and/or substrate binding, and how rotation of the C helix in the N-terminal lobe is used as a regulatory principle. The structure of the c-Src PTK in its inhibited P.Tyr527-phosphorylated state revealed that the SH2 and SH3 domains bound to P.Tyr527 and the SH2-catalytic domain linker, respectively, lie on the backside of the catalytic domain, instead of blocking substrate access to the catalytic cleft as had been expected (Figure 2). In fact, c-Src activity is inhibited indirectly as a result of a conformational change propagated through the small N-terminal lobe that leads to rotation of the C helix and distortion of the active site (Xu et al., 1999). The dimeric structure of catalytic domain 1 of RPTP$\alpha$ led to the prediction that receptor PTP activity can be negatively regulated through a symmetrical D1 dimer interaction in which a helix-turn-helix motif blocks access to the catalytic cleft (Bilwes et al., 1996). Likewise, the N-terminal SH2 domain was found to be bound to the catalytic cleft of the Shp2 PTP thus blocking substrate access, an interaction that is reversed upon the binding of a P.Tyr-containing protein ligand to the SH2 domain leading to exposure of the active site (Hof et al., 1998). The structures of Ras and trimeric G proteins bound to GTP and GDP have revealed how GTP hydrolysis results in a conformational switch that blocks binding of effector proteins. Undoubtedly additional regulatory principles will be identified through structural analysis. Moreover, as molecular docking programs become more sophisticated it may be possible to predict which signaling proteins can interact.

Structural analysis of protein kinase and phosphatase catalytic domains bound to inhibitors and protein interaction domains bound to ligands is now a routine step in the development of drugs that inhibit activity or block interactions, for therapeutic use in diseases where signaling is deregulated. The structures of protein kinases bound to protein (e.g., p16/Cdk6) and chemical (e.g., FGFR1 receptor PTK bound to SU5402 and PD173074) inhibitors have provided insights into how inhibition is brought about and how selectivity is achieved, and this promises to accelerate the pace of development of specific small molecule or peptide-based inhibitors (Mohammadi et al., 1997, 1998; Russo et al., 1998). Moreover, based on inhibitor-bound protein kinase structures it is possible to make mutant protein kinases resistant to a specific inhibitor that can be expressed and used to pinpoint which responses are a direct consequence of that protein kinase (Eyers et al., 1999).

Structures of most of the well-characterized protein and lipid interaction domains have been generated. Comparative analysis of multiple examples of these domains bound to their ligands has given us important insights into ligand binding specificity. Indeed, analysis of SH2 domains bound to P.Tyr-containing peptides has made it possible to make designer point mutations that alter SH2 domain sequence-binding specificity in a predictable way. Likewise, a point mutation in the WW domain can transform its ligand specificity (Espanel and Sudol, 1999). This heralds the possibility of designing unique signaling pathways with customized signaling components.

An area of structural understanding that has lagged behind is the molecular basis of functional regulation by protein phosphorylation. There are very few structures of proteins in both their phosphorylated and unphosphorylated states. The structures of the phospho- and dephospho-forms of glycogen phosphorylase and isocitrate dehydrogenase kinase show that addition of a phosphate can induce a local conformational change increasing substrate affinity or a steric block to substrate binding, respectively. Several protein kinases structures reveal how activation as a result of phosphorylation of residues in the catalytic domain activation loop occurs through a local conformational change that allows ATP

and protein substrate access to the active site. Structures of many more proteins in their phosphorylated and unphosphorylated states will be needed to determine the structural principles underlying functional regulation by phosphorylation.

***Protein Kinase and Phosphatase Regulation***

Protein kinases can be regulated by second messengers, by allosteric mechanisms, by intrasteric inhibition through pseudosubstrate sequences, by positive and negative phosphorylation events, by regulatory subunit binding, by inhibitor proteins and by subcellular localization through targeting domains and anchoring proteins. Will new mechanisms of protein kinase and phosphatase regulation emerge? Additional second messengers and nonprotein regulators are likely to be added to the current list, which includes cAMP, cGMP, DAG, $Ca^{2+}$/calmodulin, polyamines, double-stranded RNA, and double-stranded DNA ends. Sphingosine and ceramide are potential second messengers that reportedly regulate protein kinases; cyclic ADP-ribose and sphingosine 1-phosphate and other recently described second messengers, such as $IP_6$, may also prove to regulate protein kinases. New second messengers may be identified through mass spectrometric analysis of total low molecular weight compounds in extracts of stimulated cells. Conversely, one should be aware that second messengers known to regulate protein kinases might have other functions. For example, that most venerable of second messengers, cAMP, has recently been shown to regulate a Rap1 guanine-nucleotide-exchange factor (de Rooij et al., 1998), suggesting that cAMP, like cGMP, has multiple intracellular targets.

Protein mass spectrometry is leading to the identification of new types of posttranslational modification, and some of these modifications may be used to regulate protein kinase and phosphatase activity. An attractive possibility is that nuclear protein kinases and phosphatases involved in transcription might be regulated by acetylation. Additionally, the HIPK2 protein-serine kinase is modified by SUMO-1 (Kim et al., 1999), suggesting that modification by members of the ubiquitin family might also regulate protein kinase function.

Intracellular protein inhibitors of protein kinases have been known for many years (e.g., PKI for PKA, p21, and p16 family Cdk inhibitors). Generally, these inhibitors are highly specific for individual protein kinases, as would be expected given that they work on the principle of protein–protein interaction. New protein kinase inhibitor proteins continue to be reported, and increasingly these are likely to be of physiological importance.

***Protein Phosphatases and Signaling***

In any process governed by protein phosphorylation, regulation of the protein phosphatase(s) is just as likely to be important as regulation of the protein kinase (Hunter, 1995). Indeed, although initially regarded as constitutively active enzymes that reversed the action of inducibly activated protein kinases, the regulation of protein phosphatases is proving to be highly sophisticated. Protein phosphatase activity can regulated by posttranslational modification (e.g., phosphorylation and methylation), second messengers (e.g., $Ca^{2+}$/calmodulin and PP2B), targeting subunits, and inhibitory proteins. In particular, the PP2A protein-serine phosphatase has a large number of regulatory subunits that dictate substrate specificity and localization. There are several known inhibitor proteins for PP1 (e.g., inhibitor 1 and inhibitor 2), some of which require phosphorylation to be active, and there is some evidence for PTP inhibitor proteins. PTPs are also regulated by phosphorylation and by subcellular localization. There is a large family of receptor-like PTPs whose activity could theoretically be regulated by ligands. In some instances receptor-like PTPs appear to be negatively regulated by dimerization (e.g., RPTP$\alpha$ and CD45) (Majeti et al., 1998), in a mirror image fashion to the activation of receptor PTKs by dimerization. The identification of ligands that regulate receptor PTP dimerization will be an important step forward in understanding these enzymes.

***Spatiotemporal Aspects of Signaling***

An area that will be increasingly important is the spatiotemporal aspects of signaling by protein kinases and phosphatases. Over the past few years, it has been appreciated that protein kinases and phosphatases and their substrates are often discretely localized in the cell, and that this is critical for specificity of phosphorylation. The finding that signaling components are highly organized at the plasma membrane, in the cytoplasm, and in the nucleus suggests that there are discrete signaling domains within the cell. Traditionally, the transduction of signals was thought to involve freely diffusible entities, and this is indeed the case for second messengers like cAMP. However, pathways that involve predominantly protein components may signal in a semi–solid state fashion with minimal free diffusion. Moreover, signaling proteins may be directionally transported via filament-bound motor proteins or by vesicular transport. This leads to the idea that signals can be channeled through specific routes from the membrane to the nucleus and other destinations and that there may be specific cellular compartments that are competent to relay signals. This mechanism would be expected to greatly enhance the efficiency and specificity of signal transmission.

*Anchoring and Scaffolding Proteins*. Anchoring proteins are often used to localize protein kinases and phosphatases to their substrates in particular places in the cell (Pawson and Scott, 1997). This increases the efficiency and specificity of substrate phosphorylation and coordinates the actions of protein kinases and phosphatases. The best studied anchoring proteins are the AKAPs that are used to localize PKA, via its R subunits, and its substrates to particular places in the cell. For example, AKAP18, a myristoylated protein is localized to the plasma membrane where it binds PKA and the L-type $Ca^{2+}$ channel, a PKA target (Colledge and Scott, 1999). Anchoring proteins are also known for TGF$\beta$ RI, where SARA is used to recruit Smad proteins and to localize the signaling complex to discrete membrane regions via a FYVE domain (Tsukazaki et al., 1998), and for protein kinase C, where RACKs are used for membrane localization. Undoubtedly, many more anchoring proteins await discovery.

A different means of bringing signaling components together is through the formation of multiprotein complexes through protein–protein interaction domains. In

this regard, one important principle is the use of scaffolding proteins to assemble sequential components of a signaling pathway (Garrington and Johnson, 1999). The best characterized example is Ste5, a scaffolding protein that interacts with the Ste11, Ste7, and Fus3p/Kss1p protein kinases in the mating pheromone MAPK pathway. Scaffolding proteins are known for the JNK/SAPK and ERK MAPK pathways as well. Individual members of a MAPK pathway can also serve as scaffolds by interacting with other protein kinases in the same pathway. An important property of many scaffolding proteins is their ability to dimerize, which allows phosphorylation in *trans* between protein kinase molecules bound to different subunits in the dimer. A deeper understanding of how assemblies of signaling proteins are built up is clearly important.

*Plasma Membrane Signaling Domains*. Another higher order principle of signal localization is afforded by plasma membrane signaling domains. Membrane microdomains known as caveolae, marked by the presence of an intracellular protein coat comprised of caveolin, can be isolated in a detergent-insoluble cholesterol/glycolipid-rich fraction (Kurzchalia and Parton, 1999). By virtue of this property, caveolae are found to be enriched for many membrane signaling proteins, usually localized by GPI linkages or palmitoyl groups. These lipid rafts are estimated to contain 50–100 protein molecules, and this could be a means of concentrating signaling proteins for more efficient interaction. Another example of a discrete plasma membrane signaling domain are focal adhesions, which are organized membrane-associated structures where clustered integrins bind to extracellular matrix proteins and to the actin cytoskeleton, and which mediate integrin signaling through tyrosine and serine phosphorylation.

Yet another mechanism of organizing proteins on the inner face of the membrane is through the use of proteins with multiple PDZ domains (Fanning and Anderson, 1999). PDZ domains bind to short C-terminal E.S/T.X.V/$I_{COOH}$ motifs, and a single protein can have up to 20 PDZ domains. A good example of a submembraneous PDZ organizing protein is InaD, which contains 5 PDZ domains with different specificities and can also dimerize. InaD interacts with multiple components of the visual signaling system in photoreceptors in the *Drosophila* eye, including rhodopsin, thus concentrating all the necessary components into complexes and thereby speeding up signal transmission. An understanding of how membrane proteins involved in signaling are organized on the inner face of the membrane will be increasingly important.

*Signaling Protein Translocation*. Regulated protein localization has emerged as a fundamental principle in signaling, and spatial separation of proteins is commonly used as a mechanism for preventing spontaneous signal activation. In addition to local movement of proteins, exemplified by inducible membrane association of cytoplasmic P.Tyr-binding domain proteins, longer range movement of activated signaling proteins within the cell is essential, particularly for transcytoplasmic signal transmission, where phosphorylation triggers the translocation of proteins in and out of the nucleus. For instance, tyrosine phosphorylation of Stats induces their dimerization and nuclear import, and phosphorylation of the FKHR transcription factor by Akt/PKB results in nuclear export (Brunet et al., 1999). Vesicular protein trafficking may also be used as a mechanism of signal transduction. For instance, most ligands induce endocytosis of their receptors, and there is increasing evidence that receptors internalized in endocytic vesicles carry out specialized signaling functions.

*Temporal Aspects*. Historically, signaling has been studied in populations of cells, but it is clear that there is significant kinetic variation in the responses of individual cells to a stimulus. This underscores the importance of developing tools to study signaling at high resolution in single cells, as has been done for $Ca^{2+}$ signaling. Extensive use is already being made of GFP-fusion proteins to study the real-time movement of signaling molecules such as protein kinases in single living cells in response to extracellular stimuli. For instance, translocation to the plasma membrane of various isoforms of PKC fused to GFP can be detected in response to receptor stimulation (Sakai et al., 1997; Oancea and Meyer, 1998). SH2-GFP fusion proteins are being used to study the localization of tyrosine phosphorylation events (Stauffer and Meyer, 1997), and PH domain–GFP fusions to study where $PIP_2$ and $PIP_3$ are generated in vivo (Stauffer et al., 1998). A better knowledge of the exact timing of the onset and termination of signaling and the speed of movement of signals combined with their precise location will be a key to understanding signaling output.

***Signaling Networks and Signaling Specificity***

One of the major challenges in understanding signaling networks is to elucidate how signaling specificity is achieved when many of the same core signaling pathways are activated by receptors that elicit different cellular responses (Tan and Kim, 1999). For instance, why does activation of the PI-3 kinase pathway by the insulin receptor PTK lead to metabolic responses such as translocation of the GLUT4 glucose transporter, whereas activation of PI-3 kinase by growth factor receptor PTKs in the same cell does not? Signaling specificity can in principle result from the activation of unique signaling pathways by a receptor. Alternatively, since cellular responses reflect an integration of outputs from all the pathways activated by a single receptor, signaling specificity can also result from a unique combination of signaling pathways activated by the receptor. Receptor PTKs use not only autophosphorylation site tyrosines but also phosphorylate-specific docking proteins (e.g., IRS1/2 for the insulin receptor PTK and FRS2 for the FGF and NGF receptor PTKs) to diversify signaling and provide specificity. The exact location of ligand-activated receptors on the cell surface may dictate which pathways can be activated, thus using spatial separation as another means of generating specificity. Cell type–specific nuclear responses to activation of a given receptor can be explained by the expression of different repertoires of downstream signaling proteins including transcription factors, and by which active chromatin domains are accessible for transcriptional induction by transcytoplasmic signaling pathways in each cell type.

The apparent redundancy in signaling pathways is another challenge in understanding specificity. Redundancy is evident from the finding that mutation of individual tyrosine phosphorylation sites in a receptor PTK

often does not abolish a specific response, or even affect the spectrum of genes induced upon receptor activation (Valius and Kazlauskas, 1993; Fambrough et al., 1999). This may be explained by the existence of more than one means of activating a critical pathway (e.g., the Ras MAP kinase pathway can be activated by several distinct mechanisms). Where there are several closely related proteins in a family, there can also be functional redundancy. A good example is the Src PTK family, where c-Src, Fyn, and c-Yes are commonly coexpressed, and where any one of the three can be sufficient to propagate a signal requiring a Src family PTK (Klinghoffer et al., 1999).

Signal pathway cross-talk will become increasingly important for our understanding of signaling networks. Cross-talk can occur between pathways activated by a single receptor, or more commonly by pathways activated by different receptors. Indeed, integration of cellular responses elicited by different receptors must occur by cross-talk. There are already well-established links between G protein–coupled receptors and receptor PTKs, and between integrin adhesion receptors and receptor PTKs, to name just two examples (Luttrell et al., 1999; Moghal and Sternberg, 1999). Cross-talk can take place at many levels from the membrane to the nucleus, and involve components that are in common between two pathways, as well as positive and negative feedback signals that can act at many steps in a pathway from transcription factors to the receptors themselves. On the other side of the coin, pathway insulation to prevent cross-talk is proving an increasingly important concept. For instance, scaffolding proteins that assemble protein kinase signaling cascades insulate related MAPK pathways (Whitmarsh and Davis, 1998).

Another important concept in signaling specificity is signal thresholds. In many systems a 2-fold decrease in the level of a signaling protein can be sufficient to abrogate signaling, and conversely a 2-fold increase can initiate signaling. Moreover, signaling thresholds may be different in different cell types. Productive signaling can occur with only a few hundred activated receptor molecules per cell, and therefore there has to be significant signal amplification. In this context, one aspect of receptor PTK signaling that is not well understood is the fact that growth factor receptor signaling is needed for 6–8 hr before a cell is committed to respond, even though all the early signaling events that have been so intensively studied are over by 1–2 hr, and the receptor itself and the signaling pathways have been downregulated by degradation and negative feedback loops. It remains unclear exactly what signal(s) is sensed by the cell at later times. This also highlights the fact that most studies of ligand-induced signal transduction use acute stimulation with saturating doses of ligand, and often overexpressed receptors and heterologous cell types, and one can question whether all of the responses that are detected are physiologically relevant.

Finally, temporal aspects of signaling are also important in defining cellular responses. For instance, transient activation of ERK MAPK by a receptor PTK in PC12 cells fails to induce differentiation, whereas sustained ERK MAP kinase activation induces neurite outgrowth (Marshall, 1995). Signaling kinetics are governed by negative feedback systems that downregulate signaling through phosphorylation and dephosphorylation and also by protein degradation. Thus, ligand-induced receptor PTK signaling can be negatively regulated by receptor endocytosis, dephosphorylation, feedback serine phosphorylation, by binding of inhibitory proteins including inhibitory ligands, and through phosphorylation-dependent ubiquitination and degradation. The recruitment of nonreceptor PTPs such as Shp1 either to receptors themselves or to inhibitory receptors (e.g., KIRs) or inhibitory signaling proteins (e.g., SIRPs) is one mechanism to downregulate receptor PTK signaling (Moghal and Sternberg, 1999). Interestingly a number of SH2 domain proteins, including Socs, Cbl, and Dok family members have recently been shown to act as negative regulators of PTK signaling. Negative regulation of signaling may well turn out to be as important as positive regulation in understanding the specificity of signaling networks.

### *Role of Protein Kinases and Phosphatases in Disease*

Increasing numbers of human diseases are known to involve mutations, overexpression, or malfunctioning of protein kinases and phosphatases, and their regulators and effectors. The realization that protein kinases might play a direct role in disease came with the discovery that the v-Src oncoprotein is a PTK. Subsequently, a plethora of diseases have been show to be due to mutations that activate or inactivate PTKs and PTPs, or lead to their misexpression and/or overexpression (Hunter, 1998b). At least 18 PTK genes have been identified as oncogenes either in acutely transforming retroviruses or in human tumors. Mutations in PTKs are also involved in other diseases. In particular, mutational inactivation of nonreceptor PTKs is observed in several immunodeficiency diseases. Inactivation of both copies of ZAP70 or JAK3 causes severe combined immunodeficiency, and mutation of the X-linked *BTK* gene results in agammaglobulinemia.

Given the importance of activated PTKs in cancer, one might have anticipated that PTP genes would be found as tumor suppressor genes. So far this has not proved to be the case. However, there has been recent excitement over the finding that the *PTEN/MMAC* gene, which is mutated in a variety of sporadic cancers and in the hereditary Cowden's hamartoma cancer syndrome, encodes a member of the dual-specificity protein phosphatase family. However, PTEN's main function in negative growth regulation appears to be as a 3′ phosphoinositide phosphatase, rather than as a protein phosphatase (Maehama and Dixon, 1998).

Many genetic diseases also result from mutations in protein-serine kinases and phosphatases. For instance, the Coffin-Lowry syndrome is due to inactivation of the X-linked *Rsk2* protein-serine kinase gene, and myotonic dystrophy is due to decreased levels of expression of the myotonic dystrophy protein-serine kinase. In addition, overexpression of the aurora2 protein-serine kinase is implicated in colon carcinoma, and the Lats1 and Lkb1 protein-serine kinases have both been identified as tumor suppressors. Conversely, inactivating mutations in the Pr65 PP2A regulatory subunit are found in lung and colon cancers.

As molecular analysis of human disease proceeds,

one can predict that many additional somatic and hereditary mutations with causal roles in disease will be found in protein kinases and phosphatases, and in other signaling proteins. These enzymes and proteins will become candidates for the development of therapeutic drugs.

*Clinical Implications*. In general enzymes make good targets for drugs, and the widespread involvement of protein kinases and phosphatases in disease has led to a massive effort to develop drugs that either activate or more usually inhibit individual protein kinases and phosphatases. Drugs designed to target these enzymes fall into two categories—monoclonal antibodies or modified protein ligands, and small molecules. Significant success has been achieved with the development of small molecule inhibitors of a number of PTKs, and several PTK inhibitors are either in or are beginning to enter clinical trials. One group of small molecule inhibitors in cancer therapy trials target the EGF receptor or EGF receptor PTK family members. Another group of inhibitors is directed against the VEGF receptor PTKs that are essential for tumor angiogenesis (Fong et al., 1999). One receptor PTK antagonist, Herceptin, a monoclonal antibody that blocks the function of the ErbB2 receptor PTK, is already on the market, and is being used as an adjuvant breast cancer therapy in the significant fraction of breast carcinomas where ErbB2 is overexpressed. Another disease being treated with a PTK inhibitor is chronic myelogenous leukemia (CML), where a chromosomal translocation results in the expression of a chimeric Bcr-Abl protein that is a constitutively activated PTK (Sawyers and Druker, 1999). CGP57148, a Bcr-Abl PTK inhibitor, is proving very effective as a treatment for CML. There are also protein-serine kinase inhibitors in cancer clinical trials, including flavopiridol, a Cdk2 inhibitor.

A number of diseases are due to insufficient receptor PTK signaling, including noninsulin-dependent diabetes and peripheral neuropathies. If it were possible to enhance signaling through the receptors in question, this could serve as a viable therapy. One way to do this would be to find inhibitors of the cognate PTP for a receptor PTK. One candidate emerges from the recent exciting finding that the knockout of PTP1B nonreceptor PTP in the mouse results in insulin hypersensitivity, indicating that PTP1B is a major insulin receptor PTK phosphatase (Elchebly et al., 1999). A PTP1B-specific inhibitor has just entered clinical trials for diabetes. Another candidate is the NGF receptor PTK, and a drug that enhances NGF receptor signaling is also about to start clinical trials.

Small molecule activators of receptor PTKs have also been developed. For instance, an activator of the insulin receptor PTK has recently been reported, which could in principle act as an orally available insulin mimetic (Zhang et al., 1999). Interestingly, this pseudodimeric molecule acts intracellularly, apparently by reorienting the two subunits of the insulin receptor dimer into an active configuration in a redox-dependent manner. Likewise, small molecule activators of the G-CSF (Tian et al., 1998) and erythropoietin (Wrighton et al., 1996; Johnson et al., 1998) binary receptor PTKs have been developed.

The first small molecule drugs that act on protein kinases and phosphatases are likely to be approved for clinical use in the near future. In the next few years, we can anticipate that the rational structure-based design and development of highly specific protein kinase and phosphatase inhibitors (and activators) will become routine, and that drugs that intercede in phosphorylation-mediated signaling pathways will become a major class of drug.

***Genomics and Phosphorylation***

Complete genome sequences have the potential to reveal the totality of protein kinases and phosphatases and phosphorylation-related signaling proteins expressed by a single organism. However, a priori assignment of function to a gene product relies on its sequence being related to a protein of known function, and it clear that not all of the types of proteins that mediate phosphorylation-dependent signaling have yet been described.

*Novel Protein Kinases and Phosphatases*. Biochemical and genomic analysis has already uncovered novel types of protein kinases and phosphatases, distinct from the major protein-serine/tyrosine kinase superfamily and the known protein phosphatase families, and will undoubtedly continue to do so. For example, one newly described group of protein kinases (MHCK A, eEF-2 kinase, NFK, etc.) have a conserved catalytic domain that is completely unrelated to that of the protein-serine/tyrosine kinase superfamily (Ryazanov et al., 1999). Recent sequence analysis has uncovered a set of microbial "protein kinase" families (ABC1, piD261, RIO1 families, and aminoglycoside kinases) that are all very distantly related in sequence to the protein-serine/tyrosine kinase superfamily catalytic domain, which are also represented in budding yeast and *C. elegans* (Leonard et al., 1998). These may represent the evolutionary origin of the eukaryotic protein kinase superfamily. Histidine, lysine, and arginine can be phosphorylated in proteins; however, with the exception of a yeast histidine kinase that phosphorylates histone H4, little is known about the protein kinases that phosphorylate these residues, and this will be an important task for the future.

New types of protein phosphatase will also emerge to add to the currently known families of protein-serine, protein-tyrosine, and dual-specificity phosphatases. For instance, the recently described CTD phosphatase, which dephosphorylates the CTD of the large subunit of RNA polymerase II, is a new type of protein-serine phosphatase (Cho et al., 1999). Moreover, like PTEN, some members of the PTP superfamily may have non-protein phosphate ester substrates.

*Protein Kinase and Phosphatase Catalogs*. One exciting outcome of the ongoing genomic sequencing projects is that complete catalogs of protein kinases and phosphatases will become available for increasingly complex eukaryotic organisms. We already know that *S. cerevisiae* has 114 conventional protein kinase genes (but no bona fide PTKs) out of 6,217 genes (1.8%) (Hunter and Plowman, 1997). The recently completed sequence of *C. elegans* reveals that the worm genome encodes 400 protein kinase catalytic domains (92 are PTKs = 23%) out of 19,099 genes (2.1%) (Plowman et al., 1999). Based on the existence in public human EST databases of $>$650 distinct protein kinases ($>$98 are PTKs = $\sim$16%) and extrapolation from *C. elegans*, the human genome is predicted to encode $>$1100 protein

kinases (~150 PTKs), assuming the human genome has about 80,000 genes. Indeed, it is almost inevitable that the ballyhooed millenary of protein kinases will be reached! Analysis of the *C. elegans* genome shows that the number of protein phosphatases encoded is surprisingly high, being more than half the number of protein kinases. Indeed, there are hints that there can be one to one relationships between protein kinases and phosphatases (e.g., the Clr-1 receptor PTP and Egl-15 FGF receptor PTK in *C. elegans*) (Kokel et al., 1998).

Comparative analysis of protein kinase and phosphatases from different species will tell us more about the evolution of different protein kinases. For instance, it is already clear from the lack of bona fide PTKs in the yeasts and their presence in the simplest of multicellular eukaryotes that protein-tyrosine phosphorylation evolved hand in hand with multicellularity, presumably in response to a need for intercellular communication. In keeping with this idea, a majority of PTKs play a role in transmembrane signaling in response to ligands that bind to surface receptors. We will also learn which protein kinases have been conserved throughout evolution, and which ones have a specialized function in a particular type of organism.

Such catalogs of protein kinases and phosphatases used in conjunction with detailed cellular expression patterns derived from expression array analysis will circumscribe which protein kinases and phosphatases are present in a cell and can therefore be involved in any particular phosphorylation event. This information, in combination with the available repertoire of protein kinase target proteins and algorithms for simulating signaling networks, may allow predictions of signaling outcomes in response to individual stimuli or combinations of stimuli.

*Signal Network Modeling: E-Phosphorylation*. Analogies have been drawn between cellular signaling networks and electronic circuits. Indeed cellular signaling pathways have many of the attributes of electronic circuits. Individual proteins can act as amplifiers or switches, and protein kinase cascades can act as serial amplifiers or switches. Signal pathways can have positive and negative feedback loops, and networks can be built up out of multiple signaling pathways.

Even though cell signaling networks are multidimensional rather than two dimensional, their properties have encouraged attempts to develop predictive algorithms. Efforts have been made to model MAPK cascades and the regulation of cell cycle transitions, and informative predictions have emerged. For instance, theoretical analysis of the ERK MAPK cascade has shown that it acts as a switch to provide a very sharp activation curve in response to increasing levels of stimulus, rather than acting an amplifier (Huang and Ferrell, 1996). Ultimately, however, useful prediction of signaling pathways and networks will require a knowledge of all the players, their kinetic properties, their interaction partners and mechanisms of positive and negative regulation, and their subcellular localizations and concentrations. With the availability of complete genome sequences, efforts are underway to define all of the proteins involved in signaling responses induced by specific receptors (e.g., G protein coupled receptors). These efforts will be aided by the use of expression array analysis to define which genes are expressed in a particular cell, and sophisticated bioinformatics analysis. The new discipline of proteomics, where protein modifications and interactions are analyzed in a high throughput format to provide protein linkage maps and other information, will be of fundamental importance in this endeavor. Databases that catalog protein–protein and protein–small molecule interactions will also be an important step in this direction. The availability of consensus sequences for inducible and constitutive protein–protein interactions and for phosphorylation by particular protein kinases will also be useful in predicting signaling connections.

One of the major challenges will be to model protein–protein interactions in vivo, where protein concentrations are much higher than those used in vitro. High protein concentrations favor low affinity interactions, which are hard to measure in vitro and yet are likely to be important in signal propagation. Indeed, many signaling assemblies may rely on a combination of multiple low-affinity interactions for their existence. Knowledge of the concentration of a given protein at a particular location in the cell will be critical information to obtain. Incorporation of kinetic and particularly spatial aspects of signaling into models will also be a serious challenge; and given that several thousand different proteins could be involved in signaling in a single cell, the computational difficulties will be immense. Nonetheless, successful in silico analysis of signaling pathways and networks must be a major goal for the future.

***Protein Phosphorylation Methodology***

Advances in methodology play a key role in progress in most fields of biology, and this has certainly been true in the field of signal transduction in general and protein phosphorylation in particular. New methods will surely also play a key role in future advances.

*Genetic Analysis*. Our understanding of signaling pathways has benefited enormously from the use of organisms where genetic analysis is feasible. The highly conserved nature of most of the major signaling pathways in eukaryotes allows use of genetic information obtained in one organism to predict the existence of pathways and specific components in other organisms. A good example is the elucidation of the MAPK pathways where genetic analysis in yeast, *C. elegans*, and *Drosophila* was combined with biochemical analysis of growth factor–stimulated protein kinases in mammalian cells to provide a complete picture of this pathway.

Genetic analysis of signaling systems will increasingly be used to investigate the nature and function of signaling pathways in vivo. Gene disruption through homologous recombination, selection of null mutants, or the powerful new interfering double-stranded RNA (RNAi) "knockout" technology can be used to determine whether a protein is essential for a given response. In vertebrates, increasing use will be made of tissue-specific conditional knockouts, to circumvent potential defects in embryogenesis. Future genetic analysis of signaling pathways will rely more and more on making subtle germline mutations in organisms or cells (e.g., inactivating point mutations) so that the gene product is expressed at physiological levels at the correct time and place, thus mitigating the potential problems with using overexpressed proteins and heterologous cell

types. In the phosphorylation arena, the function of individual phosphorylation sites will be studied by mutating them to a nonphosphorylatable residue to block phosphorylation or an acidic residue that can mimic phosphorylation. Such an analysis of the functions of individual tyrosine phosphorylation sites in the PDGF β receptor PTK has recently been carried out by making knockin mutations in the mouse germline (Heuchel et al., 1999). Finally, genetic screens for suppressors and enhancers of sensitized conditional mutations, synthetic lethal screens, and analysis of modifier genes will remain important tools in our armamentarium for analyzing signaling pathways.

*Biochemical Analysis*. Many new techniques have been developed in the protein phosphorylation arena. Traditionally, $^{32}$P labeling has been used to study protein phosphorylation both in vivo and in vitro, but techniques that emphasize nonradioactive detection of phosphorylation are becoming more and more prominent (e.g., measurements of protein kinase activity using fluorescence anisotropy with fluorescently tagged peptides that bind to phosphospecific antibodies upon phosphorylation). Anti-P.Tyr antibodies have been widely used to detect tyrosine phosphorylation, but attempts to develop anti-P.Ser and anti-P.Thr antibodies for similar uses have been less successful, because these antibodies tend to recognize other phosphate esters, and are generally of low affinity. Of much greater value have been antibodies directed against specific P.Ser- and P.Thr-containing peptide sequences, corresponding to known or suspected sites of phosphorylation. Phosphospecific antibodies against single or closely spaced sites will be increasingly useful tools for studying serine/threonine and tyrosine phosphorylation of individual proteins by immunoprecipitation, immunoblotting, and immunofluorescence staining. Analysis of histidine, lysine, and arginine phosphorylation would be greatly facilitated by the development of antibodies against these phosphoamino acids.

The mapping of phosphorylation sites is a critical endeavour, which traditionally has been done using $^{32}$P-labeling either in vivo or in vitro. However, within the past few years mass spectrometry has emerged as the best method for identifying phosphorylation sites, and, in the future, phosphorylation site mapping and measurements of phosphorylation stoichiometry will be accomplished routinely using mass spectrometry.

*Single Cell Analysis*. It is imperative that we devise better methods to study the kinetics and intracellular localization of signaling processes in single living cells, so that spatiotemporal aspects of signaling can be determined. New assays to determine in real time where in the cell a protein kinase is active using fluorescent reporter proteins will be important (e.g., using engineered GFP derivatives with a grafted protein kinase consensus sequence that exhibit fluorescence changes upon phosphorylation by a specific protein kinase). The location and kinetics of specific phosphorylation events can be monitored simultaneously in living cells using expressed or microinjected fluorescent reporter proteins (e.g., natural or artificial P.Ser or P.Tyr binding proteins and phosphospecific antibodies). Better yet, one can use fluorescence resonance energy transfer (FRET) to monitor the timing and location of an intramolecular interaction dependent upon protein kinase activation or phosphorylation. For instance, the localization of activated protein kinase Cα has been imaged by expressing a GFP-tagged PKCα and microinjecting Cy3-labeled anti-P.Thr250 antibodies, which recognize activated phosphorylated PKCα (Ng et al., 1999).

Specific cell-permeant inhibitors of protein kinases and phosphatases, and other signaling proteins are extremely powerful tools for analyzing signal transduction processes in intact cells. Many such inhibitors are already available and are extensively used. For instance, there are nearly 2000 papers reporting the use of the Parke-Davis MEK inhibitor PD98059, and over 1500 where wortmannin has been used as a PI-3′ kinase inhibitor. Phosphopeptides or other peptides that block protein–protein interactions are also increasingly used to interdict signaling pathways. Conversely, rapid and reversible activation of exogenously expressed protein kinases and phosphatases can be achieved by using chemical inducers of homo- or heterodimerization, or using 4-hydroxy tamoxifen to activate protein kinases and phosphatases fused to the estrogen receptor hormone binding domain.

The ongoing efforts of the pharmaceutical industry to develop drugs that act as highly specific inhibitors (and activators) of protein kinases and phosphatases has already had significant benefits in basic research by providing inhibitors as research tools. In many cases a potent inhibitor of a target protein is developed, but for a variety of reasons is not taken forward into clinic trials or fails in trials. Such inhibitors as well as early derivatives of successful drugs are perfectly suited for analytical research. In the coming years, we can expect to see increasing use of compounds from the pharmaceutical industry as tools for investigating signaling processes.

*Protein Kinase and Phosphatase Substrates*. Protein kinases recognize their substrates in part through the primary sequence surrounding the phosphorylatable residue. An emerging theme is that protein kinases also recognize substrates via secondary docking sites distinct from primary phosphorylation sites (Holland and Cooper, 1999). This concept is well developed for PTKs where SH2 and PTB domains are used to recruit substrates to specific P.Tyr residues in activated receptor PTK dimers, triggering their phosphorylation. Protein-serine kinases also recognize substrates through docking sites. For instance, JNK1/2 MAPKs interact specifically with a short LXL motif in the δ region of c-Jun via a loop in their C-terminal lobe, and ERK MAPKs recognize substrates via at least two distinct motifs (e.g., FXFP and LAQRRXXXXL/I) (Kallunki et al., 1994; Yang et al., 1998; Gavin and Nebreda, 1999; Jacobs et al., 1999; Smith et al., 1999). In addition, the G1 cyclin/Cdks select substrates through interaction of the cyclin subunit via a ZRXL motif (Adams et al., 1996), and the L45 loop in the N-terminal lobe of the TGFβ type I receptor catalytic domain interacts with a short sequence in the Smad2/3 MH2 domain (Chen et al., 1998). The emerging picture is that a variety of surface loops on the catalytic domain as well as sites elsewhere in a protein kinase can be used for selection of substrates. Protein kinase catalytic domains interact with many

types of protein including activators, inhibitors, targeting proteins, and substrates. An important challenge for the future will be to understand the structural bases for these multiple protein kinase catalytic domain interactions.

One major problem in the protein phosphorylation field is the difficulty in identifying true substrates for individual protein kinases and phosphatases in vivo. Specific protein kinase inhibitors can be useful, but it is hard to prove that they are truly selective in vivo. A new substrate detection method has recently been developed in which the ATP-binding site of a protein kinase is "enlarged" by mutation to accept an $N^6$-modified ATP analog, which can be used as a phosphate donor for the mutant protein kinase when expressed in vivo but which cannot be used by other normal cellular protein kinase (Liu et al., 1998). This method has been validated with members of the Src PTK family, and efforts are currently being made to extend this to other PTKs and to protein-serine kinases.

The analysis of degenerate oriented peptide libraries for sequences that can be phosphorylated by purified protein kinases continues to be a valuable method for deducing primary sequence consensuses for newly identified protein kinases (Songyang et al., 1994). Such consensus sequences, combined with compilations of phosphorylation sites in known targets, can be used to screen sequence databases for potential substrates. Physiological targets have already been identified by this strategy in a number of instances. Since many protein kinases have secondary docking sites on their substrates, database searches for potential substrates that contain both consensus phosphorylation site and docking site sequences should have greatly increased chances of finding physiological targets.

The identification of physiological substrates for protein phosphatases is equally difficult. Cell permeant phosphatase inhibitors (e.g., okadaic acid) can be used as a method of identifying substrate proteins through increased in vivo phosphorylation. For PTPs catalytically impaired "substrate-trapping" mutant PTPs that bind stably to their phosphoprotein substrates have successfully been used to isolate substrates (Flint et al., 1997). For identification of a PTP for a known phosphoprotein, in gel phosphatase assays are proving useful. Like protein kinases, protein phosphatases appear to have secondary docking sites on their substrates, recognized either by associated targeting subunits or protein interaction domains on the catalytic subunit itself, and these interactions can be used to identify potential substrates.

## Futurescope

Phosphorylation touches on most aspects of cell physiology. Which areas are most likely to be important in the near future? Emergent areas are transcriptional control, apoptosis, phosphorylation-dependent protein degradation, phosphorylation-dependent nuclear import and export, cytoskeletal regulation, and checkpoint signaling. However, it is in the function of the vertebrate central nervous system where studies of phosphorylation seem likely to have the greatest impact. The majority of protein kinases are expressed in the brain, and many of the novel vertebrate protein kinases revealed by genomic sequencing will probably be expressed and function in specific neurons. From a technical standpoint a major requirement is the development of assays that allow temporal analysis of signaling events at high spatial resolution in single cells. Also better methods for rapid detection of phosphorylation, phosphorylation site mapping, and protein kinase and phosphate substrate identification are essential. The evolution of theoretical methods for analyzing signaling networks and sophisticated protein interaction databases to support modeling efforts is another obvious need. From a practical standpoint, one can foresee that the accumulating knowledge about signaling networks and the proteins involved will permit development of potent and specific pharmacological modulators of signaling that can be used therapeutically. Finally, we should not be so arrogant as to think that we already know all the possible principles of signaling, and we should certainly expect totally new types of signaling systems to be uncovered.

### Acknowledgments

The important findings in the history of signal transduction are adequately covered in many reviews, and I have therefore cited reviews that discuss the seminal papers. Space constraints have unfortunately meant I have had to omit many pertinent citations in the forward-looking part of this review. I thank Tony Pawson for providing the basis for Figure 1.

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