

REVIEW

New insights into protein-tyrosine kinase receptor signaling complexes

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Since the discovery of the existence of a large family of cell surface growth factor receptors possessing intrinsic protein-tyrosine kinase (PTK) activity, considerable effort has been made to identify their substrates and the intracellular signaling pathways that enable these receptors to induce changes in gene expression, cell division, and differentiation. Early efforts were focused on the search for protein substrates that were tyrosine phosphorylated in either growth factor-stimulated cells, or in cells that were transformed by oncogenic forms of PTKs. At the time, this approach met with minimal success because many of the proteins identified by these studies appeared not to be key substrates (the MAP kinases, p42/44, being the most notable exception). Many false leads were pursued because of the considerable amount of apparently fortuitous phosphorylation of cellular proteins, particularly in cells containing activated oncogenic PTKs. However, a seminal observation emerged from these studies: a major substrate for tyrosine phosphorylation was the PTK oncogene product, or the activated PTK growth factor receptor itself. The significance of this autophosphorylation was not immediately clear because in some cases it apparently did not regulate PTK activity, but in recent years this has come to be seen as an important observation (reviewed in Ullrich & Schlessinger [1990]).

Toward the end of the 1980s, the emphasis of PTK research shifted from looking directly for tyrosine-phos-

phorylated substrates to examining which of the known or possible second messenger-generating signaling pathways were coupled to, and activated by, specific PTK receptors. These studies paved the way for our current understanding of how cell-surface PTK receptors are linked to these intracellular signaling pathways. Among the key observations were that a minor phosphoinositide kinase activity (which subsequently turned out to be phosphatidylinositol [PI] 3-kinase) and phospholipase C γ (PLC γ) were found in anti-phosphotyrosine and in PTK receptor immunoprecipitates following stimulation with appropriate growth factors (Whitman et al., 1985; Wahl et al., 1988). Both PI 3-kinase and PLC γ generate known second messenger molecules involved in growth factor signaling pathways. These discoveries were rapidly followed by reports that other signaling molecules were recruited into receptor complexes upon growth factor stimulation. Implicated in these studies were the GTPase-activating protein (GAP) for Ras (Kazlauskas et al., 1990) and members of the Src family of nonreceptor PTKs (Kypta et al., 1990). These observations were noted at about the same time as a report that the viral oncogene product v-Crk was directly associated with a wide range of phosphotyrosine-containing proteins in v-Crk-transformed cells (Matsuda et al., 1990). A common feature of these proteins (PI 3-kinase, GAP, PLC γ , Src, and Crk) is that they possess two short regions of sequence similarity known as the Src-homology 2 (SH2) and SH3 domains. These are named in this way because they were described originally as protein sequence motifs present in the noncatalytic N-terminal region of the Src family of PTKs—SH1 being the PTK domain itself (reviewed in Pawson [1988], Koch et al. [1991], Musacchio et al. [1992a], and Pawson

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& Gish [1992]). The structure and function of these domains have been the subjects of intense investigation, and the concepts that have emerged have completely altered our understanding of the ways in which PTKs function to bring about coordinated, specific activation of the signaling pathways that lie downstream.

It is possible to divide currently known PTK targets into two broad classes: classical substrates for tyrosine phosphorylation and SH2 domain-containing targets. The classical substrates do not participate in the formation of multiprotein signaling complexes, and their activities or functions are modified directly by tyrosine phosphorylation (e.g., the MAP kinases or ERKs; Cobb et al., 1991). The SH2 domain-containing family of proteins uses phosphotyrosine as a recognition signal for complex formation, e.g., Grb-2 (Lowenstein et al., 1992), PLC γ (Wahl et al., 1988), or the 85-kDa subunit of PI 3-kinase (Escobedo et al., 1991b; Otsu et al., 1991; Skolnik et al., 1991). The formation of these signaling complexes with specific receptors leads ultimately to alterations in the activities of the bound proteins through a diverse set of mechanisms (see later). Some, but not all, of these bound SH2 domain-containing proteins may also be substrates for phosphorylation by the PTKs with which they interact. The remainder of this review will concentrate on SH2/SH3 domain-containing proteins associated with signal complexes because it is with this class of targets that most progress has been made.

The SH2 domain: A phosphotyrosine recognition module

SH2 domains have been identified in many proteins with seemingly diverse functions (reviewed in Pawson [1988], Koch et al. [1991], and Pawson & Gish [1992]) and can be classified into two broad subfamilies. The first group includes proteins that possess a known intrinsic enzymatic or regulatory activity (e.g., pp60^{c-Src} and PLC γ); the second group includes proteins that lack intrinsic enzymatic activity and have modules that mediate protein–protein interactions, and therefore presumably play some form of adaptor role (e.g., the 85-kDa subunit of the PI 3-kinase, Crk, Grb-2, Nck, and Shc). Many of the proteins from both groups also contain SH3 domains, but these two protein modules can exist independently and serve functionally distinct roles. Mutational studies performed on the v-Crk oncogene and the Abl PTK, both of which possess SH2 and SH3 domains, have shown that in each case the SH2 domain was of primary importance for their association with phosphotyrosine-containing proteins (Matsuda et al., 1991, 1993; Mayer et al., 1991, 1992). This observation led to the suggestion that the function of the SH2 domain was to recognize phosphotyrosine residues within a particular peptide sequence context. This novel role for protein phosphorylation in the regulation of intracellular events has since been shown to

function in diverse systems (Anderson et al., 1990; Moran et al., 1990; Escobedo et al., 1991a), and this overall concept has revolutionized our thinking about the range of possible regulatory and interactive functions of protein phosphorylation within the cell.

Structural features of SH2 domains

Knowledge of SH2 domain function at the molecular level has been greatly enhanced by recent structural studies on several different SH2 domains—including those of Abl (Overduin et al., 1992), Src (Waksman et al., 1992), and Lck (Eck et al., 1993) PTKs—and on the N-terminal SH2 domain of the p85 subunit of the PI 3-kinase (Booker et al., 1992). It is clear from a comparison of the published solution and X-ray structures that the SH2 domain represents a well-conserved protein fold consisting of a central antiparallel β sheet with an α helix packing onto each face of the sheet. Variations in structure between different members of the SH2 domain family are found mainly in the length of the loops joining these secondary structural elements (Booker et al., 1992; Overduin et al., 1992; Waksman et al., 1992, 1993; Eck et al., 1993). A nomenclature for the α helices (α A and α B) and β strands (β A– β G) has been proposed that allows these insertions and deletions to be placed when making comparisons of the different SH2 domain structures (Fig. 1; Eck et al., 1993).

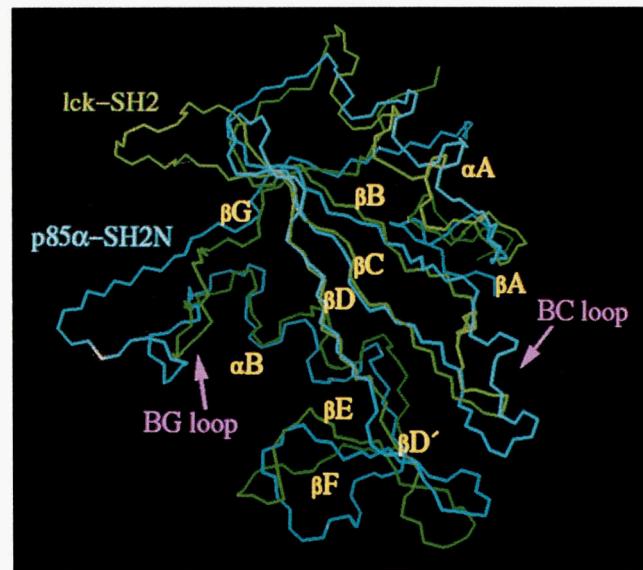


Fig. 1. Structural features of the SH2 domain. Comparison of p85 α (blue) and Lck (green) SH2 domains. The backbone atoms for the SH2 domains from p85 α (Booker et al., 1992) and Lck (Eck et al., 1993) are shown superimposed on the conserved region of the central β sheet (root mean square deviation 0.79 Å). Also indicated are the α helices and β strands following the convention of Eck et al. (1993) and the two loops (BC and BG) thought to undergo conformational change upon phosphotyrosine-peptide binding.

The structures of SH2 domain complexes with phosphotyrosine peptides that bind with high affinity have been solved for the Src (Waksman et al., 1993) and Lck domains (Eck et al., 1993). These studies provide important insights into the nature of phosphotyrosine recognition. It becomes clear that the side chains of conserved residues Arg- α A2 and Arg- β B5 make hydrogen bonds with the phosphate oxygens, whereas Arg- α A2 and Lys- β D6 make contact with the aromatic ring of the phosphotyrosine residue from opposite sides. These structures have also shed light on the role of the amino acid side chains of the residues at position +3 relative to the phosphotyrosine residue within the ligands. This side chain protrudes into a deep pocket seen in the two crystal structures, a feature that is likely to be conserved in many SH2 domains. However, whether this interaction occurs with some variation in all SH2 domains remains to be determined. Preliminary evidence suggests that the key residue for specific binding of the Grb-2 SH2 domain is in fact an asparagine residue at the +2 position (Songyang et al., 1993).

Contrary to expectation, the crystal structures of v-Src alone and in complex with a high-affinity ligand are distinguished by only minor conformational changes (Waksman et al., 1993). As indicated in Figure 1, two loops in particular, loop BC and loop BG, appear to differ in the complexed conformation (Lck-SH2) compared with the uncomplexed conformation (p85-N-terminal SH2). This is consistent with the observation that these two loops are poorly defined in the two solution structures and are likely to be relatively mobile (Booker et al., 1992; Overduin et al., 1992). It would appear, therefore, that the role of the SH2 domain is largely one of sequence-specific phosphotyrosine recognition, although it will be necessary to study the structure of SH2 domains within the context of the rest of appropriate proteins before an allosteric mechanism for SH2 domain-mediated signal transduction can be excluded.

Formation of PTK receptor complexes

The best studied PTK receptors with respect to signal complex formation are those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin, which form the prototypes for three of the PTK receptor classes (Schlessinger & Ullrich, 1992). Although these three receptors will be used to illustrate key points, the underlying principles described are true for other PTK receptors that have been examined.

Following the binding of an appropriate growth factor, receptor PTKs become activated and are autophosphorylated at a number of discrete sites within their C-terminal cytoplasmic domains (reviewed in Ullrich & Schlessinger [1990] and Schlessinger & Ullrich [1992]). In the case of the EGF and insulin receptors, these sites are mainly located in the domain of the receptor, which lies C-terminal

to the catalytic domain. However, in the case of the PDGF and related receptors (e.g., the receptor for colony stimulating factor-1 [CSF-1] and the receptor c-Kit), phosphorylation also occurs at sites within the kinase insert region, a domain that probably forms a distinct surface feature of the kinase domain. Following autophosphorylation of these sites, specific SH2 domain-containing proteins are recruited from the surrounding cytosol into complexes with the activated PTK receptor. This recruitment is mediated by phosphotyrosine-SH2 domain interactions that exhibit both high-affinity binding and rapid dissociation and exchange (Felder et al., 1993; Panayotou et al., 1993). The critical requirement for the presence of phosphotyrosine has been demonstrated by mutation of key tyrosine residues to phenylalanine residues at the autophosphorylation sites on PTK receptors. Such changes abolish both receptor autophosphorylation at these sites and the recruitment of the SH2 domain-containing protein that would normally bind to the particular phosphorylation sites (Kashishian et al., 1992; Valius & Kazlauskas, 1993). The binding of SH2 domain-containing proteins to phosphotyrosine-containing sequences is therefore not a random event, but is a precise interaction between specific SH2 domains and a limited range of phosphorylated target sequences. Short tyrosine-phosphorylated peptides based on autophosphorylation sites of the PDGF β -receptor have been shown to be able to competitively inhibit the binding of specific SH2 domain-containing proteins to phosphorylated PDGF β -receptors (Escobedo et al., 1991a; Fantl et al., 1992). Phosphopeptides derived from autophosphorylation sites (e.g., the sequence surrounding Y751 of the PDGF β -receptor) have also been used to affinity purify their appropriate SH2 domain-containing protein, e.g., the PI 3-kinase (Fry et al., 1992).

SH2 domain-specific binding sites

Specific binding sites have been identified for a number of SH2 domain-containing signal complex molecules. One of the best studied sites is that which mediates interactions with the SH2 domains of the p85 subunit of the PI 3-kinase. This enzyme has been shown to associate with the human PDGF β -receptor via tyrosines 740 and 751, which lie in the kinase insert region of this receptor (Fantl et al., 1992; Kashishian et al., 1992); with the murine CSF-1 receptor via tyrosine 721 (Reedijk et al., 1992); with tyrosine 315 of polyoma middle T antigen in the mT/pp60^{c-src} transforming complex (Talmage et al., 1989); and with several phosphorylated tyrosines on the insulin receptor substrate, IRS-1 (Backer et al., 1992; Yonezawa et al., 1992). All of these tyrosines lie within the consensus sequence YxxM (where x can be a wide range of possible residues). This motif appears therefore to be important for PI 3-kinase binding (Cantley et al., 1991) and recent structural studies on the N-terminal SH2 domain of p85 α provide a molecular basis for this

specificity (Booker et al., 1992). Studies with the recombinant N- and C-terminal SH2 domains of p85 α have shown that both are capable of binding to sequences containing YxxM motifs. However, the two domains show distinct affinities for such related sequences, suggesting that they may have independent roles (Panayotou et al., 1993). Although both SH2 domains of p85 α have been shown to interact with high affinity with YxxM sequences, it should be noted that a recent study has found that the lower affinity binding sites for PI 3-kinase on the hepatocyte growth factor/scatter factor receptor (c-Met) fall into the consensus YVxV (Ponzetto et al., 1993). It is possible that other lower affinity SH2 domain–phosphotyrosine sequence interactions may also occur and have a role to play in vivo.

Other SH2 domain-binding sites have been identified on the human PDGF β -receptor. These include tyrosine 771 in the kinase insert region, the site of interaction with RasGAP (Kazlauskas et al., 1992); tyrosines 1,009 and 1,021 in the C-terminal tail for interaction with PLC γ (Rönnstrand et al., 1992; Kashishian & Cooper, 1993; Valius et al., 1993); tyrosine 1,009, the binding site for the phosphotyrosine phosphatase, Syp (Feng et al., 1993; Valius et al., 1993); and tyrosines 579 and 581 in the juxtamembrane segment N-terminal to the catalytic domain, as the binding site for Src family PTKs (Mori et al., 1993). The observation that a single receptor can use the same mechanism to interact with such a diverse range of signaling molecules (and this list is probably not complete) demonstrates the flexibility and functional diversity of SH2 domain–phosphotyrosine interactions.

The binding sites that have been identified for SH2 domain-containing molecules on a number of other tyrosine-phosphorylated proteins are summarized in Figure 2. Unlike PI 3-kinase, other SH2 domain-binding sequence motifs have yet to be so precisely defined. This is mainly

because insufficient sites of interaction have been identified to date. However, in a recent study, Songyang et al. (1993) attempted to address this issue by binding short random phosphotyrosyl peptide mixtures to recombinant SH2 domains and then identifying by protein sequence analysis those peptide species that bound. This approach to derive a consensus binding sequence correctly identified the major PI 3-kinase binding site as the YxxM motif. Thus, the sequences predicted in this study as binding sites for other SH2 domains are clearly worth careful consideration. Proposed consensus binding sequences are also shown in Figure 2; however, for most SH2 domain-containing proteins, these are still undergoing a process of experimental refinement.

Finally, it should also be noted briefly that there have been reports that the range of sequences with which SH2 domains can interact may be much broader than was believed initially. Two papers have suggested that certain SH2 domains are also capable of binding to specific sequences through high-affinity phosphotyrosine-independent interactions (Pendergast et al., 1991; Muller et al., 1992). This type of interaction was demonstrated originally between the BCR (the product of the breakpoint cluster region gene) sequences essential for transformation by the BCR-Abl oncogene and the Abl SH2 domain, and was similarly documented subsequently for several other SH2 domains, including the C-terminal SH2 of PLC γ , the N-terminal SH2 of GAP, and the Src SH2 domain (Muller et al., 1992). This interaction was shown to be dependent on the presence of phosphoserine/phosphothreonine and not phosphotyrosine (Pendergast et al., 1991). If SH2 domain interactions do occur in vivo with sequences containing phosphorylated residues other than tyrosines, then the range of potential interactions between SH2 domains and their target proteins would be greatly extended because the degree of cellular serine/threonine phosphorylation is manyfold larger than tyrosine phosphorylation. However, the affinities of these interactions are estimated to be two orders of magnitude lower than high-affinity SH2 domain–phosphotyrosine interactions. In light of this, and in the absence of any in vivo role for SH2 domain–phosphoserine/phosphothreonine interactions, their significance remains speculative.

How does complex formation lead to changes in activity of signaling molecules?

Once a specific SH2 domain protein has been recruited into a signaling complex, there are a number of possible consequences that may result in a change in the activity of the associated protein. The basic modes of regulation have been reviewed recently by Panayotou and Waterfield (1993) and will be considered only briefly here. Possible regulatory mechanisms include translocation, phosphorylation, and conformational changes induced by binding to phosphorylated sequences. These mechanisms are

PI 3-kinase	
YXXM	
PDGF β R	Y740
PDGF β R	Y751
CSF-1R	
CSF-1R	Y721
mT	Y315
IRS-1	8 sites
(HGFR) (HGFR)	Y1349) Y1356)
p64/Syp	
PDGF β R	Y1009

PLC γ	
FGFR	Y766
EGFR	Y992
NGFR	Y785
PDGF β R	Y1009
PDGF β R	Y1021

Grb-2	
YXNX	
EGFR	Y1068
Shc	Y317
IRS-1	Y895

Src	
PDGF β R	Y579
PDGF β R	Y581
Src	Y527

GAP	
YMAP	
PDGF β R	Y771

Fig. 2. Known binding sites and deduced consensus binding sequences for SH2 domain-containing proteins. Other more speculative SH2 domain-binding sites can be found in Cantley et al. (1991) and Songyang et al. (1993).

clearly not mutually exclusive; regulation of the function and binding of many SH2 domain-containing proteins probably occurs by a combination of two or more of the above acting in concert.

Translocation

Complex formation between SH2 domain-containing proteins and membrane-bound receptors results in their movement from the cytosol to a juxtamembrane location. In many cases this brings them into close proximity with their known substrates, or with other proteins with which they are known to interact, e.g., membrane phosphoinositides for PLC γ and PI 3-kinase and Ras in the case of GAP. Such translocations have been reported for PI 3-kinase, which moves to the plasma membrane following PDGF stimulation (Susa et al., 1992) and to low-density membrane vesicles following insulin stimulation (Kelly & Ruderman, 1993). Although translocation alone might be sufficient to cause activation of a signaling pathway, it is likely that secondary events are required for more specific regulation; this has indeed been shown to be the case with PLC γ (see below).

Phosphorylation

Phosphorylation and dephosphorylation are regarded as classical mechanisms for modulating enzyme activity. There is ample evidence to support a role for tyrosine phosphorylation operating in concert with SH2 domain binding in the regulation of PLC γ activity. In addition to binding to activated receptors, PLC γ has also been shown to be phosphorylated at residues Y783 and Y1254. This phosphorylation appears to be essential in order to achieve full activation of PLC γ (Kim et al., 1991). A mechanism to account for this activation has been proposed. The suggestion is that phosphorylation of tyrosine residues induces an intramolecular SH2 domain–phosphotyrosine interaction, resulting in a more active form of PLC γ , possibly through a conformational change (reviewed in Rhee [1991]). An SH2 domain-containing phosphotyrosine phosphatase has also been reported to be activated by tyrosine phosphorylation (Vogel et al., 1993), but to date tyrosine phosphorylation of other receptor-associated proteins has not been shown clearly to play an important regulatory role.

Conformational changes

A number of reports now exist demonstrating that a small but reproducible activation of PI 3-kinase can be observed following the binding of short phosphotyrosine-containing peptides to its SH2 domains (Backer et al., 1992; Carpenter et al., 1993). Similar activation can be achieved by the addition of tyrosine phosphorylated IRS-1 to preparations of PI 3-kinase (Backer et al., 1992; Myers

et al., 1992; Giorgetti et al., 1993); PI 3-kinase is known to bind to IRS-1, which is itself a substrate of the insulin receptor, via the SH2 domains of p85 both in vitro and in vivo. This interaction mediates the indirect activation of PI 3-kinase by the insulin receptor following insulin stimulation of appropriate cells (Hadari et al., 1992; Yonezawa et al., 1992; Backer et al., 1993). Presumably, following binding of the phosphopeptide to one or both of the p85 SH2 domains, a conformational change takes place and is transmitted to the associated p110 catalytic subunit, resulting in an increase in PI 3-kinase activity. Small conformational changes in p85, and an isolated N-terminal SH2 domain from this protein, have been observed upon phosphopeptide binding using circular dichroism and fluorescence studies (Panayotou et al., 1992; Shoelson et al., 1993).

Finally, regulation that may involve conformational changes has been suggested recently to explain the observed regulation of the Src PTK. It has been found that the Src SH2 domain is able to bind to the known negative regulatory site of tyrosine phosphorylation in its C-terminal tail, Y527, which is phosphorylated by the kinase Csk (Courtneidge, 1985; Roussel et al., 1991; Lui et al., 1993). This interaction suggests a possible mechanism for the regulation by phosphorylation at this site whereby the Src protein is held in a conformation unfavorable for kinase activity toward its normal substrates (Roussel et al., 1991; Lui et al., 1993). This intramolecular SH2 domain–Y527 interaction appears to be of only moderate affinity, which would fit with the negative regulation being alleviated once a suitable high-affinity binding site becomes available. In support of this, high-affinity binding phosphopeptides have been shown to be able to stimulate Src PTK activity in vitro (Lui et al., 1993). These data fit well with the earlier finding that point mutations within the SH2 domain (which from the crystal structure are predicted to disrupt the overall structure of the domain, or which involve key residues in binding phosphotyrosine) were both able to activate the proto-oncogene pp60^{c-src} (O'Brien et al., 1990).

The available evidence would seem to support the concept that recruitment of signaling proteins to receptors mediated by phosphotyrosine–SH2 domain interactions provides a “switch” that initiates signaling cascades, but that other events are required for full activation of the signaling molecule. This is most clearly seen in the case of PLC γ . The PDGF β -receptor mutants that lack the critical tyrosine residues at the PLC γ binding site fail to bind this protein upon receptor stimulation. The PLC γ becomes tyrosine phosphorylated in cells expressing these mutant receptors, but this is not sufficient for full activation (Valius et al., 1993). Similarly, as described above, mutants of PLC γ lacking the tyrosine phosphorylation sites Y783 and Y1254 can still bind to an activated PDGF β -receptor but cannot be activated fully by it (Kim et al., 1991).

The SH3 domain: A regulator of G-protein function?

SH3 domains are found in a wide range of proteins with no obvious common underlying function. The wealth of information on signaling complexes gained from the study of SH2 domains has spurred great interest in the more enigmatic SH3 domain. The SH3 domain, until recently, was suggested to have a role either at the cell membrane or in actin cytoskeleton function (Koch et al., 1991; Musacchio et al., 1992a). However, there was little direct experimental evidence to support such proposed roles. As with the SH2 domain, studies on the SH3 domain have involved both structural and functional analyses, and interesting and important advances have recently been made through these approaches.

Functional analysis of SH3 domains

SH3 domains, like the SH2 domains, have no fixed topological position within their host proteins; it was therefore thought that this conserved sequence element was also likely to be a discrete protein module. Several groups took the approach of expressing SH3 domains from various proteins as recombinant glutathione S-transferase (GST) fusion proteins, which were then used to screen for potential SH3 domain binding proteins. Two such approaches have been described. Cicchetti et al. (1992) used a biotinylated GST-SH3 domain from Abl to screen a murine pre-B cell line expression library. Two novel cDNA clones were described: 3BP-1 (for SH3 binding protein) and 3BP-2. The protein products of these two cDNAs both bound to the Abl SH3 domain but showed selective and distinct binding properties with respect to the SH3 domain of Src. Further characterization of the 3BP-1 protein revealed a region with recognizable sequence similarity to the BCR gene product, *n*-chimaerin, and RhoGAP. This sequence similarity lies in the region of these proteins that recently has been implicated functionally as a GAP domain for Rac- and Rho-related small G-proteins (reviewed in Fry [1992]). This observation led to the suggestion that SH3 domains might function in the regulation of small GTP-binding proteins (Pawson & Gish, 1992; Mayer & Baltimore, 1993). By a combination of deletion mutagenesis and the use of short peptide sequences, the binding site for the Abl SH3 domain on 3BP-1 was localized to a short proline-rich region (Cicchetti et al., 1992; Ren et al., 1993). A 10-amino acid peptide containing the putative consensus sequence XPXXPPP Ψ XP (where X is any amino acid and Ψ is a hydrophobic amino acid) seems to be the minimum requirement for Abl SH3 domain binding to 3BP-1. A similar proline-rich sequence is found in 3BP-2, and a peptide containing this sequence is also capable of binding to SH3 domains (Ren et al., 1993). Presumably the prolines form a core for the binding motif and the adjacent amino acids provide some de-

gree of binding specificity for different SH3 domains, but this remains to be demonstrated rigorously.

The second approach involved using a panel of GST-SH3 domain fusion proteins immobilized on glutathione-Sepharose as affinity ligands to purify potential binding partners. Screening a bovine brain extract in this manner revealed a number of proteins that were able to bind specifically to distinct subsets of this protein panel (Gout et al., 1993). One of these proteins, which bound with high affinity to the SH3 domains of p85 α , PLC γ , and Grb-2, was sequenced and revealed as the bovine homologue of dynamin (Booker et al., 1993; Gout et al., 1993). Dynamin was originally described as a microtubule-binding protein with a GTPase activity of uncertain function (Obar et al., 1990). However, a *Drosophila* homologue of dynamin, *Shibire*, has been characterized and implicated in vesicle trafficking (Chen et al., 1991; van der Bliek & Mayerowitz, 1991). Like 3BP-1, dynamin (and *Shibire*) contains several proline-rich sequences in its C-terminus that appear to mediate its binding to SH3 domains. A 21-amino acid peptide that incorporated one of these proline-rich sequences was able to compete with dynamin for SH3 domain binding (Booker et al., 1993; Gout et al., 1993). These proline-rich sequences show similarity to the consensus binding sequence described above for 3BP-1 but are not a perfect match, suggesting that some sequence variation may be tolerated by different SH3 domains. This may add to the specificity of their possible binding interactions. Providing further support for the possibility of a G-protein regulatory role for SH3 domains, it was found that several of the SH3 domains that were able to bind to dynamin were also able to stimulate its intrinsic GTPase activity (Gout et al., 1993). The key issue now is to determine which SH3 domain-containing proteins are able to interact with binding proteins such as 3BP-1 and dynamin *in vivo*.

Structural features of SH3 domains

The three-dimensional structure of the SH3 domain has also been the subject of intense interest. Solution structures for the domains from pp60 c -src (Yu et al., 1992), PLC γ (Kohda et al., 1993), and p85 α (Booker et al., 1993; Koyama et al., 1993), together with the crystal structures of α -spectrin (Musacchio et al., 1992b) and c-Fyn (Noble et al., 1993), have been reported recently. It can be seen from Figure 3 that the published structures are strikingly similar and represent a compact β barrel. The most noteworthy differences between the different domains studied to date are the insertion of a long loop in the p85 α SH3 domain between β strands B and C (Booker et al., 1993) and the length of the loop between β strands C and D. The conformation of the PLC γ SH3 domain differs slightly from the other three shown in the figure, although this may be due in part to the relatively low resolution of this structure (Kohda et al., 1993).

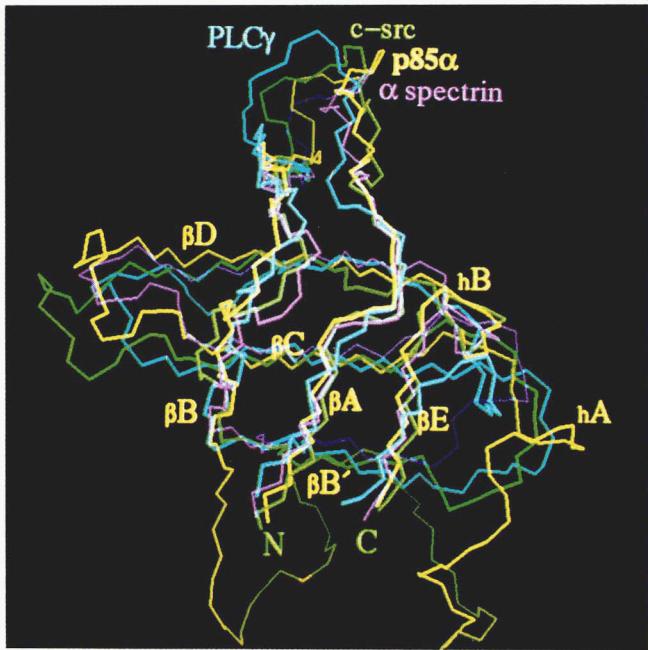


Fig. 3. Structural features of the SH3 domain. Comparison of SH3 domains is shown. The backbone atoms from p85 α (Booker et al., 1993), α spectrin (Musacchio et al., 1992b), c-Src (Yu et al., 1992), and PLC γ (Khoda et al., 1993) are shown superimposed on the conserved regions of β sheet with root mean square deviations relative to spectrin of 0.85 Å (p85 α), 0.92 Å (c-Src), and 1.7 Å (PLC γ).

Initial characterization of the ligand binding site of the SH3 domain has been undertaken using synthetic peptides derived from 3BP-1 and dynamin and observing the effect of complex formation on the chemical shift values of SH3 domain protons (Yu et al., 1992; Booker et al., 1993). These experiments, together with analysis of sequence conservation and point mutations (Clark et al., 1992), have indicated that the ligand binding site consists of a hydrophobic binding surface surrounded by highly charged loops. It is likely that the conserved hydrophobic surface provides a general platform for binding the proline-rich sequence, whereas the selectivity of the interaction arises from interactions of the ligand with charged groups in the loops between the strands. Identification of domain-specific, high-affinity SH3 ligands and the determination of the structure of the resulting complexes will be required to confirm these initial observations.

Coordinate regulation of Ras function through SH2 and SH3 domains

We have seen examples of how the SH2 and SH3 domains function by binding specific motifs and thus regulate protein-protein interactions, but how do these domains function together in the regulation of the transmission of intracellular signals? The most complete description of

concerted SH2 and SH3 domain function within cellular signaling pathways has come from studies on a mechanism of activation of the small G-protein Ras. For some time Ras has been known to function downstream of PTKs (Mulcahy et al., 1985; Smith et al., 1986), but the way in which they were linked had proved elusive. However, recently one widely utilized pathway for Ras activation by PTKs has become clear. This work has been aided greatly by the genetic dissection of invertebrate developmental signal transduction pathways in both *Drosophila melanogaster* and *Caenorhabditis elegans*.

The elucidation of this pathway started with the identification of a cell-signaling gene in *C. elegans*, *sem-5*, which encodes a protein composed of a central SH2 domain flanked by two SH3 domains (Clark et al., 1992). Genetic analysis suggested that in *C. elegans* this gene product functioned downstream of the *let-23* receptor PTK and upstream of the *let-60* Ras-related gene product in a developmental pathway leading to the induction of vulval cell fate (Horvitz & Sternberg, 1991). Independent mutations in the SH2 and SH3 domains were shown to impair *sem-5* activity, strongly supporting a role for both of these domains in signaling. A mammalian homologue of *sem-5*, Grb-2, was isolated recently by an expression library screening technique designed to identify SH2 domain-containing proteins (Lowenstein et al., 1992). This screening technique involves the probing of expression cDNA libraries with radiolabeled phosphorylated sequences derived from receptor tyrosine phosphorylation sites (Skolnik et al., 1991; Margolis et al., 1992). This method has allowed the isolation of cDNAs for proteins that are able to bind to these phosphorylated sequences and has proved a successful approach for isolating novel SH2 domain-containing proteins including the p85 subunit of the PI 3-kinase, Grb-2, and several other SH2 domain-containing Grb proteins of unknown function (Skolnik et al., 1991; Margolis et al., 1992).

Grb-2 was shown to bind to both EGF and PDGF receptors in a phosphotyrosine/SH2 domain-dependent manner, but did not itself become phosphorylated on tyrosine in growth factor-stimulated cells (Lowenstein et al., 1992). It was also shown in the same study that Grb-2 was able to stimulate DNA synthesis, but only when introduced into cells together with a functional Ras protein. Neither of these proteins introduced alone into cells had any effect on cell proliferation. These results suggested that Grb-2/*sem-5* plays a critical role in the regulation of Ras function. An extensive amount of data published recently has filled in the remaining gaps by providing a clear link between Ras and receptor PTKs. It was assumed that if the SH2 domain of Grb-2 was involved in its recruitment by receptor PTKs, then it was likely that the SH3 domains provided the link to Ras. The proteins mediating this interaction in *Drosophila*, *C. elegans*, and mammalian systems turn out to be members of the Sos (Son of sevenless) protein family, which bind via proline-rich

regions of sequence in their carboxy-termini to the SH3 domains of Grb-2 (Buday & Downward, 1993; Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Olivier et al., 1993; Rozakis-Adcock et al., 1993; Simon et al., 1993). This is the first clear description of a physiological ligand for an SH3 domain. The Sos protein was described originally as a signaling molecule that acted downstream of the Sevenless PTK receptor, a requirement for the proper specification of R7 photoreceptor cells in the eye of *Drosophila*. In addition to proline-rich motifs, Sos was shown to contain sequences related to known Ras guanine-nucleotide exchange proteins (GNRP) (Simon et al., 1991). It would appear that Grb-2 and Sos proteins are associated constitutively, but only function to activate Ras upon receptor stimulation. This leads to recruitment of the Grb-2/Sos complex to the membrane and results in the phosphorylation of the Sos protein (Rozakis-Adcock et al., 1993). Presumably Sos is then able to convert the inactive GDP-bound form of Ras to an active GTP-bound form by nucleotide exchange. The exact mechanism by which recruitment of the Grb-2/Sos complex to the membrane results in Ras activation remains to be determined. However Grb-2 and the RasGNRP, Sos, clearly seem to be the missing components between receptor PTKs and Ras in a wide variety of cell types.

A further degree of complication is added to this pathway by the fact that Grb-2 is known to complex with tyrosine-phosphorylated proteins other than autophosphorylated receptors. Grb-2 has been shown to form complexes with tyrosine-phosphorylated forms of the Shc protein, another SH2 domain-containing family member (Pellicci et al., 1992; Rozakis-Adcock et al., 1992; Egan et al., 1993; Skolnik et al., 1993). The Shc proteins have a carboxy-terminal SH2 domain and an amino-terminal glycine/proline-rich region and have been shown to be transforming when overexpressed in fibroblasts (Pellicci et al., 1992). In addition, Shc is known to become tyrosine phosphorylated in response to growth factor stimulation (Pellicci et al., 1992; Ruff-Jamison et al., 1993) and in v-Src- and v-Fps-transformed cells (McGlade et al., 1992; Egan et al., 1993). It has been shown that Shc/Grb-2/Sos complexes exist in Src-transformed Rat1 cells and it may be that this provides a mechanism for activating Ras in the absence of a PTK receptor to which Grb-2 can bind directly (Egan et al., 1993). This would appear to be the case in cells stimulated by insulin, where Grb-2 and Shc have been found in complexes with IRS-1 (Skolnik et al., 1993). Such multiple pathways for Ras activation by a single receptor have been predicted for some time. Interestingly, recent studies on the PDGF β -receptor have suggested that this receptor can mediate activation of Ras through distinct pathways in different cell types examined (Satoh et al., 1993), and that several of the known PDGF receptor-activated signaling pathways may function upstream of Ras within a single cell type (Valius & Kazlauskas, 1993).

The remaining black box in this pathway between the cell surface and the nucleus also appears to have been resolved because complexes containing activated (Gly 12 Val mutants) or GTP-bound forms of Ras, together with the downstream protein kinase, Raf-1, and MAP kinase kinase have been isolated (Moodie et al., 1993). It is not clear whether this is a direct interaction between activated Ras proteins and these protein kinases, or whether the interaction is mediated by an as yet unidentified Ras target protein. However, the specificity and likely relevance of this interaction are supported by the fact that effector mutants of Ras (e.g., Ile36Ala) fail to interact with these kinases. This would link Ras directly into a protein kinase cascade resulting in the activation of MAP kinase and the pp90 ribosomal S6 kinase, leading ultimately to the phosphorylation of known nuclear proto-oncogenes, such as c-Fos, SRF, and c-Jun (reviewed in Roberts [1992]). This pathway is represented schematically in Figure 4.

The pleckstrin homology (PH) domain: Another putative module in signaling proteins?

The preceding sections illuminate the manifold possibilities for interactions between SH2 domain- and SH3 domain-containing proteins. However, the number of potential interactions between signaling proteins may be increased further by another putative domain found within diverse proteins involved in intracellular signaling and/or in cytoskeletal function (Haslam et al., 1993; Mayer et al., 1993). Because this domain was identified initially as an internal N- and C-terminal sequence duplication in the major platelet substrate of protein kinase C, pleckstrin (Tyers et al., 1988), it has been named the pleckstrin homology, or PH, domain. In addition to pleckstrin, proteins that exhibit sequence similarity to this putative domain and that have been identified to date include the SH3 domain-binding protein 3BP-2, RasGAP and a RasGRF, the RAC family of serine/threonine protein kinases, the *Drosophila* and murine Sos proteins, Sec7p, the GTPase dynamin, the SH2 domain-containing protein Grb-7, oxysterol-binding protein, the yeast protein Bem3p, a trypanosomal protein kinase NrkA, the *C. elegans* Unc-104 kinesin-related product, the phospholipases PLC δ and PLC γ , and the β -spectrins (Haslam et al., 1993; Mayer et al., 1993). The PH domain is approximately 100 amino acids in length, with family members noted so far exhibiting 21–25% sequence identity. No clear role for the PH domain can be suggested as yet. However, based on the large number of signaling proteins in which it is found, it is tempting to speculate that, like SH2 and SH3 domains, the PH domain may also mediate some form of protein-protein interaction during signaling events. The PH domain will certainly be the focus of intense study following the rich vein of information that has been revealed through the examination of the structure and function of SH2 and SH3 domains. If the PH domain proves to have

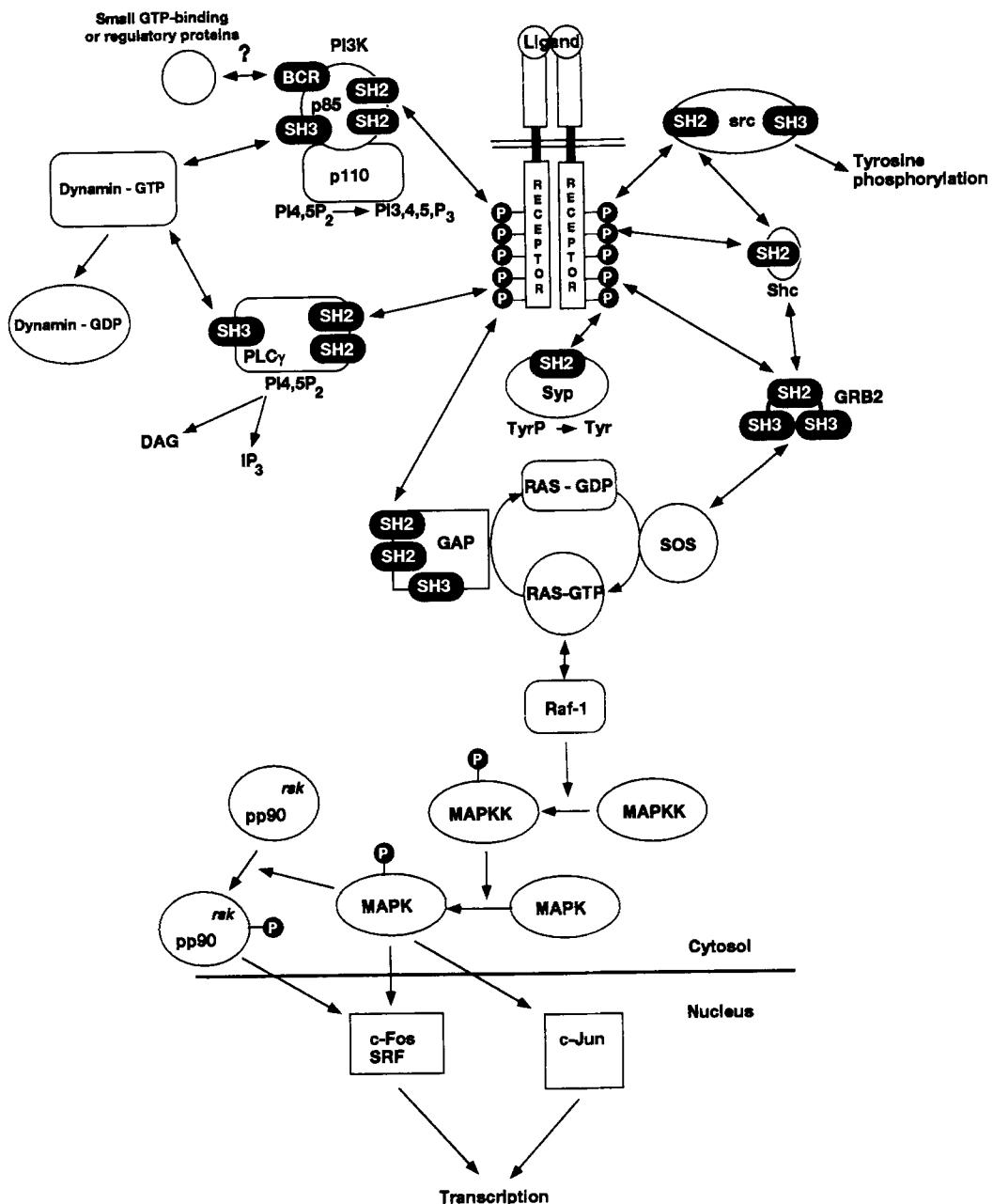


Fig. 4. The SH2 and SH3 domain-mediated signal transduction pathways. Schematic representation of the many signal outputs that can potentially be achieved by a single class of activated PTK receptor. The initial receptor signaling molecule interactions are mediated by SH2 domain-phosphotyrosine interactions, and the subsequent steps are mediated either by further protein-protein interactions involving SH2 and SH3 domain interactions or by second messenger molecules generated by the primary interacting SH2 domain-containing proteins, e.g., PLC γ and PI 3-kinase. The recently described pathway mediating the flow of signaling information from an activated PTK receptor via Ras to the nucleus is included. Two-headed arrows indicate direct protein-protein interactions, whereas single-headed arrows indicate enzymatic transformations.

an independently folding structure, then interesting results are likely to follow rapidly.

Summary and perspectives

Much has been learned over the last 5 years about the way in which receptor PTKs at the plasma membrane couple

to intracellular signaling pathways. The general mechanisms by which SH2 domains couple ligand-activated, tyrosine-phosphorylated PTK receptors to diverse signaling pathways are now well established. The SH3 domains have also been linked intimately into this network of signaling pathways, and a picture of the SH3 domains functioning to regulate interactions via proline-rich sequences

has begun to emerge. It remains to be seen whether the general features observed thus far for SH3 domain regulation of various aspects of G-protein function will be a unifying theme for this domain in intracellular signaling. Interest in both SH2 and SH3 domains remains high, and already these modules are seen as potential targets for therapeutic drug design (Brugge, 1993). However, there is still much left to be discovered. The *in vivo* binding partners for most of the known SH2 and SH3 domain-containing proteins remain to be identified. With the possible addition of a third such protein module in the form of the PH domain, the permutations of possible interactions for a protein such as GAP, which contains a PH, an SH3, and two SH2 domains, become very large indeed. It will be some time before a full understanding of the incredibly complex network of the protein–protein interactions that are set in motion following activation of PTK receptors, and that appear to regulate many aspects of cell function, is achieved.

The key question at the forefront of current research has now shifted from what are the targets to how do the different PTK receptors produce their diverse effects within a single cell type? For example, stimulation of PC12 cells with EGF leads to mitogenesis, whereas stimulation of the same cells with nerve growth factor (NGF) leads to cell differentiation. Both of the receptors for these factors are PTKs and seem to interact with a very similar range of signaling pathways, but they produce dramatically different effects. The answer to the question “Which key pathways are required for mitogenesis?” has also remained elusive, with different studies in different cell types producing contradictory results. This issue remains very confusing, in part due to the high level of complexity involved. It has been assumed that different PTK receptors will have unique SH2 domain-binding proteins that will confer specific signaling properties to receptors. Preliminary data on signaling by the PDGF α - and β -receptor subtypes in fibroblasts suggest that differences in their substrate specificities (in this case their ability to interact with RasGAP) correlate with specific receptor functions (Heidaran et al., 1993). However, the alternative is that the specificity of a given PTK receptor may come from the particular subset of common targets with which it is able to interact and, in turn, the ability of these targets to interact with cell type-specific molecules that lie further downstream in the pathway. These are now clearly the most important issues to be addressed.

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