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Review

Specificity in Signal Transduction: From Phosphotyrosine-SH2 Domain Interactions to Complex Cellular Systems

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Over the last two decades, a new and unifying concept of cellular organization has emerged in which modular protein-protein interactions provide an underlying framework through which signaling pathways are assembled and controlled. In this scheme, posttranslational modifications such as phosphorylation commonly exert their biological effects by regulating molecular interactions, exemplified by the ability of phosphotyrosine sites to bind selectively to SH2 domains. Although these interactions are rather simple in isolation, they can nonetheless be exploited to generate complex cellular systems. Here, I discuss experiments that have led to this view of dynamic cellular behavior and identify some current and future areas of interest in cell signaling.

The Past

Tyrosine Phosphorylation

Thirty years ago, when I started my PhD, we lacked most of the experimental and computational tools that are now taken for granted. Fortunately, we had no idea at the time of our sublime state of ignorance, or the very notion of trying to understand the underlying principles of cellular organization and specificity in signal transduction would have seemed like so much tilting at windmills. We did, however, have RNA and DNA viruses capable of inducing malignant transformation, whose genomes could be readily characterized. Their profound and rapid effects on the host cell suggested that they could teach us not only about malignancy, but also about the properties of normal cells. For example, Rous sarcoma virus (RSV) promotes changes in cell shape, adhesion, motility, growth, proliferation, gene expression, metabolism, and survival, raising the possibility that these distinct facets of cellular behavior might all be interconnected. Learning how a virus such as RSV interacts with the cellular machinery might therefore reveal unifying themes in cell biology. Indeed, work in the 1970s showed that the transforming activity of RSV could be mapped to a single viral gene (*v-Src*) (Martin, 1970; Lai et al., 1973), which was shown by Michael Bishop and Harold Varmus to represent a pirated copy of a normal cellular gene, *c-Src* (Stehelin et al., 1976). This reduced the problem to a deceptively simple question—what are the protein products of *v-Src* and similar viral oncogenes, and what do they do? Joan Brugge and Ray Erikson were the first to identify the elusive 60 kDa v-Src protein by inoculating rabbits with a strain of RSV that causes small tumors that subsequently regress, leaving anti-Src antibodies in their wake (Brugge and Erickson, 1977). The v-Src protein was subsequently shown by the Erikson and Bishop labs to have protein kinase activity, a finding that was intuitively appealing because protein phosphorylation seemed a logical way in which a single polypeptide could exert such a pervasive influence on the cell (Levinson et al., 1978; Collett and Erikson, 1978). Initial phosphoamino acid analysis suggested that the relevant modification might be phosphothreonine.

So things stood until Tony Hunter, working with Walter Eckhart in 1979, explored the biochemical properties of an entirely different viral oncogene product, the enigmatic polyomavirus middle T antigen (PyMT). Primed with the idea that viral oncoproteins might be protein kinases, Hunter tested immunoprecipitates of PyMT for kinase activity and indeed found that PyMT itself became phosphorylated in such an assay. More importantly, he identified the phosphorylated amino acid as phosphotyrosine (pTyr), a modification that had not been previously found in proteins (Eckhart et al., 1979). Pursuing these observations, Hunter and Bart Sefton found that v-Src itself has associated tyrosine kinase activity, which induces an elevation in cellular tyrosine phosphorylation and correlates with transformation (Hunter and Sefton, 1980; Sefton et al., 1980). Later work from Sara Courtneidge and Alan Smith showed that PyMT is not itself a protein-tyrosine kinase but rather is a virally encoded scaffold that binds and activates endogenous Src family kinases (Courtneidge and Smith, 1983) (Figure 1). Consistent with the findings regarding PyMT, Owen Witte and David Baltimore reported in 1980 that the v-Abl oncoprotein encoded by Abelson murine leukemia virus becomes autophosphorylated on tyrosine in vitro (Witte et al., 1980). A preliminary answer to the issue of v-Src function was therefore that it transforms cells through tyrosine phosphorylation.

In short order, a veritable avalanche of experiments suggested that the receptors for several growth factors and insulin are tyrosine kinases, and that the aberrant activation of such receptors, or ectopic expression of their extracellular ligands, could induce cellular transformation (Ushiro and Cohen, 1980; Ek et al., 1982; Petruzzeli et al., 1982; Downward et al., 1984; Hunter and Cooper, 1981; Waterfield et al., 1983; Doolittle et al., 1983). Therefore, receptor tyrosine kinase (RTK) signaling is important for the normal cellular response to mitogenic and metabolic hormones, and the pathological activation of such signaling pathways can provoke a cancerous phenotype. This raised the next critical issues—how do tyrosine kinases exert their effects on cellular behavior, and how is specificity maintained in signaling from different receptors?

Protein kinases were thought to achieve specificity through the ability of the active site of the kinase to recognize short motifs for phosphorylation (Kemp et al., 1975) and to employ phosphorylation to induce a conformational change in regulatory enzymes. This paradigm seemed sufficient to explain the regulation of tyrosine kinases themselves. Ora Rosen found that

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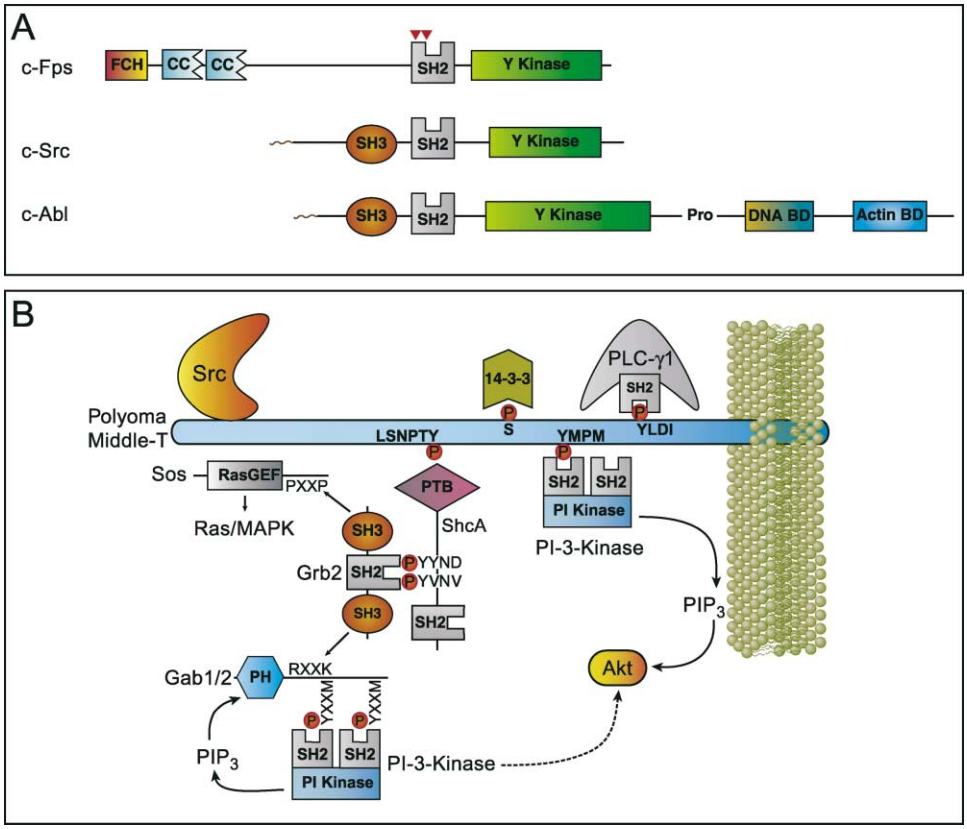

Figure 1. Molecular Organization of Signaling Proteins and Complexes

(A) The domain organization of the Fps, Src, and Abl cytoplasmic tyrosine kinases. FCH: Fps/Cip4 homology domain; CC: coiled coil; Pro: proline-rich SH3 binding motifs; BD: binding domain. The sites of dipeptide insertions that led to the identification of the v-Fps SH2 domain are shown by arrowheads.

(B) A signaling complex assembles around PyMT. This illustrates several concepts, including the ability of a scaffold to recruit a tyrosine kinase and downstream targets, the ability of SH2 domains to distinguish different pTyr motifs, the capacity of serine phosphorylation to create protein binding sites, and the formation of a signaling network through the reiterated use of modular interactions. A positive feedback loop is evident in the ability of the Gab1 scaffold to both activate PI 3′ kinase and respond to its product, PIP_3, which binds the Gab1 PH domain (Rodrigues et al., 2000). PTB = phosphotyrosine binding domain.

phosphorylation of purified insulin receptor stimulates its in vitro kinase activity (Rosen et al., 1983); we pinpointed an autophosphorylation site within the kinase domain of the cytoplasmic v-Fps tyrosine kinase, a relative of Src and Abl, and showed that its substitution with phenylalanine suppressed both v-Fps kinase and transforming activities (Weinmaster et al., 1984). Coupled with a body of work from Joseph Schlessinger showing that growth factors can activate receptors through dimerization (e.g., Schreiber et al., 1983), such data indicated that transphosphorylation within the catalytic domain stimulates tyrosine kinase activity. However, the search for downstream substrates of tyrosine kinases produced the surprising result that the most abundant pTyr-containing protein in a growth factor-stimulated cell is usually the receptor itself. To us, this finding suggested that tyrosine phosphorylation might have unsuspected biochemical functions, and that tyrosine kinases might have ways of recruiting their targets in addition to the transient binding of a substrate to the active site of the enzyme.

SH2 Domains and Protein-Protein Interactions

In pursuit of this idea, we introduced short dipeptide insertions throughout the length of the coding sequence for the oncogenic v-Fps/Fes cytoplasmic tyrosine kinase, with the notion that we might locally disrupt the folding of globular domains and thus identify any noncatalytic elements that regulate the kinase domain and its interactions with cellular proteins (Stone et al., 1984). Mutations in the kinase domain led to an expected loss of catalytic and transforming activities. However, through this approach we identified, in 1986, a region N-terminal to the kinase domain that was not required for catalytic activity per se but that modified both kinase activity and substrate recognition and was necessary for cellular transformation (Sadowski et al., 1986). We termed this element the Src homology 2 (SH2) domain because inspection of the sequences of the Src and Abl kinases revealed that the stretch of approximately 100 amino acids identified as functionally important by the mutagenesis of v-Fps is conserved in Src and Abl and similarly positioned adjacent to the kinase (SH1) domain (Figure 1). Partial proteolysis indicated that the SH2 domain has a folded structure (Sadowski et al., 1986; Koch et al., 1989). These results suggested that specificity in signaling by tyrosine kinases requires protein-protein interactions that are mediated by a dedicated noncatalytic domain (Sadowski et al., 1986; DeClue et al., 1987; Koch et al., 1989). I proposed that the regulation of cell

signaling by modular protein interactions might be a more general principle (Pawson, 1988), and indeed the SH2 domain now serves as the prototype for a diverse collection of interaction domains that recognize not only proteins but also phospholipids, nucleic acids, and small molecules (Pawson and Nash, 2003). This view of tyrosine kinase action has led to a new way of thinking about cellular regulation, in which molecular interactions, mediated by protein interaction domains, provide a fundamental means of organizing signaling pathways and networks.

Important support for this model came from the cloning by Hidesaburo Hanafusa and Bruce Mayer of the viral *Crk* oncogene, which encodes a small protein composed exclusively of an SH2 domain, and a second noncatalytic element also found in Src and Abl that Hanafusa termed SH3 (Mayer et al., 1988). v-Crk associated with a 130 kDa tyrosine phosphorylated protein ($p130^{cas}$) in transformed cells, raising the possibility that it activates latent tyrosine kinase signals. At the same time, cloning of cDNAs for the cytoplasmic signaling enzymes phospholipase C (PLC) γ1 and Ras GTPase activating protein (GAP) revealed the presence of SH2 and SH3 domains, indicating that these domains might be common elements of seemingly disparate proteins (Stahl et al., 1988; Trahey et al., 1988; Vogel et al., 1988). Indeed, PLCγ and RasGAP were found to be substrates for tyrosine kinases (Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989; Molloy et al., 1989; Ellis et al., 1990), and we also found that RasGAP associates inducibly with other pTyr-containing proteins (Ellis et al., 1990). Such data were consistent with a scheme in which SH2-containing proteins couple tyrosine kinase signals to intracellular effectors such as the Ras GTPase and phospholipid metabolism.

In a parallel set of experiments, interest was gathering around the idea that growth factor receptors might physically interact with their cytoplasmic targets and that autophosphorylation of the receptor was important for these interactions (Margolis et al., 1989; Kumjian et al., 1989). In 1989, Jonathan Cooper and Andrius Kazlauskas found that autophosphorylation of the platelet-derived growth factor β receptor on Tyr751 was important for the selective recruitment of phosphatidylinositol (PI) 3′ kinase (Kazlauskas and Cooper, 1989). Joining these two lines of research, on RTK signaling and SH2-containing proteins, we found in 1990 that the isolated SH2 domains of proteins such as PLC-γ1, RasGAP, and Src have the common property of binding specifically to autophosphorylated RTKs and tyrosine-phosphorylated cytoplasmic proteins following growth factor stimulation (Anderson et al., 1990; Moran et al., 1990). These data showed that SH2 domains are independently folding modules that provide otherwise distinct proteins with the ability to specifically bind pTyr-containing RTKs and intracellular docking proteins following growth factor stimulation. We found that different isolated SH2 domains had distinct binding preferences for pTyr-containing proteins, leading us to conclude that the specificity of SH2-ligand interactions determines which signaling proteins associate within a growth factor-stimulated cell (Moran et al., 1990). Meanwhile, the Hanafusa lab showed that recombinant v-Crk mediates the pTyr-dependent recognition of varied proteins from transformed cells, consistent with the theme that such interactions are important in the deregulated signaling induced by oncogenic tyrosine kinases (Matsuda et al., 1990; Mayer and Hanafusa, 1990). An answer to the question of how tyrosine kinases signal was therefore that tyrosine phosphorylation creates binding sites for proteins with SH2 domains. This led to the next problem—what is the molecular basis for an SH2-pTyr interaction?

SH2 domains appeared to bind directly to pTyr sites (Margolis et al., 1990; Mayer et al., 1991; Escobedo et al., 1991; Koch et al., 1992). In a seminal study in 1992, John Kuriyan and Gabriel Waksman solved a structure of the Src SH2 domain that revealed in molecular detail how it recognizes the pTyr moiety of a phosphopeptide (Waksman et al., 1992). This analysis uncovered several features that are now recognized as common themes of many interaction domains. The SH2 domain itself has a cassette-like design, with the N and C termini closely juxtaposed in space, so that it can be inserted into a loop on a pre-existing polypeptide while leaving the ligand binding surface exposed. The pTyr of a phosphopeptide ligand fits into a conserved pocket and is captured by an invariant arginine at the base of the pocket. These findings begged the question of how an SH2 domain can preferentially bind a specific pTyr-containing sequence, exemplified by the ability of individual SH2 domains to select defined RTK autophosphorylation sites (Escobedo et al., 1991; Reedijk et al., 1992; Ronnstrand et al., 1992). The basis for this discrimination was suggested by Steven Shoelson in 1992, who used synthetic phosphopeptides to show that the SH2 domains of the p85 subunit of PI 3′ kinase bind preferentially to phosphorylatyed YMXM motifs, indicating that the sequence context of the tyrosine phosphorylation site is important for SH2 domain recognition (Domchek et al., 1992).

The broader repertoire of SH2 binding specificity, however, remained mysterious until an elegant experiment by Zhou Songyang and Lewis Cantley in 1993 (Songyang et al., 1993). Using an early example of a proteomics approach, Cantley probed a degenerate peptide library, containing a fixed pTyr, with a large number of SH2 domains. The results indicated that SH2 domains recognize residues C-terminal to the pTyr in a fashion that varies from one domain to another, and this provided a general classification for SH2 domains based on their phosphopeptide binding preferences. Significantly, known SH2 binding sites showed a remarkable consonance with motifs identified in the library screen, typified by the finding that the SH2 domain of the Grb2 adaptor binds phosphorylated YXN sequences (Songyang et al., 1994) (Figure 1). Not only did Cantley's technique provide a set of preferred residues at each position of an SH2 binding site, it also identified residues that are *disfavored*. This is important, as selectivity in modular protein interactions stems from a combination of permissive and nonpermissive forces, both of which contribute to biological specificity within the cell (e.g., Larose et al., 1993; Kay et al., 1998; Zarrinpar et al., 2003b). The identification of preferred SH2 binding motifs allowed the primary sequences of proteins such as RTKs to be scanned for potential SH2 domain interaction sites (Yaffe et al., 2001), and this bioinformatic approach has been widely useful in predicting modular protein-protein interactions.

Pursuing these investigations into SH2 binding prefer-

ences, Waksman and Kuriyan solved the structure of the Src SH2 domain bound to an optimal phosphopeptide with a YEEI motif (Waksman et al., 1993). At the same time, Michael Eck and Steven Harrison reported the structure of the SH2 domain of the Src family kinase Lck, also bound to a phosphorylated YEEI peptide (Eck et al., 1993). These data revealed that SH2 domains have a second binding surface, more variable than the pTyr binding pocket, that engages the C-terminal specificity residues. This concept has also proved to be wide ranging, in the sense that families of interaction domains often bind a core element in their peptide ligands (i.e., pTyr in the case of SH2 and PTB domains, PXXP or RXXK for SH3 domains, phosphothreonine for FHA domains) and achieve specificity through the variable recognition of flanking amino acids (Pawson and Nash, 2000). Indeed, somewhat surprisingly, we found that it was possible to change the binding properties of an SH2 domain by substitution of a predicted specificity residue and thus to alter its biological activity in an intact organism (Marengere et al., 1994).

The generality of these ideas was supported by an exploration of SH3 domains. The yeast *S. cerevisiae* has 28 SH3 domains that function in pathways controlling events such as osmosensing, cytoskeletal organization, and polarity, but lacks conventional tyrosine kinases and SH2 domains (Tong et al., 2002). SH2 domains make an evolutionary appearance in protozoa and Dictyostelium discoideum, coincident with the emergence of conventional tyrosine kinases (Kawata et al., 1997; King et al., 2003). This suggested that SH3 domains evolved to regulate basic aspects of cellular organization and were later co-opted by SH2-containing proteins to link newly evolved pTyr signals to SH3-regulated pathways. In 1993, David Baltimore's lab found that SH3 domains bind preferentially to proline-rich motifs (Ren et al., 1993), and this has led to an extensive pursuit of the varied structures, binding properties, and functions of SH3-containing proteins and to the identification of a larger family of domains that recognize proline-rich sequences (i.e., WW, EVH1, GYF domains) (Zarrinpar et al., 2003a).

In sum, receptors with intrinsic tyrosine kinase activity, or with associated cytoplasmic tyrosine kinase subunits, mediate the effects of hormones, cytokines, adhesion molecules, guidance cues, and antigens and are aberrantly activated in a range of human cancers (Hunter, 2000; Schlessinger, 2000). They signal through SH2 proteins; the synergy between tyrosine kinase and SH2 domains extends to the therapeutic arena since the kinase inhibitor Gleevec selectively recognizes a conformation of the autoinhibited Abl catalytic domain that is imposed by the adjacent SH2 domain (Nagar et al., 2003). The ability of RTKs to recognize specific targets through regulated protein-protein interactions has proven to be a very general feature of specificity in signaling from cell surface receptors (Figure 2) and intracellular cues (Pawson and Scott, 1997). Consistent with our original identification of the SH2 domain in the context of pTyr signaling, interaction modules can recognize numerous protein modifications (including phosphorylation, methylation, acetylation, hydroxylation, and ubiquitination), and in this sense, they control the dynamic state of the cell (Yaffe and Elia, 2001; Jaakkola et al., 2001; Ivan et al., 2001; Marmorstein, 2001; Hicke and Dunn, 2003). In a more general context, they provide a framework for the protein machines and interaction networks that organize cellular behavior.

The Present

How do rather simple events, such as posttranslational modifications and protein-protein interactions, yield specific biological responses in the crowded environment of the cell? Some general principles have started to emerge, building on the themes enunciated above.

Trafficking and Location

Biological specificity requires that receptors and their cytoplasmic targets be delivered to the appropriate site in the cell so that they are activated at the right time and in the right place. Cell surface receptors, for example, can be directed to specific compartments of the plasma membrane through selective binding of their C-terminal motifs to the PDZ domains of polarity proteins. In *C. elegans*, the EGF receptor LET-23 is sorted to the basolateral surface of epithelial cells through an association with the PDZ domain of the adaptor protein LIN-7 (Kaech et al., 1998). Lin-7 also regulates the basolateral location of human ErbB RTKs in epithelial cells, first by delivering the receptor to the basolateral surface through an interaction with the kinase domain and in addition by stabilizing the receptor at this site through PDZ binding to the C terminus (Shelly et al., 2003). Human airway epithelial cells express both the ErbB2/4 RTKs and their ligand Heregulin, but localize Heregulin to the apical surface so that it is segregated from its receptors by cellular tight junctions until polarity is disturbed, for example, by damage to the epithelial monolayer (Vermeer et al., 2003). Thus, location of ligand and receptor control when and where signaling takes place. Loss of cell polarity, or overexpression of a receptor such as ErbB2 in breast cancer, can abrogate the fine control imposed by spatial restrictions, activating signaling pathways at the wrong time and in the wrong place.

Because the duration of a signal can also be critical for the nature of the cellular response, mechanisms must exist to attenuate receptor signaling. Once activated, RTKs are internalized in clathrin-coated vesicles and can traffic through endosomes to the multivesicular body and thus to the lysosome for degradation (Figure 3). This process involves a series of regulated protein-protein and protein-phospholipid interactions that in aggregate form a rather sophisticated machinery. Initially, a pTyr motif on the activated receptor binds to the SH2 domain of the E3 protein-ubiquitin ligase c-Cbl, leading to monoubiquitination of the receptor at multiple sites (Joazeiro et al., 1999; Haglund et al., 2003; Mosesson et al., 2003). The monoubiquitinated receptor can then be recognized by endocytic proteins with ubiquitin interaction motifs (UIM), such as epsin, and be recruited into budding vesicles (Polo et al., 2002; Shih et al., 2002). Epsin also has an ENTH domain that binds $PI(4,5)P_2$, a phospholipid enriched in the plasma membrane, in a fashion that induces a new α helix in the ENTH domain. This helix has a hydrophobic outer surface that is inserted into the outer leaflet of the membrane bilayer, thereby triggering deformation of the membrane re-

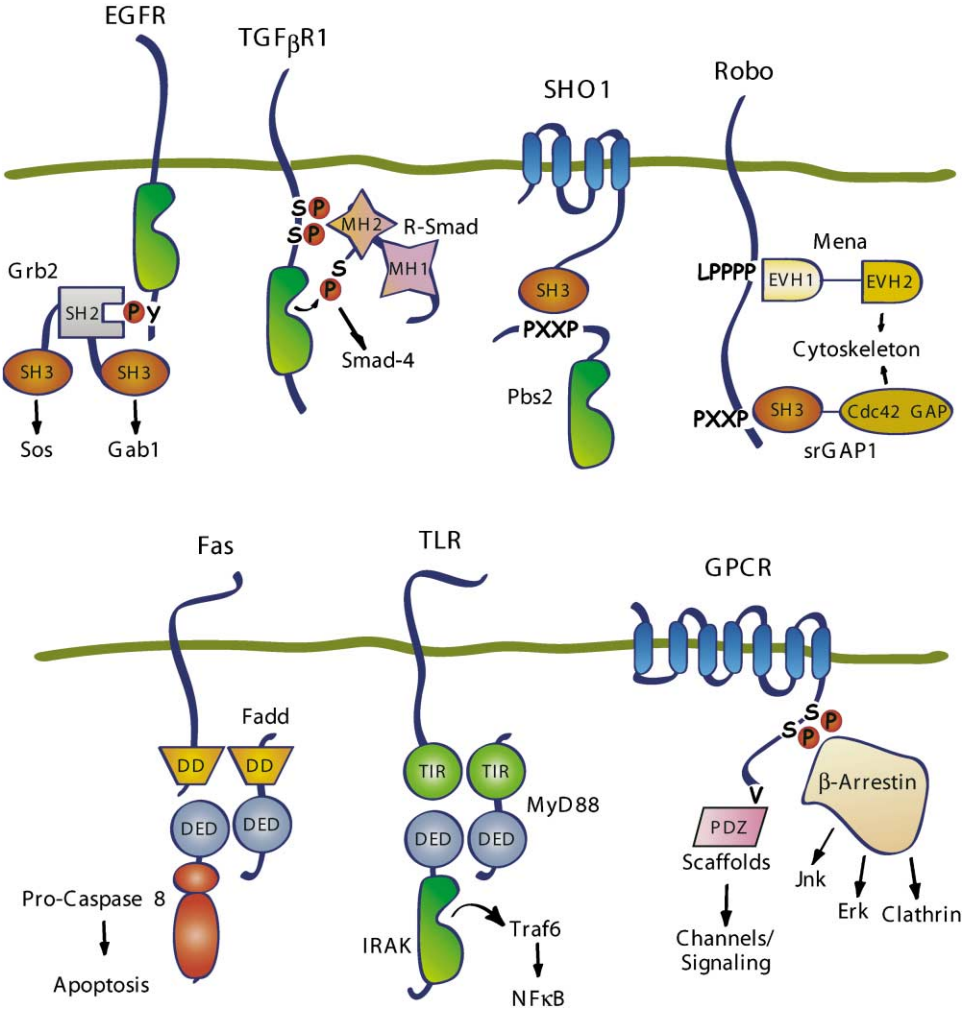

Figure 2. Cell Surface Receptors Signal through Modular Protein Interactions

Many receptors bind intracellular targets and regulators through interaction domains, typified by the binding of RTK autophosphorylation sites to SH2 domains, such as those on adaptor proteins. The TGFßR1 serine/threonine kinase recruits the MH2 domain of R-Smad proteins through juxtamembrane autophosphorylation sites. Proline-rich motifs and their cognate interaction domains (i.e., SH3, EVH1) are employed in diverse settings, such as binding of the yeast osmosensor Sho1 to the MAP kinase kinase/scaffold Pbs2 and recruitment of cytoskeletal regulators such as Mena and srGAP1 to the axon guidance receptor Robo. Domains that form homo- or hetero-oligomers are commonly used to recruit and cluster the targets of death and inflammatory receptors, such as Fas and Toll-like receptors (TLR). These receptors couple through multidomain adaptors to targets such as initiator caspases or IRAK kinases/scaffolds (which in turn bind the Traf6 adaptor). G protein-coupled receptors (GPCR), in addition to conventional signaling through heterotrimeric G proteins, can recruit multifunctional scaffolding proteins that control receptor internalization, association with targets, and interactions with other receptors. Examples include β-arrestin, which binds many GPCRs and in turn interacts with the endocytic machinery and cytoplasmic signaling pathways. GPCRs can also interact through their C termini with the PDZ domains of adaptor proteins, which in turn scaffold multiple signaling proteins or position the receptor adjacent to ion channels. Details have been omitted for the sake of clarity. DD = death domain; DED = death effector domain; green bilobe = kinase domain, MH = MAD homology, EVII = Ena/Vasp homology, TIR = Toll/IL-1 receptor domain.

quired for invagination and vesicle formation (Stahelin et al., 2003; Ford et al., 2002).

UIM-containing proteins can induce their own monoubiquitination, potentially generating a network of low-affinity UIM-mediated interactions that support endocytic trafficking (Polo et al., 2002). Indeed, the receptor for monoubiquitinated cargo on endosomes (Hrs/Vps27) has a UIM domain (Shih et al., 2002; Raiborg et al., 2003; Katzmann et al., 2002) and also a FYVE domain that selectively binds PI(3)P, a phospholipid enriched in the endosomal compartment (Burd and Emr, 1998; Raiborg et al., 2001). Thus spatial segregation of UIM trafficking proteins is maintained through their modular interactions with distinct membrane phosphoinositides. Monoubiquitinated cargo is transferred to Hrs/Vps27 and then to three successive multiprotein complexes (ESCRT-I, -II, and –III) before being internalized into the multivesicular body of late endososmes (Katzmann et al., 2001, Katzmann et al., 2003; Bache et al., 2003). Not only are Hrs-mediated interactions important for the physiological downregulation of cell surface receptors (Jekely and Rorth, 2003), but they can also be subverted to ease the passage of viruses out of infected cells. Intriguingly, enveloped viruses such as HIV and Ebola have hijacked

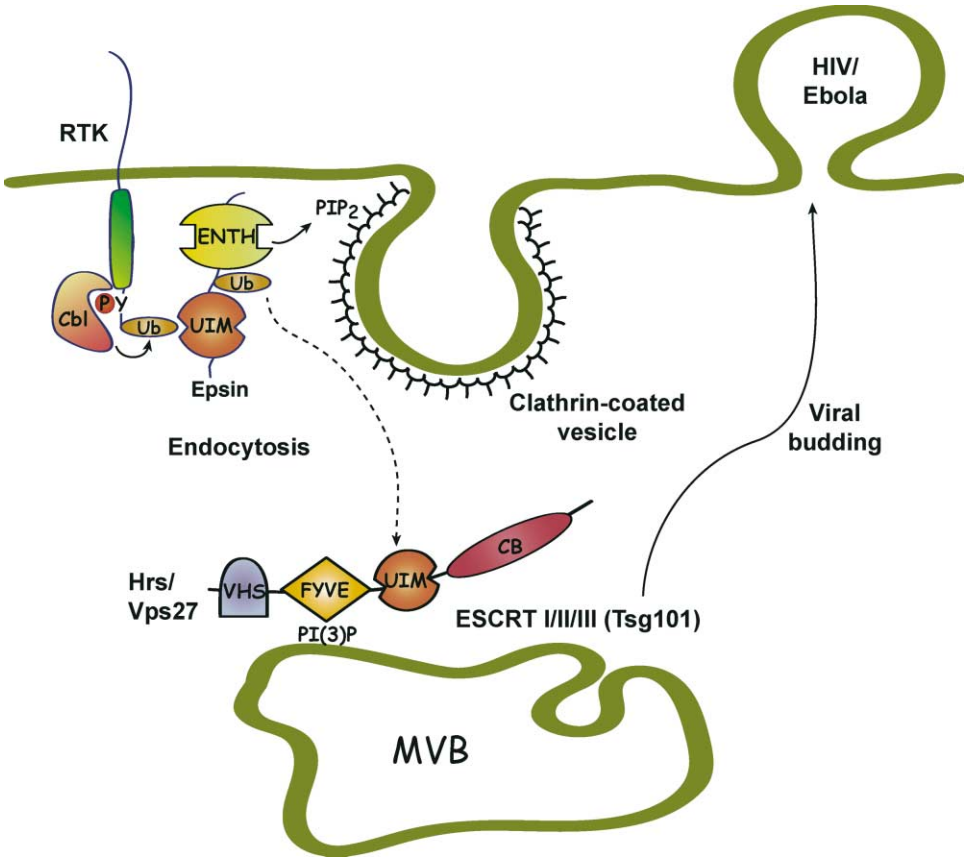

Figure 3. Simplified Illustration of Protein-Protein and Protein-Phospholipid Interactions during Internalization of a Receptor

CB: clathrin binding region; Ub: ubiquitin; Tsg101: Ubiquitin and Hrs binding component of the ESCRT-I complex; MVB: endosome multivesicular body.

this endosomal network to promote budding from the plasma membrane, a process that can be viewed as similar to internalization into late endosomes (Garrus et al., 2001; Martin-Serrano et al., 2001). The HIV Gag protein interacts directly with the trafficking machinery, in part by mimicking an interaction motif in Hrs/Vps27 (Pornillos et al., 2003; von Schwedler et al., 2003; Strack et al., 2003). The ability of pathogens to reorganize cellular behavior by recruiting a network of regulatory proteins to an inappropriate location speaks to the plasticity of cellular signaling systems.

The trafficking of receptors has stimulated debate as to whether a component of receptor-mediated signaling occurs on internal membranes (Gonzalez-Gaitan and Stenmark, 2003), as appears to be the case for TGFβ receptor serine/threonine kinases (Di Guglielmo et al., 2003). These receptors recruit and phosphorylate R-Smads (e.g., Smad2) and induce an R-Smad/Smad4 complex that is retained in the nucleus to regulate gene expression. This process is facilitated by a scaffolding protein, SARA, that binds to R-Smads and the activated receptor, as well as to PI(3)P through a FYVE domain. The TGFβ receptor is recruited into clathrin-coated vesicles and sorted to EEA1-positive endosomes. The retention of TGFβ receptors in endosomes requires binding of the FYVE domain of SARA to PI(3)P and stimulates signaling through the Smad2/4 pathway. An alternative pathway involves the localization of TGFβ receptors to caveolin-positive vesicles in lipid rafts at the plasma membrane and recruitment of inhibitory proteins that induce receptor ubiquitination and degradation. In this cell signaling system, the specialized scaffold SARA directs receptors to an internal site for signaling and away from the pathway for downregulation. In a related vein, distinct scaffolding proteins may target components of the Erk MAP kinase pathway to either the plasma membrane or late endosomes, resulting in differential Erk activation at these sites. For example, the endosomal p14 adaptor appears to recruit the MP1 scaffold with its associated MEK1 and Erk1 kinases, thereby eliciting Erk1 activation at late endosomes (Teis et al., 2002), whereas the KSR scaffold stimulates the Erk MAP kinase pathway at the plasma membrane (Muller et al., 2001).

Similar issues surround the sites of activation of Ras GTPases. These proteins act as molecular switches, through the exchange of GDP for GTP and, once in the GTP bound state, can potentially interact with one of a number of targets. A prominent pathway from RTKs to Ras involves recruitment of the Grb2 SH2/SH3 adaptor and the associated guanine nucleotide exchange factor (GEF) Sos, which elicits activation of Ras at the plasma membrane. Another pathway to Ras activation requires the SH2-containing phospholipase C-γ, which cleaves $PI(4,5)P_2$ to yield diacylglycerol and IP_3, with the latter

inducing release of calcium from intracellular stores. This stimulates a calcium/diacylglycerol-sensitive Ras GEF (RasGRP1) that localizes through a C1 domain to golgi and potentially induces selective Ras activation at the golgi membrane (Bivona et al., 2003). Simultaneously, the calcium-sensitive RasGAP CAPRI inhibits Ras activation at the plasma membrane and thus accentuates the stimulatory effect of RasGRP1 at the golgi. These two pathways may therefore activate separate pools of Ras at the plasma membrane or golgi, which could interact with different effectors or stimulate a common pathway at distinct locations or with different kinetics. While there is still much debate about these ideas, the spatial segregation of signaling pathways is likely to be an important topic for the future.

Docking Sites and Scaffolds

The early work on RTKs and SH2 domains suggested that tyrosine kinases select their targets through noncatalytic docking interactions. SH2 proteins can then be activated by juxtaposition to substrates at the membrane, by a conformational change induced by SH2 binding, or by phosphorylation once tethered to a tyrosine kinase. Similar mechanisms are employed by protein-serine/threonine kinases, which can bind docking sites on their targets and then select compatible motifs for phosphorylation within the anchored substrate. Alternatively, such kinases can employ dedicated substrate binding subunits, or the kinase and its substrate can associate with a common scaffold. MAP kinases, for example, bind docking motifs on their substrates, regulators, and scaffolds in a way that confers pathway specificity (Holland and Cooper, 1999; Sharrocks et al., 2000). Structural analysis of the p38 MAP kinase reveals a docking groove in the C-terminal lobe of the kinase for the motif R/K-X_4-ϕ-X-ϕ, found in substrates such as the MEF2A transcription factor and regulators such as the kinase MKK3b (Chang et al., 2002). Of interest, this interaction induces a conformational change in the active site and activation segment of the kinase, suggesting that docking may be coupled to regulation of kinase activity.

The active conformation of cyclin-dependent kinases (CDK) is dependent on the association of a regulatory cyclin subunit with the kinase. However, cyclins also provide selective docking sites for substrates and regulators, as in the case of cyclin A, which binds through a hydrophobic patch to RXL motifs in targets (e.g., p107, E2F-1) and inhibitors (e.g., p21, p27) (Schulman et al., 1998). Extending this concept, the protein kinase mTOR, which integrates external and intrinsic signals that control cell growth, is targeted to substrates through scaffolds, such as the WD40 repeat protein Raptor (Kim et al., 2002; Hara et al., 2002). Raptor interacts with the mTOR kinase and a conserved TOR signaling (TOS) motif on substrates such as 4E-BP1 and S6 kinase that regulate protein synthesis (Schalm et al., 2003; Schalm and Blenis, 2002; Nojima et al., 2003). These interactions are complex since Raptor has been reported to suppress mTOR kinase activity when nutrients are limiting (Kim et al., 2002), while an additional motif (RAIP) is important for 4E-BP1 phosphorylation (Choi et al., 2003; Beugnet et al., 2003).

These data indicate that both the activity and the substrate specificity of serine/threonine kinases are regulated through protein-protein interactions analogous to those identified in tyrosine kinase signaling. This principle is exemplified by Polo serine/threonine protein kinases, such as Plk1, that play multiple roles during mitosis. Plk1 has an N-terminal kinase domain that preferentially phosphorylates E/D-X-S/T-ϕ motifs and a C-terminal Polo box domain (PBD) that binds phosphorylated S-pS/pT-P sequences (Elia et al., 2003a, 2003b; Cheng et al., 2003). Phosphopeptide binding to the PBD simultaneously activates the kinase and targets it to cellular substrates (i.e., Cdc25C) that have been previously phosphorylated by a proline-directed kinase such as Cdc2/cyclin B. This provides an elegant means to couple the activities of distinct kinases in a complex process such as cell division (Figure 4). In outline, this mechanism is remarkably similar to cytoplasmic tyrosine kinases such as Src and Abl, in which the SH2 and SH3 domains maintain the kinase in an autoinhibited state but target the activated kinase to substrates.

The requirement for scaffolds to translate rather general signals into specific biological responses becomes particularly acute for enzymes regulated by a diffusible second messenger, such as cAMP. A paradigm is provided by A-kinase anchoring proteins (AKAPs). These are large polypeptides with the common ability to bind the regulatory RII or R1 subunits of cAMP-dependent protein kinase (PKA) and thus to hold PKA in an inactive state, from which the active catalytic subunit can be released by cAMP (Michel and Scott, 2002). AKAPs have two other important properties. First, they have targeting motifs that bind to subcellular determinants or receptors. Furthermore, they possess binding sites for other signaling enzymes, including protein kinase C (PKC) and the phosphatase PP2B (Bauman and Scott, 2002). An AKAP therefore provides a signaling hub that can be localized to a specific site in a cell and through the combined activity of its associated partners can convert a generic cAMP signal into a specific localized response, which is integrated with other regulatory inputs.

A further sophistication is that the same AKAP scaffold can employ distinct binding partners to regulate different ion channels in neurons. AKAP-79/150 is recruited to the GluR1 subunit of AMPA receptors by MAGUK adaptor proteins and as a consequence localizes PKA and PP2B to regulate phosphorylation of Ser845 in the tail of the receptor; this in turn determines the stability and surface expression of the channel (Colledge et al., 2000, 2003). In contrast, the same AKAP-79/150 is recruited to membranes in the vicinity of M type KCNQ2 potassium channels by binding to $PI(4,5)P_2$ and suppresses the inhibitory M current by facilitating channel phosphorylation by PKC (Hoshi et al., 2003). Such observations emphasize that signaling by protein kinases involves the physical tethering of the kinase and substrate at a defined site in the cell and highlight the dynamic nature and combinatorial possibilities of signaling scaffolds.

Switches, Timing, Memory, and Asymmetry

For cells to function, biochemical pathways must integrate multiple signals, incorporate a temporal dimension, and yet preserve an ability to react in a decisive fashion. Regulation of actin polymerization and branching by the modular WASP/N-WASP proteins provides an example of such complex regulation. The isolated C

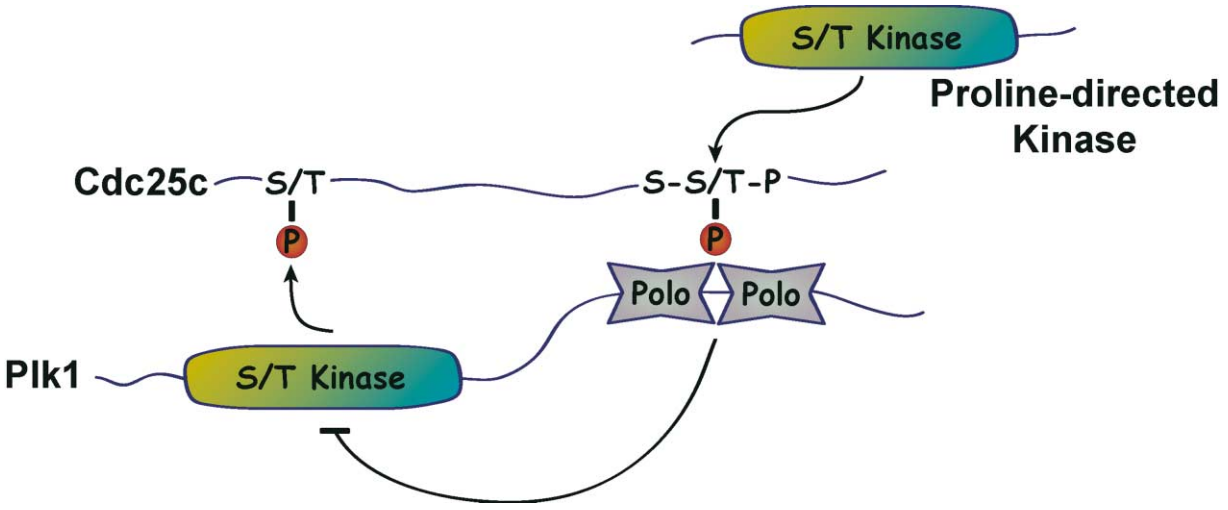

Figure 4. Regulation of the Plk1 Serine/Threonine Kinase by the Polo Box Domain, Containing Two Polo Motifs

The inhibitory interaction between the Polo box doamin and the kinase domain is relieved by phosphopeptide binding, which also targets the kinase to its substrates.

terminus of WASP interacts constitutively with the Arp2/3 complex to promote actin polymerization and branching, but this is inhibited in the full-length protein by an intramolecular interaction with the N terminus (Caron, 2002). This inhibition can be relieved by binding of the Cdc42 GTPase to a binding domain (GBD) in the N-terminal regulatory region, which induces a conformational reorganization that liberates the active C terminus (Kim et al., 2000). Furthermore, this activation is greatly potentiated by binding of PIP_2 to a basic region preceding the GBD, and the SH3 domains of SH2/SH3 adaptors can also stimulate WASP activity (Rohatgi et al., 2000, 2001). It has been argued WASP is an AND gate, in that it only fires effectively in the presence of multiple converging signals (Prehoda et al., 2000). Such an arrangement can suppress noise and allow the cell to make switch-like responses. Of interest, artificial proteins containing the N-WASP C terminus flanked ectopically by heterologous interaction domains and binding motifs can exhibit a range of switch-like responses, including cooperative behavior (Dueber et al., 2003). This supports the notion that proteins with complex properties can evolve by combining simple catalytic and interaction domains within a single polypeptide. The native WASP protein can also be regulated by phosphorylation at Y291 within the GBD. Y291 phosphorylation requires prior Cdc42 binding but stabilizes actin polymerizing activity following dissociation of the Cdc42 GTPase (Torres and Rosen, 2003). Torres and Rosen have therefore argued that Y291 phosphorylation detects coincident Cdc42 and tyrosine kinase inputs to WASP and drives a long-lived response to transient agonists, thereby endowing WASP with a form of molecular memory.

Transitions through the cell cycle provide a useful testing ground for these ideas since these must only occur at the right time and under the right conditions, but be irreversible. Here, phosphorylation of a key protein may monitor the activity of a cell cycle kinase, while polyubiquitination provides a means of rapidly destroying the phosphorylated regulator and advancing the cell cycle. One example of this mechanism involves Sic1, an inhibitor of S phase CDK activity in the yeast *S. cerevisiae*. Phosphorylation of Sic1 on Ser/Thr sites by the G1 CDK leads to its recognition by an SCF ubiquitin ligase complex, and consequent ubiquitination and degradation, permitting entry into S phase. However, binding to the Cdc4 component of the SCF complex requires at least six Sic1 phosphorylation sites, and this may necessitate sustained G1 CDK activity (Nash et al., 2001). Multisite phosphorylation of Sic1 may therefore provide a timing device for progression through the cell cycle.

A transient signal may also be converted into an irreversible, sustained response through a positive feedback loop (Xiong and Ferrell, 2003). Such a process has been proposed to mediate the induction of cell asymmetry and polarity in *S. cerevisiae* by the Cdc42 GTPase. In this scheme, GTP bound Cdc42 binds and opens up the scaffold protein Bem1, which has a series of interaction domains (PB1, SH3, PX) that direct its association with a guanine nucleotide exchange factor (GEF) for Cdc42, downstream polarity proteins, and phosphoinositides. The cycling of Cdc42, promoted by the activated GEF, and cooperative interactions between recruited polarity proteins themselves can potentially establish a positive feedback loop that induces a single supramolecular assembly at the plasma membrane to polarize the cytoskeleton and break cellular symmetry (Irazoqui et al., 2003).

Affinity and Specificity

In signaling networks, there is no optimal affinity for protein-protein interactions, but rather a wide range of dissociation constants that are tailored for distinct forms of biological regulation. It is natural to assume that tight protein-protein interactions yield a high degree of specificity and are more biologically relevant than interactions with relatively weak affinities. Strong interactions are long lived, and this can be advantageous, as in the

tethering of inactive PKA to an AKAP in readiness for a cAMP signal. However, such interactions cannot always provide the flexibility that a cell needs to respond dynamically to changing external conditions or internal programs. Indeed, protein-protein interactions that are dependent on posttranslational modifications must by definition have relatively modest affinities since much of the binding energy must come from the modified residue itself (Bradshaw et al., 2000). However, affinities in the micromolar range do not necessarily mean an absence of specificity. Indeed for both SH2 and SH3 domains, increased affinity for a particular motif can (somewhat paradoxically) come at the expense of specificity (Kessels et al., 2002; Zarrinpar et al., 2003b). A resolution to this conundrum may be that enhanced binding to an optimal sequence may at the same time interfere with a barrier to recognition of ectopic motifs and thus decrease specificity.

The yeast osmosensor Sho1, for example, has a C-terminal cytoplasmic SH3 domain that binds to a proline-rich motif in the scaffold (and kinase) Pbs2 (Figure 2) in a fashion that is required for activation of the Hog1 MAP kinase in response to high salt. Recent data indicate that Pbs2 binds specifically in vitro and in vivo to the Sho1 SH3 domain if tested against the entire set of yeast SH3 domains. However, it is rather promiscuous if presented with metazoan SH3 domains. The Sho1 SH3-Pbs2 interaction is therefore not optimized for affinity, but rather has apparently been selected for specificity in the context of competing SH3-mediated interactions within the yeast cell (Zarrinpar et al., 2003b). While specificity in vivo apparently can be generated through simple binary interactions, it is likely that many signaling complexes utilize multiple contacts to ensure fidelity (Bardwell et al., 2001). To this end, individual domains can be combined in a single polypeptide to enhance binding specificity and to generate allosteric control of signaling proteins and multiprotein complexes.

The Future

Networks and Systems

We can now start to explore biochemical function and signaling at a cell-wide level. The theme of posttranslational modifications and protein-protein interactions provides a powerful lens through which to investigate how protein complexes are assembled and connected to form larger networks. Experimentally, the yeast two-hybrid system and mass spectrometry have been used to probe protein-protein interactions and modifications on a large scale in organisms such as yeast, *C. elegans*, and *Drosophila* (Uetz et al., 2000; Ito et al., 2001; Ho et al., 2002; Gavin et al., 2002; Walhout et al., 2002; Giot et al., 2003). In general, the resulting data reveal hubs of highly connected proteins, distributed in a more random network with relatively few links required to connect any two proteins. Such scale-free networks may facilitate communication between disparate elements of the cell so that the different cellular subsystems can be organized into a functioning unit, yet rapidly respond to changing conditions in a coherent fashion. A comparison of interaction data from different species may also be valuable in identifying conserved subcellular systems and more recently evolved complexes (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/116/2/191/DC1).

Although the rules that underlie cellular networks have yet to be clearly established, clues have started to emerge from an analysis of yeast transcription factors (Lee et al., 2002). Importantly, the integration of large-scale proteomic data with transcriptional analysis, and with genetic interactions, promises to be a powerful aid in understanding how cells work in normal and disease states and may provide new avenues for manipulating cellular function that are therapeutically valuable (Walhout et al., 2002).

Present proteomic studies give us only a rather static picture of a cellular environment that is in reality extraordinarily dynamic. It will be intriguing and important to learn in more detail the extent to which signaling proteins assemble in distinct combinations in different cells and even within a single cell. This type of analysis will require single cell biochemistry, new analytical techniques to follow the changing composition of multiprotein complexes in living cells, and computational modeling to extract the biological meaning of dynamic behavior (Lee et al., 2003). Imaging of the movement, interactions, and locations of proteins in living cells will become especially important; small fluorescent probes such as semiconductor quantum dots, which can be used to track protein dynamics over extended periods in vivo, will likely prove valuable in this regard (Dahan et al., 2003).

Understanding the rules by which cells operate, and co-operate, raises the intriguing possibility of building new cellular functions. Thus, one can envisage a new era of synthetic biology, in which the cell's own regulatory and organizational prowess is harnessed to treat disease.

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