SH2 and PTB Domains in Tyrosine Kinase Signaling

Joseph Schlessinger[1]* and Mark A. Lemmon[2]

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Intracellular signaling pathways that involve protein tyrosine kinases (PTKs) are critical for the control of most cellular processes. Dysfunctions in PTKs, or in the signaling pathways that they regulate, result in a variety of diseases such as cancer, diabetes, immune deficiency, and many others. SH2 (Src homology region 2) and PTB (phosphotyrosine-binding) domains are small protein modules that mediate protein-protein interactions involved in many signal transduction pathways. Both domains were initially identified as modules that recognize phosphorylated tyrosines in receptor tyrosine kinases and other signaling proteins. Subsequent studies have shown that, while binding of SH2 domains to their target proteins is strictly regulated by tyrosine phosphorylation, most PTB domains actually bind to their (non-phosphorylated) targets constitutively. The functions of SH2 and PTB domains include targeting of their host proteins to different cellular compartments, assembly of key components of signaling pathways in response to extracellular signals, and the control of autoinhibition, activation and dimerization of their host proteins. The information flow from the cell surface to different cellular compartments to regulate the cell cycle, cell shape and movement, cell proliferation, differentiation, and cell survival are all controlled in part by SH2 and PTB domains that can recognize phosphotyrosine or particular amino acid sequence motifs in a wide variety of target molecules.

Introduction

Many proteins involved in intracellular signaling contain multiple small modules (of 50 to 200 amino acids) that direct their constitutive and/or signal-regulated association with other proteins, with membranes, and with other cellular components. It is believed that understanding the individual function of each class of domain found in these proteins, preferably across the whole genome, may ultimately permit the construction of cellular wiring diagrams (*1*, *2*). Of the different signaling domains in tyrosine kinase signaling, arguably the most important are those that recognize phosphotyrosine itself: the Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains. Furthermore, the discovery of the central role played by SH2 domains in tyrosine kinase signaling marked the beginning of our view of intracellular signaling molecules as multidomain proteins with an array of interaction modules that define their function. In this review, we briefly discuss how SH2 and PTB domains were identified, consider the structural basis for their recognition of different phosphotyrosine-containing (and other) binding sites, and survey the different roles these domains play in the functions of their host molecules.

[1]Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520, USA. [2]Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

*Corresponding author. Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, SHM B-204, New Haven, CT 06520, USA. Telephone, 203-785-7395; e-mail, joseph.schlessinger@yale.edu

SH2 Domains

SH2 domains were first defined as conserved sequences of about 100 amino acids (*3*) that play a role in regulating the tyrosine kinase activity of v-Fps and members of the Src family of oncogenic tyrosine kinases. SH2 domains have central roles in mediating intracellular signaling by receptor tyrosine kinases (RTKs) and cytoplasmic protein tyrosine kinases (PTKs). Signaling proteins such as phospholipase C-γ (PLC-γ) and Ras-GTPase (guanosine trisphosphatase)-activating protein (Ras-GAP), which become tyrosine phosphorylated in response to stimulation of cells with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), also form a complex with the tyrosine-phosphorylated EGF-receptor (EGFR) or PDGF-receptor (PDGFR) upon growth factor stimulation (*4-9*). Because both PLC-γ and Ras-GAP contain SH2 domains, these domains were implicated as being responsible for the formation of complexes between these proteins and the tyrosine-phosphorylated EGF or PDGF receptors (*10-12*). This idea was further reinforced by experiments demonstrating that Crk, a retroviral oncogenic protein composed entirely of SH2 and SH3 (Src homology 3) domains, binds to various phosphotyrosine-containing proteins in lysates prepared from v-Crk-transformed cells (*13*, *14*). Taken together, these studies argued that tyrosine-phosphorylated regions in RTKs and other proteins could function as specific binding sites for the SH2 domains of cellular signaling proteins. Indeed, direct binding experiments using isolated SH2 domains confirmed the notion that SH2 domains bind to specific phosphotyrosine (p-Tyr)-containing sequences in RTKs (*15-17*). Moreover, a variety of SH2 domain-containing signaling proteins were discovered in screens of bacterial expression libraries in which the tyrosine-phosphorylated tail of EGFR was used as a specific probe (*18*). This approach enabled the cloning of new SH2 domain-containing proteins, including the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) (*18*); the adaptor protein Grb2 (*19*) and its *Drosophila* homolog Drk (*20*); the signaling proteins Grb7, Grb10, and Grb14; and previously known SH2 domain-containing proteins such as the tyrosine kinase Fyn, PLC-γ, Ras-GAP, and Crk (*21*).

The phosphotyrosine-containing binding sites for SH2 domains were mapped in several RTKs, and SH2 domain binding to tyrosine-phosphorylated RTKs was shown to be blocked specifically by short p-Tyr-containing peptides [reviewed in (*22-24*)]. These studies demonstrated that SH2 domains bind p-Tyr only within the context of particular peptide sequences C-

terminal to the p-Tyr moiety [reviewed in (*1*, *22*, *23*)]. For example, the SH2 domain of Grb2 binds to a pYXN [pY, phosphotyrosine; N, asparagine; X represents any amino acid (*25*)] sequence motif that is found in the tyrosine-phosphorylated tail of EGFR, in the oncogenic protein tyrosine kinase Bcr-Abl, in the adaptor protein Shc, in the docking protein FRS2, and in the protein tyrosine phosphatase Shp2, among many other signaling proteins. On the other hand, the two SH2 domains from p85 (the regulatory subunit of PI3K) bind specifically to pYMXM (M, methionine) sequence motifs that are found in the kinase insert region of the PDGF receptor; in the docking proteins (insulin receptor substrates) IRS1 and IRS2, as well as Gab1 and Gab2; and in various other signaling proteins that recruit and activate PI3K. Although the first binding motifs for SH2 domains were identified by direct mapping of tyrosine phosphorylation sites and by site-directed mutagenesis [reviewed in (*1*, *22*, *23*)], the majority of the peptide-binding motifs for individual SH2 domains were identified by in vitro screening of combinatorial phosphopeptide-containing libraries (*24*). Many SH2 domains bind to tyrosine autophosphorylation sites located in noncatalytic regions of RTK cytoplasmic domains, or to tyrosine phosphorylation sites in docking proteins (membrane-linked proteins that assemble various signaling proteins after tyrosine phosphorylation). When phosphorylated, these sites provide a platform for the recruitment and activation of multiple signaling pathways through SH2 domain-mediated interactions. Each activated RTK and tyrosine-phosphorylated docking protein recruits a distinct complement of signaling molecules from a common preexisting pool of membrane-attached and cytoplasmic proteins. The specificity of SH2 domain interactions defines which proteins are recruited by a given RTK or docking protein. Thus, SH2 domains function as an important element for control of RTK signaling specificity (*26*-*28*).

The three-dimensional structures of several SH2 domains bound to cognate p-Tyr-containing peptide ligands revealed the common protein fold of the SH2 module and the molecular basis for ligand-binding specificity [reviewed in (*1*, *22*, *23*)]. The SH2 domain fold is composed of two α helices that surround a conserved antiparallel β sheet containing three or four β strands (Fig. 1). The bound p-Tyr moiety lies in a conserved positively charged binding pocket on the SH2 domain surface, and it forms hydrogen bonds with several residues, including an invariant arginine in strand βB of the central β sheet. The surface of the SH2 domain that surrounds the p-Tyr-binding pocket contains variable residues that interact with sequences immedi-

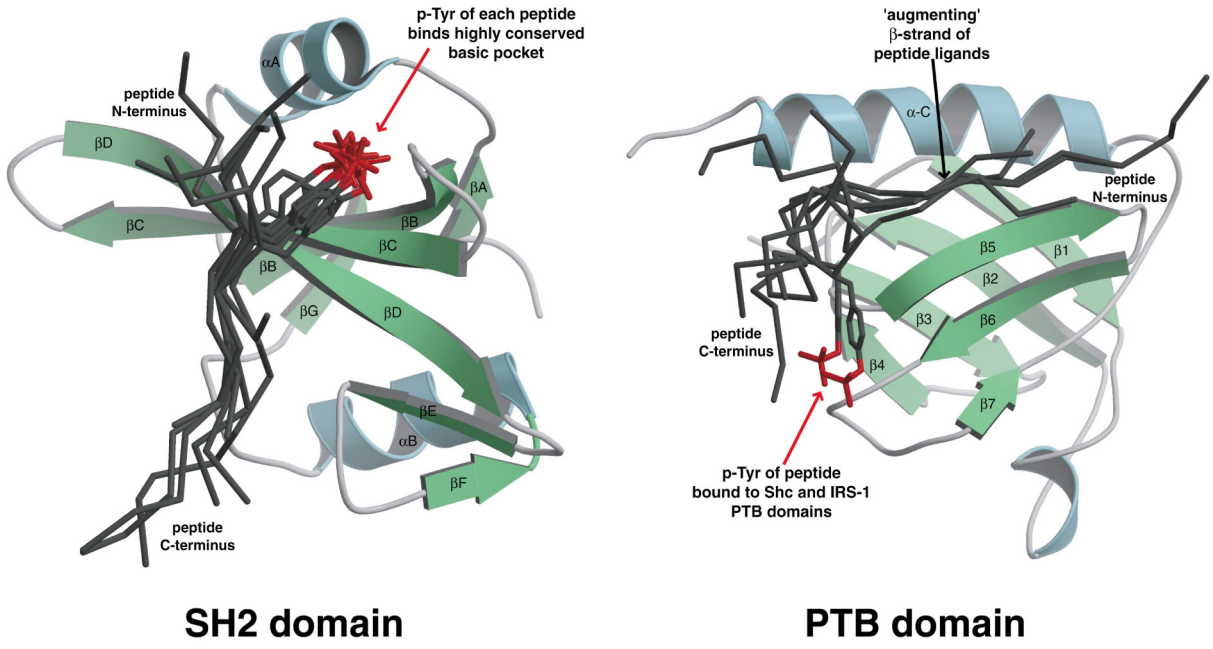

Fig. 1. Different modes of peptide recognition by SH2 and PTB domains. Ribbons representations are shown on the left for the Src SH2 domain [PDB code 1SPS (*116*)] and on the right for IRS-1 [PDB code 1IRS (*95*)]. Superimposed in each case are multiple different ligands, to show how they bind with respect to the SH2 or PTB domain fold. For SH2 domains, eight different SH2/phosphopeptide complex structures were overlaid in the program "O" by using the region from the beginning of strand βA to the end of αA as the point of reference. Peptide coordinates from each of the overlaid structures were superimposed on a representation of the Src SH2 domain. For PTB domains, the six available PTB domain/peptide complex structures were overlaid in O by using as the reference the region extending from the beginning of β7 to the middle of α-C. Peptide coordinates were superimposed on a representation of the IRS-1 PTB domain. The SH2 domain structures used were Src (*116*), Lck [PDB code 1LCJ (*117*)], C-terminal p85 SH2 domain [PDB code 1H9O (*118*)], C-terminal PLC-γ1 SH2 domain [PDB code 2PLD (*119*)], Fyn [PDB code 1AOT (*120*)], Grb2 [PDB code 1TZE (*121*)], SAP [PDB code 1D4W [*102*)], N-terminal SH2 domain from Syp [PDB code 1AYA (*122*)]. The PTB domain structures used were IRS-1 [PDB code 1IRS (*95*)], Shc [PDB code 1SHC (*83*)], X11 [PDB code 1X11 (*90*)], Numb [PDB code 1DDM (*91*)], talin [PDB code 1MIZ (*110*)], and radixin [PDB code 1J19 (*111*)].

ately C-terminal to the p-Tyr moiety (which bind in an extended conformation) and play a critical role in determining the binding specificity of SH2 domains for different phosphotyrosine-containing peptide ligands (*1*, *22*, *23*). For example, the Src SH2 domain binds preferentially to peptides with the sequence pYEEI (E, glutamic acid; I, isoleucine), largely because of a hydrophobic pocket on the Src SH2 domain surface into which the isoleucine side chain can project (*1*, *22*, *23*). By contrast, the surface of the C-terminal PLC-γ SH2 domain contains a groove that accommodates several hydrophobic residues that follow p-Tyr in its preferred peptide ligand. It should be noted that even the preferred p-Tyr-containing peptides bind to isolated SH2 domains with only modest affinities. The dissociation constants range from about 0.2 to 5 μM for a preferred SH2/p-Tyr peptide interaction, compared with a K_d greater than ~20 μM for binding of the same SH2 domain to a random peptide sequence containing p-Tyr (*24*, *29*, *30*).

Crystallographic studies of Cbl, a scaffold protein with an E3 ubiquitin ligase activity that is recruited by RTKs and promotes their ubiquitination and degradation [reviewed in (*31*)], showed that not all SH2 domains are immediately apparent from sequence analysis. Meng *et al*. (*32*) determined the structure of the conserved N-terminus of Cbl, bound to a phosphopeptide representing its binding site in the tyrosine kinase ZAP-70. Surprisingly, the phosphopeptide was found to bind in a canonical manner to an SH2 domain in Cbl that had gone undetected. Although the Cbl SH2 domain differs in several ways from other SH2 domains (lacking one small characteristic β sheet, for example), its phosphotyrosine-binding site is very well preserved. This finding hints that there may be additional so-far-unidentified SH2-like modules in other signaling molecules that could provide new clues to understanding RTK signaling specificity.

Families of Signaling Molecules That Contain SH2 Domains

SH2 domain-containing enzymes. Various enzymes contain one or two SH2 domains, in some cases alongside SH3, PH (pleckstrin homology), or other modules that direct interactions with additional proteins or with the cell membrane. A few examples of SH2 domain-containing enzymes, including protein tyrosine kinases, (for example, Src, Btk, and ZAP-70), protein tyrosine phosphatases (for example, Shp1 and Shp2), PLC-γ, and Ras-GAP are depicted in Fig. 2. Ligand-induced RTK autophosphorylation provides a switch for the recruitment and activation of SH2 domain-containing signaling proteins. For example, binding of the PLC-γ SH2 domains to their canonical sites in the autophosphorylated tails of EGFR or the fibroblast growth factor receptor (FGFR) facilitates translocation of PLC-γ to the plasma membrane, where its substrate PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) resides. Membrane association of PLC-γ is also aided by binding of its N-terminal PH domain to PtdIns(3,4,5)P_3 (phosphatidylinositol-3,4,5-trisphosphate) molecules that are simultaneously generated in response to growth factor stimulation of PI3Ks (*33*). After its tyrosine phosphorylation, activated PLC-γ (*34*) hydrolyzes

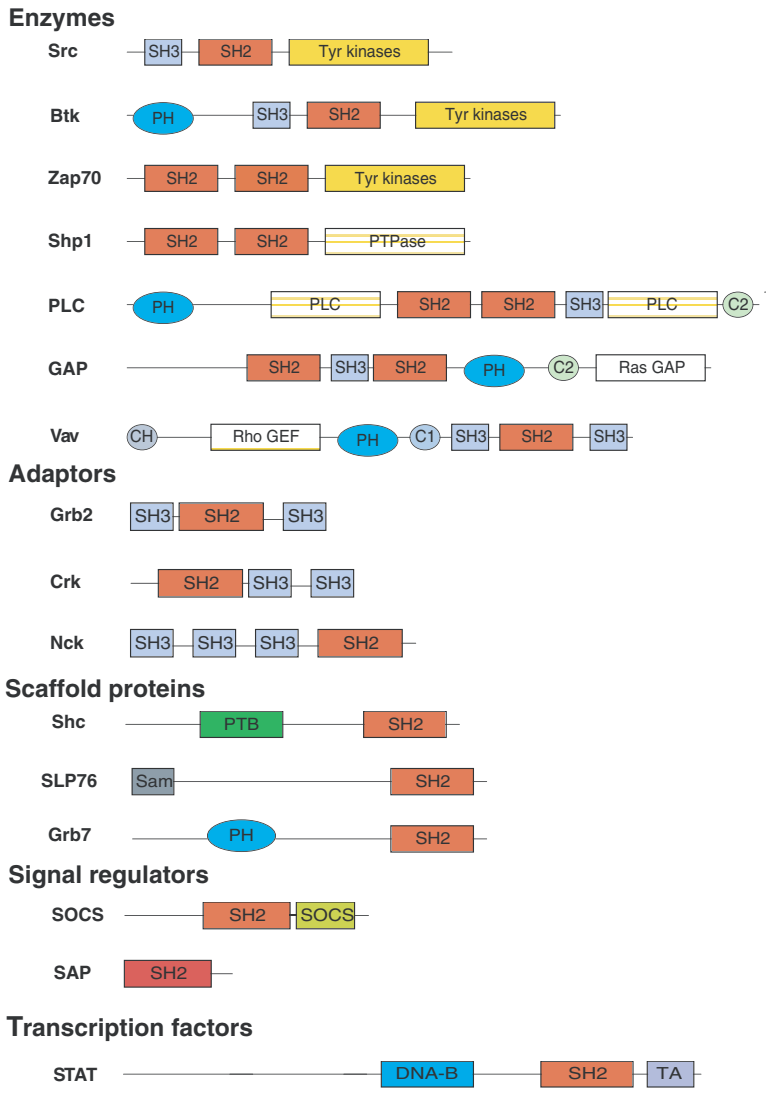

Fig. 2. Families of SH2 domains containing proteins. *Enzymes*. Examples include protein tyrosine kinases (Src, Btk, ZAP), protein tyrosine phosphatases (Shp1), phospholipase C-γ (PLC-γ), Ras-GAP (GAP) and Rho-GEF (Vav). Many enzymes contain one or two SH2 domains alongside SH3 (Src homology 3), PH (pleckstrin homology), C1 (phorbol ester binding region), C2 (Ca^{+2} and phospholipid binding region), and CH (calponin homology) domains. *Adaptors*. The adaptor proteins Grb2, Crk, and Nck are composed exclusively of SH2 and SH3 domains. *Scaffold proteins*. The scaffold proteins Shc, SLP76, and Grb7, contain SH2, PTB, PH, and Sam (Sterile alpha motif) domains. *Signal regulators*. While members of the SOCS family of signal regulators contain an SH2 and SOCS (suppressor of cytokine signaling) domains, SAP is composed of almost exclusively an SH2 domain. *Transcription factors*. Members of the STAT family of transcription factors contain in addition to an SH2 domain, a TA (transcriptional activation) domain, and a DNA-B (DNA binding) domain.

PtdIns(4,5)P_2 to generate the second messengers diacylglycerol and Ins(1,4,5)P_3 (inositol 1,4,5-trisphosphate). FGF-induced PLC-γ activation and second messenger production are strictly dependent on binding of PLC-γ SH2 domains to pY^{766} on the FGFR (*35*). Mutation of this amino acid prevents fibroblast growth factor (FGF)-induced association of PLC-γ with the receptor and its consequent tyrosine phosphorylation. Moreover, FGF-induced PtdIns(4,5)P_2 hydrolysis and intracellular Ca^{+2} release are impaired in cells expressing the FGFR $Y^{766}F$ point mutant (*35*).

SH2 domain-containing adaptor proteins. Another group of proteins contain only SH2 and SH3 domains and have no intrinsic enzymatic activities. Members of this family of so-called adaptor proteins (such as Grb2, Crk, and Nck) use their SH2 and SH3 domains for assembling and regulating the activities of signaling protein complexes in response to tyrosine kinases activated by extracellular cues (*1*, *22*, *23*, *26*). These adaptors utilize their SH2 domains for binding to tyrosine-phosphorylated proteins, and their SH3 domains for binding to proline-rich regions in downstream effector proteins. Grb2, for example, is composed of a single SH2 domain flanked only by two SH3 domains (*19*). The SH2 domain of Grb2 binds to pYXN motifs in activated EGFR and other receptors, in oncogenic tyrosine kinases such as Bcr-Abl, or in docking proteins such as FRS2 and IRS1 (*1*, *22*, *23*, *26*). The SH3 domains of Grb2 bind to a proline-rich region in SOS, a guanine nucleotide-exchange factor (GEF) for Ras. The SH3 domain-mediated Grb2-SOS complex is recruited (through the Grb2 SH2 domain) to tyrosine-phosphorylated receptors or docking proteins. The result is that SOS is brought close to its target Ras, which leads in turn to activation of the Ras/MAP (mitogen-activated protein) kinase signaling cascade, an evolutionarily conserved cellular signaling pathway for the control of cell proliferation and differentiation in response to various extracellular stimuli. The N-terminal SH3 domain of Grb2 is the primary site of SOS interaction and binds to a proline-rich sequence in SOS (*26*, *36-42*). The C-terminal SH3 domain of Grb2 can simultaneously bind to a proline-rich region in the docking protein Gab1 (Fig. 3), which is thus also recruited to activated RTKs by Grb2. Gab1 becomes tyrosine phosphorylated (*43-45*), thus generating additional binding sites (in Gab1) for the SH2 domains of the p85 regulatory subunit of PI3K (pYMXM motif). The p85 protein is then recruited to Gab1, leading to stimulation of PI3K and activation of the antiapoptotic PI3K/Akt-dependent cell survival pathway. Grb2 thus functions as a versatile adaptor protein that is capable of simultaneously recruiting several components that are essential for activation of both the Ras/MAPK and the PI3K/Akt signaling pathways in response to RTK stimulation at the cell membrane (*44*) (Fig. 3).

SH2 domains in regulatory subunits of signaling proteins. A third group of SH2 domain-containing proteins share some of the functions of adaptor proteins, but also act as regulatory subunits or allosteric regulators of a separate catalytic subunit. For example, the p85 regulatory subunit of PI3K contains two SH2 domains and one SH3 domain, in addition to a Rho GAP-like domain and a region that is responsible for its binding to the p110 subunit that has catalytic PI3K activity [reviewed in (*46*, *47*)]. The p85 regulatory subunit is constitutively associated with the p110 catalytic subunit. Binding of the p85 SH2 domains to tyrosine-phosphorylated receptors, or to tyrosine-phosphorylated membrane-linked docking proteins like IRS1 or Gab1, drives the translocation of the p85/p110 PI3K complex to the cell membrane where its substrate PtdIns(4,5)P_2 is located. Furthermore, p85 binding to tyrosine-phosphorylated proteins appears to relieve an autoinhibited configuration of PI3K, thus stimulating its catalytic activity [reviewed in (*46*, *47*)]. In addition to its function as a signal adaptor (recruiting PI3K to the membrane) and an allosteric regulator of PI3K activity, p85 may also function as a scaffold protein through association of its SH3 domain with proline-rich regions in other cellular proteins.

SH2 domains in scaffold proteins. Another variation on this theme is exemplified by SH2 domain-containing scaffold proteins (proteins that regulate the assembly of signaling proteins), such as Shc (*48*). Shc is tyrosine phosphorylated in response to various extracellular stimuli. Tyrosine phosphorylation occurs on two YXN sequence motifs, resulting in the recruitment of Grb2 (*49*) together with the downstream effector molecules

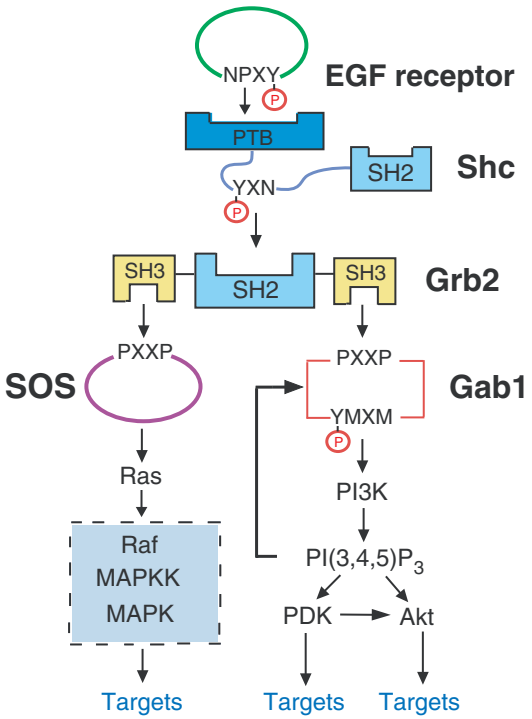

Fig. 3. An intracellular wiring diagram controlled by the action of SH2, PTB, and other cell-signaling protein modules. In response to activation of the EGFR at the cell membrane, the PTB domain of the scaffold protein Shc (Fig. 2) binds to an NPXpY motif in activated EGFR, resulting in tyrosine phosphorylation of Shc on at least two canonical binding sites (pYXN), for the SH2 domain of the adaptor protein Grb2 (Fig. 2). Grb2 is bound primarily via its N-SH3 domain to a proline-rich region in the guanine nucleotide-releasing factor SOS and via its C-SH3 domain to a proline-rich region in the docking protein Gab1. Grb2-mediated membrane recruitment of SOS results in activation of the Ras/MAP-kinase intracellular signaling pathway. Grb2-mediated recruitment of Gab1 leads to tyrosine phosphorylation of the docking protein on multiple sites, including the canonical binding site for the SH2 domains of the p85 regulatory subunit of PI3K (pYMXM). Recruitment of p85 results in stimulation of PI3K and activation of the antiapoptotic Akt-dependent signaling pathway. Binding of the PH domains of PDK and Akt to PtdIns(3,4,5)P_3 leads to membrane translocation, followed by stimulation of the protein kinase activities of PDK and Akt. In addition, PtdIns(3,4,5)P_3 binds to the PH domain of Gab1, which results in a positive-feedback mechanism mediated by membrane translocation of the docking protein.

constitutively bound to the Grb2 SH3 domains (i.e., SOS, Gab1). Shc contains both an SH2 domain and a PTB domain (*50*, *51*), which enables it to interact with a variety of tyrosine-phosphorylated receptors and signaling proteins (Fig. 3). Shc is thought to function as a scaffold protein that integrates and amplifies signals generated at the cell surface by tyrosine phosphorylation induced by a variety of extracellular cues.

SLP76 and its homolog BLAK are two members of a family of scaffold proteins that play prominent roles, reminiscent of that described for Shc, in the regulation of cellular signaling after engagement of the T cell receptor (TCR) and the B cell receptor, respectively (*52*). SLP76 contains an SH2 domain, a proline-rich region, and tyrosine phosphorylation sites that serve as binding sites for the SH2 domains of the guanine nucleotide-exchange factor Vav, the adaptor protein Nck, and the adhesion and degranulation-promoting adaptor protein (ADAP) [reviewed in (*52*)]. It has been shown that Gads (a hematopoietic Grb2 homolog) binds through its SH3 domains to a proline-rich region in SLP76. When the TCR is stimulated, the membrane-linked docking protein LAT becomes tyrosine phosphorylated and recruits the SH2 domain of Gads. A ternary LAT-Gads-SLP76 complex is thus assembled at the cell membrane (*52*). Similarly, PLC-γ1 becomes associated with phosphorylated LAT through its SH2 domains and interacts via its SH3 domain with a proline-rich region in SLP76. The resulting LAT-PLCγ1-SLP76 membrane-linked complex, which is nucleated by tyrosine phosphorylation of LAT, is necessary for TCR-mediated activation of PLC-γ, intracellular Ca^{+2} release, and stimulation of MAP kinase (*52*, *53*). The scaffold protein SLP76 thus plays a critical role in the assembly and regulation of multiple signaling pathways critical for signaling in T cells. Indeed, T cell development is impaired in mice deficient in either SLP76 or LAT, which underscores the importance of both scaffold and docking proteins in this case (*54*-*56*).

SH2 domains in intramolecular autoinhibitory interactions. In addition to their role in assembling activated complexes of signaling proteins, SH2 domains control the enzymatic activity of certain proteins that contain them by mediating autoinhibitory intramolecular interactions. For example, the cytoplasmic protein tyrosine kinase Src is maintained in an inactive autoinhibited configuration by intramolecular interactions mediated by its SH2 and SH3 domains [reviewed in (*57*)]. The Src SH2 domain is bound to p-Tyr^{527} in the C-terminal tail of the same molecule. In addition, the Src SH3 domain binds intramolecularly to a PXXP motif in the linker region between the SH2 domain and the kinase domain (*58*, *59*). These intramolecular interactions lock the tyrosine kinase domain of Src into an inactive configuration. This autoinhibition of Src can be relieved by several mechanisms that break the intramolecular SH2 domain/p-Tyr^{527} or the SH3 domain/PXXP motif interactions. For example, binding of other phosphotyrosine-containing sequences to the Src SH2 domain can compete with the intramolecular autoinhibitory interaction. Alternatively, dephosphorylation of p-Tyr^{527} by tyrosine phosphatases or oncogenic mutation of the Tyr^{527} autoinhibitory phosphorylation site prevents the intramolecular interaction and activates the kinase. The active extended configuration of Src can also be promoted by the binding of proline-rich sequences to its SH3 domain.

A distinctly different example in which intramolecular SH2 domain interactions maintain an enzyme in an autoinhibited configuration is seen for the N-terminal SH2 domain (N-SH2) of the cytoplasmic protein tyrosine phosphatase (PTPase), Shp2 (*60*). A loop from the N-SH2 domain binds intramolecularly to the catalytic cleft of Shp2 and prevents access of substrates to the PTPase domain (*60*). In this case, binding of specific phosphotyrosine-containing sequences to the N-SH2 domain disrupts its interaction with the phosphatase domain and thus relieves the autoinhibited configuration, which results in enhancement of PTPase activity. Once in the open or active configuration, the two tandem SH2 domains of Shp2 mediate complex formation with tyrosine-phosphorylated receptors (PDGFR) or docking proteins (FRS2, Gab1).

SH2 domains in transcription factor dimerization. STATs (signal transducers and activators of transcription) represent a family of latent transcription factors that become tyrosine phosphorylated at the cell membrane in response to various cytokines or growth factors [reviewed in (*61*)]. All STAT proteins contain an SH2 domain, a tyrosine phosphorylation site, and DNA binding and transcriptional activation domains. Stimulation of cytokine receptors at the cell membrane leads to receptor dimerization and activation of members of JAK (Janus kinase) family of tyrosine kinases, which results in tyrosine phosphorylation of STATs. Dimerization of phosphorylated STATs is mediated by their SH2 domains, through mutual intermolecular interactions that involve a single tyrosine-phosphorylation site on each STAT molecule. Dimerization triggers STAT translocation into the nucleus, where it participates in the regulation of gene expression (*61*).

SH2 domain-containing proteins that function as competitive signal attenuators. The eight known members of the SOCS (suppressor of cytokine signaling) family are small proteins composed of an SH2 and a SOCS homology domain [reviewed in (*62*)]. Members of the SOCS family participate in a negative-feedback loop for the control of intracellular signaling pathways activated by cytokines. SOCS negatively regulates cytokine signaling by binding to and inhibiting the tyrosine kinase activity of JAK family tyrosine kinases. SOCS proteins are also proposed to exert negative effects by competing with STATs for binding to cytokine receptors. Furthermore, SOCS proteins use the BC box in the SOCS homology domain for recruitment of a multiprotein complex with E3 ubiquitin ligase activity, resulting in ubiquitination and degradation of JAKs by the proteasome (*62*).

SOCS family members also attenuate the intracellular signaling pathways activated by RTKs [reviewed in (*63*)]. Attenuation of the intracellular signal generated in response to insulin or insulin-like growth factor 1 (IGF1) stimulation is mediated in part by SOCS-dependent ubiquitination and degradation of IRS1 and IRS2, docking proteins that function as important mediators of signaling by insulin or IGF1 receptors. This finding is consistent with the enhanced insulin responsiveness observed in SOCS1-deficient mice, and the sustained insulin-induced tyrosine phosphorylation of IRS1 detected in fibroblasts isolated from $SOCS1^{-/-}$ embryos. SOCS family members represent a class of proteins that attenuate the signals generated at the cell membrane in response to cytokines, growth factors, and hormones (*63*, *64*).

Another example of a negative regulator of RTK signaling that contains an SH2 domain is the scaffold protein Cbl [reviewed in (*31*, *64*, *65*)]. Cbl is recruited to RTKs through phosphotyrosine binding by its once-cryptic SH2 domain. Cbl appears to play multiple roles in RTK signaling (*66*). Some of its adaptor or scaffold functions potentiate RTK signaling. However, Cbl also appears to have E3 ubiquitin ligase activity, and Cbl recruitment to RTKs can

lead to their ubiquitination and targeting for degradation by the proteasome and/or in lysosomes (after sorting into the lumen of multivesicular bodies). It should be noted, however, that the effect of Cbl on ubiquitination and degradation of EGFR can only be detected in cells that ectopically overexpress Cbl. Moreover, EGFR internalization and degradation are not detectably altered in fibroblasts isolated from Cbl-deficient embryos (*66*).

Proteins with tandem SH2 domains. Proteins such as the tyrosine kinases ZAP-70 and Syk, as well as the protein tyrosine phosphatases Shp1 and Shp2, contain two tandem SH2 domains followed by either a tyrosine kinase or a tyrosine phosphatase domain. In response to engagement of the TCR and stimulation of tyrosine phosphorylation, ZAP-70 binds via its tandem SH2 domains to two phosphotyrosine motifs in the cytoplasmic domains of the ε and ξ chains of the TCR complex, designated TAMs (tyrosine-based activation motifs), which contain two phosphotyrosines with a defined spacing. A protein containing the tandem SH2 domains of ZAP-70 binds to the pair of phosphorylated tyrosines in a TAM motif 500 to 1000 times as tightly as an individual SH2 domain binds to either single p-Tyr-containing peptide [reviewed in (*23*)]. The enhanced binding affinity of the tandem SH2 domains results from an "avidity-effect" (increased binding affinity of a bivalent "ligand" to a bivalent "receptor") that arises when two covalently linked SH2 domains bind simultaneously to the two phosphotyrosines (also covalently linked) in the bivalent TAM motif (*67*-*72*). This is illustrated in Fig. 4, which shows the structure of the tandem Syk SH2 domains bound to a dually phosphorylated peptide corresponding to its TAM-binding site in CD3ε (*72*).

Interestingly, the proteins for which it has been most straightforward to "map" the phosphotyrosine-binding sites responsible for SH2 domain-mediated interactions (using site-directed mutagenesis or peptide inhibition experiments) are those that contain tandem or multiple SH2 domains. These include the p85 subunit of PI3K, PLC-γ1, ZAP-70, Syk, Shp1, and Shp2. It may well be that these examples, for which phosphotyrosine-mediated binding can have a very high affinity because of the avidity effect mentioned above, are the only cases in which SH2 domains are entirely responsible for recruitment to activated RTKs or docking proteins. The structural complementarity of the two phosphotyrosines in the target and the two SH2 domains in the recruited protein also allows for a greater degree of specificity in such interactions (*71*, *72*). The combination of higher specificity and higher affinity may explain why these interactions are more effectively abolished by a single Y to F mutation than examples in which a single SH2 domain is involved. In those cases that do not involve tandem SH2 domains, greater promiscuity in phosphotyrosine binding (or decreased reliance on phosphotyrosine binding) may result in reduced sensitivity to the mutation of an individual tyrosine-phosphorylation site.

PTB Domains

The first phosphotyrosine binding (PTB) domain was identified in the scaffold protein Shc. Shc was found among numerous SH2 domain-containing clones when bacterial expression libraries were screened with a tyrosine-phosphorylated form of the EGFR intracellular domain. Although it seems reasonable to expect that Shc should have been identified in this screen, because it has an SH2 domain at its C-terminus, N-terminal fragments of Shc (which lack the SH2 domain) were shown also to bind efficiently to the tyrosine-phosphorylated EGF receptor (*50*, *51*). Similar observations were made in biochemical studies of Shc binding to tyrosine-phosphorylated proteins and in yeast two-hybrid studies of Shc binding to the phosphorylated insulin receptor intracellular domain (*72*-*74*). Refinement of these observations led to the identification of a domain of about 200 amino acids (the PTB domain) from the Shc N-terminus that binds to tyrosine-phosphorylated proteins.

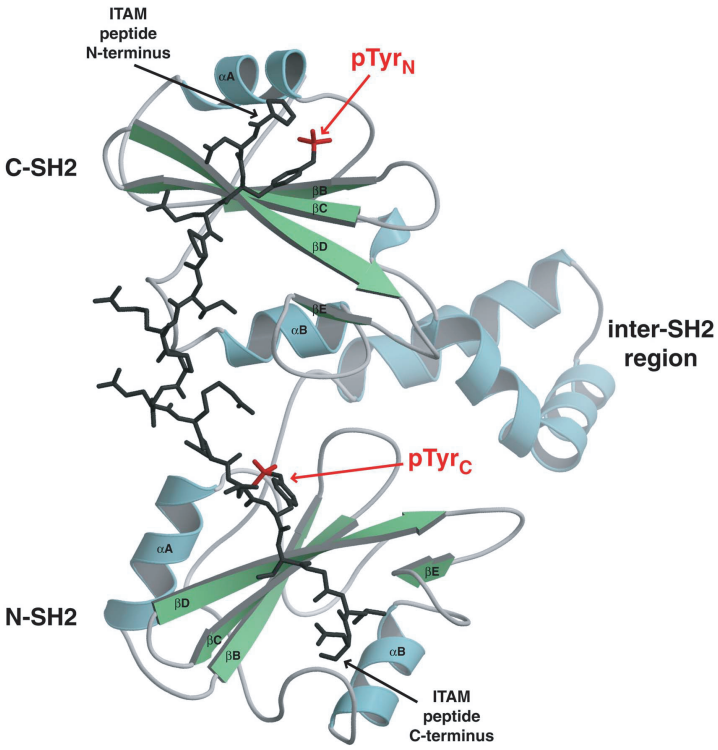

Fig. 4. Binding of tandem Syk SH2 domains to a dually phosphorylated TAM. The tandem SH2 domains from Syk are shown in ribbons representation, with N-SH2 and C-SH2 marked. The CD3ε TAM peptide runs from top (N-terminus) to bottom (C-terminus), with both phosphotyrosines projecting into equivalent basic pockets close to the C-terminal end of βB in each case (see also Fig. 1). This figure illustrates how the spacing between the two phosphotyrosines in the TAM will be critical for simultaneous engagement of both phosphotyrosines with the two SH2 domains in a tandem pair. Another Syk target, FcγRII, has more residues separating the two phosphotyrosines in its TAM. Flexibility in the relationship between the SH2 domains shown in this structure is believed to allow recognition by the tandem Syk SH2 domains of a subset of TAM ligands (*71*). PDB code 1A81 (*71*) was used to generate this figure.

Shc binds to an unusual phosphotyrosine-containing sequence in activated RTKs and other proteins, with the consensus NPXpY (*75*-*77*). This motif differs from that recognized by SH2 domains, in that the specificity-defining residues occur to the N-terminal side, rather than to the C-terminal side, of the

phosphotyrosine. After identification of the Shc PTB domain, a number of studies showed that this domain specifically recognizes the NPXpY sequence in both proteins and peptides (*78-80*). Together with the finding that the SH2 domain is dispensable for Shc binding to phosphoproteins, this indicated that the PTB domain plays a primary role in recruiting Shc to its tyrosine-phosphorylated targets. These studies also demonstrated that the PTB domain resembles the SH2 domain in binding phosphorylated tyrosine, but recognizes quite different features of the p-Tyr sequence and structural contexts.

Around the same time that the Shc PTB domain was discovered, a domain with similar properties (but with little sequence similarity) was recognized in the insulin receptor substrates IRS-1 and IRS-2 (*74*, *81*). It was already well appreciated that an NPXpY motif in the insulin receptor (including phosphorylated Y960) is critical for coupling the activated receptor to the IRS-1 docking protein (*82*). The apparent (but limited) sequence relationship to Shc-PTB of the IRS-1 region responsible for this binding, together with biochemical and yeast two-hybrid demonstrations of phosphotyrosine recognition (*74*, *81*), suggested that IRS-1 has a PTB domain resembling that seen in Shc. Subsequent structural studies (summarized below) showed that there is indeed a remarkable structural resemblance between the Shc and IRS-1 PTB domains (*83*, *84*), reminiscent of the close similarity between the Cbl SH2 domain and others to which it shows little sequence similarity (*85*).

PTB domains, related to that of Shc (*86*), exist in several other proteins such as Disabled (a tyrosine-phosphorylated adaptor protein in *Drosophila*) and Numb. The SMART database (*87*) now includes two PTB domain designations (*88*). One is the group of Shc-like PTB domains (about 25 to 30 domains in humans), which includes those from Shc, Disabled, and Numb (and their close relatives), as well as Jip1 (a scaffolding protein for the Jun N-terminal kinase); X11 (a protein involved in intracellular trafficking); FE65 (amyloid precursor protein-binding protein); tensin; Eps8 (EGFR pathway substrate 8); RGS12 (a regulator of G-protein signaling); a GTPase-activating protein for Rab6; and ICAP-1 (integrin cytoplasmic domain-associated protein-1). The second group is that of IRS-1-like PTB domains (approximately 10 of them in humans), which includes those from IRS-1, IRS-2, the $p62^{Dok}$ docking protein, and FRS2 (fibroblast growth factor receptor substrate-2).

PTB domain structure. Nuclear magnetic resonance (NMR) and/or crystal structures have been described for six different PTB domains bound to cognate peptides: four from the Shc PTB domain family (*83, 89-91*) and two from the IRS-1 PTB domain family (*84*, *92*). Regardless of whether they are Shc-like or resemble that from IRS-1, all of the PTB domains share the same β-sandwich structure (Fig. 1), termed the pleckstrin homology (PH) domain "superfold" (*93*), which is seen in PH domains (some of which bind phosphoinositides), as well as Enabled/VASP homology-1 (EVH-1) domains, Ran-binding domains, and in other proteins [reviewed in (*94*)]. In the core of this fold, two nearly orthogonal β sheets (of four- and three-strands, respectively) form a β-sandwich that is capped off at one closed corner by a characteristic C-terminal α helix. The first two PTB domain structures, those of the Shc and IRS-1 PTB domains in complex with cognate phosphopeptides, showed that the bound NPXpY-containing peptide (from a TrkA sequence that binds Shc and from the insulin or interleukin 4 (IL-4) receptor that binds IRS-1 (*83*, *92*, *95*) has the type-1 β-turn structure that was expected (*79*, *96*). The region of the peptide N-terminal to the phosphotyrosine (the residues preceding proline in the NPXpY motif) adopts an extended conformation and forms hydrogen bonds with strand β5 of the PH-fold core (Fig. 1). The peptide effectively adds a fourth strand to the three-stranded antiparallel sheet (β5 through β7) of the core β sandwich, filling a "cleft" between strand β5 and the C-terminal α helix. This mode of binding has been termed antiparallel β-sheet augmentation (*97*). The asparagine of the NPXpY motif appears to form critical side-chain hydrogen bonds both within the peptide and between the peptide and the PTB domain. The NPX motif positions the subsequent phosphotyrosine so that it can make hydrogen bonds to basic side chains on the surface of the PTB domain. The side chains involved in p-Tyr binding are not conserved in the sequences of the Shc and IRS-1 PTB domains, although they occur in approximately similar places in the three-dimensional structures of the two domains (*98*). Thus, the mode of phosphate group recognition appears to be significantly less well conserved between different PTB domains than between different SH2 domains (which all share the "FLVR" sequence). Instead, it can be argued that the general mode of peptide recognition is similar in the Shc and IRS-1 PTB domains, but that its phosphorylation-dependence arises from quite different sets of interactions.

Binding of most PTB domains is independent of phosphotyrosine. Consistent with the idea that the mode of peptide binding is better conserved between PTB domains than are modes of phosphate group ligation, it now appears that phosphotyrosine binding is actually a property of only a minority of so-called PTB domains (*98*, *99*). Structural and biochemical studies of the PTB domains from X11 (*90*), Numb (*91*), FRS2 (*84*), and Disabled-1 (*89*) have all shown that nonphosphorylated peptides can bind to these domains with high affinity in a mode that closely resembles peptide binding to the IRS-1 and Shc PTB domains (Fig. 1). In each case, much of the peptide lies between strand β5 and the C-terminal α helix, and extends (or augments) the β5-β7 antiparallel sheet of the PH β-sandwich core.

Intriguingly, the PTB domains from FRS2 (*100*) and Numb (*91*) both bind with relatively high affinity to quite distinct phosphorylated and nonphosphorylated target sequences. The FRS2 PTB domain binds strongly to the same (NPXpY) site in nerve growth factor (NGF)-receptor ($pTyr^{490}$) that is bound by Shc, and this interaction is phosphorylation-dependent. However, FRS2 PTB also recognizes a very different site in the juxtamembrane region of FGFR, which has a sequence [AVHKLAKSIPLRRQVTVS (*101*)] that contains no tyrosines. An NMR structure of the complex between this FGFR peptide and the FRS2 PTB domain shows that binding occurs again through β augmentation. However, additional distinct interactions are made, with the peptide "wrapping" around the β sandwich (*84*). These interactions presumably play a role in enhancing binding affinity and take the place of hydrogen bonds to p-Tyr that occur with the Shc and IRS-1 PTB domains. Along very similar lines, the Numb PTB domain binds strongly to a nonphosphorylated GFSNMSFEDP sequence from the Numb-associated kinase (Nak). The β-augmentation mode of binding is seen once again, but the interaction surface appears to extend beyond the central NMSF sequence (*91*), which presumably adds to the interaction strength, just as p-Tyr interactions do in the case of Shc and IRS-1.

The X11 PTB domain provides perhaps the best illustration of how an alternative feature of a peptide ligand can "replace"

phosphotyrosine in promoting peptide-PTB interactions (*90*). The X11 PTB domain binds to a region from the cytoplasmic tail of the amyloid β-protein precursor protein (βAPP), recognizing a sequence that includes the motif NPTY. Studies of peptide-binding affinities demonstrated that phosphorylation of the tyrosine in this motif has little or no effect on binding. A crystal structure of a nonphosphorylated NPTY-containing peptide bound to X11 PTB showed that is bound through β-sheet augmentation. However, a region of the bound peptide that is C-terminal to the NPTY motif (and contains residues important for binding) forms a portion of 3_{10} helix that contacts a unique hydrophobic surface on the X11 PTB domain. This portion of 3_{10} helix may have a role in peptide binding to the X11 PTB domain that is analogous to the role of p-Tyr in peptide binding to the Shc PTB domain.

PTB domains versus SH2 domains. Structural studies of PTB domains have revealed a picture of ligand recognition that is much more diverse than that obtained for SH2 domains. The binding of SH2 domains to phosphopeptides is determined primarily by the presence of phosphotyrosine and only secondarily by the surrounding amino acid sequence. Indeed, SH2 domains bind significantly to free phosphotyrosine and phenylphosphate. By contrast, PTB domains appear primarily to recognize the propensity of their ligand to form a β-strand antiparallel to β5 in the cleft between strand β5 and the C-terminal α helix of the PH/PTB core β-sandwich. PTB domains recognize other features, such as the presence of a phosphorylated tyrosine, as a second priority. Thus, phosphotyrosine is only relevant for a subset of PTB domains, whereas it is central to the function of all (or nearly all) SH2 domains. This distinction is illustrated in Fig. 1, where eight different phosphopeptides bound to their SH2 domain targets are seen to be most similar in the position of the phosphotyrosine when complexes are overlaid. By contrast, six different PTB domain ligands are found to be most similar in the strand that lies between β5 and α-C when complexes are overlaid. The C-terminal part of PTB binding peptides, including the region that contains p-Tyr (where relevant), is much more diverse in conformation.

This general division of the characteristics of SH2 and PTB domains does become blurred slightly by the example of the SH2 domain of SAP [SLAM (signaling lymphocyte activation molecule)-associated protein], a protein that is altered in X-linked lymphoproliferative syndrome (XLP). The SAP SH2 domain, which constitutes the entire protein, is highly unusual in being able to bind tyrosine-containing peptides that have not been phosphorylated. Structural studies (*102*, *103*) have shown that this SH2 domain is unusual in making significant interactions with sequences N-terminal to the tyrosine in its binding site, in addition to the C-terminal interactions seen in all other SH2/peptide interactions. Tyrosine phosphorylation of the SAP binding site does increase its affinity for the SH2 domain (*103*), but only by a factor of about 5. Whereas most SH2 domains are completely reliant on phosphate interactions for binding to their ligands, the SAP SH2 domain can substitute interactions with N-terminal parts of the peptide. In this sense, the SAP SH2 domain appears to be much more PTB domain-like than any other SH2 domain that has been extensively studied to date.

PTB domains compared with other domains with PH domain folds. The fact that PTB domains contain the same fold as PH domains, some of which bind and recognize phosphoinositides (*94*), prompted the suggestion that PTB domains may also share this property (*83*). The Shc and IRS-1 PTB domains are both electrostatically polarized, like PH domains, and have both been reported to bind phosphoinositides (albeit weakly), likely through their positively charged surface (*83*, *104*, *105*). It is possible that a combination of protein binding and phospholipid interactions is involved in membrane recruitment of PTB domains, although some studies indicate that these two ligands compete for overlapping binding sites.

A recent crystal structure of the Disabled-1 (Dab1) PTB domain has provided the first view of a phosphoinositide headgroup bound to a PTB domain (*89*). The Dab1 PTB domain binds to internalization signals in the cytoplasmic tails of low density lipoprotein (LDL) receptors, but also interacts with PtdIns(4,5)P_2 (*106*). Dab1 PTB binds NPXY motifs in the cytoplasmic tails of its targets, and the affinity of this binding is reduced if the tyrosine is phosphorylated (*106*). Crystals of a Dab1 PTB complex with a peptide were soaked with the PtdIns(4,5)P_2 headgroup, which was then found to bind in a basic "patch" highly reminiscent of the location of the inositol phosphate ligand bound to PH domains (*94*). In Dab1 PTB (by contrast with reports for Shc PTB), peptide binding and phosphoinositide binding can occur simultaneously and so could cooperate in driving the Dab1 PTB domain to the plasma membrane (*89*). A similar cooperation between weak phosphoinositide binding and weak peptide-protein binding has also been suggested for PH domains (*94*), and good evidence for such a collaboration of multiple types of ligand has been provided in the case of the β-adrenergic receptor kinase (βARK) PH domain (*107*). It might therefore be argued that the Dab1 PTB domain represents one of the most PH domain-like PTB domains. Similarly, the βARK PH domain could represent one of the most PTB domain-like PH domains.

Other modules that exhibit the PH domain fold also share PTB domain-like characteristics despite an absence of obvious sequence similarity. The FERM domain (for four point one, ezrin, radixin, moesin) contains a PH or PTB-like subdomain as one of its three modules (the F3 subdomain) (*108*). The FERM domain of talin binds to the short cytoplasmic tails of integrin β subunits, thus linking integrins to the cytoskeleton. The PH- or PTB-like subdomain appears to be able to bind to, and activate, integrins (*109*), which contain an NPXY motif in their cytoplasmic tails. A crystallographic study (*110*) showed that a sequence derived from the integrin-β3 tail interacts with the PH- or PTB-like subdomain of the talin FERM domain in a manner highly reminiscent of peptide binding to the X11 PTB domain. A similar mode of binding was observed in a crystal structure of the radixin FERM domain bound to a peptide with a sequence from the ICAM-2 cytoplasmic tail (*111*). This peptide, which contained the motif RXXTYXXVXXA, binds in the β-sheet augmentation mode seen for all ligand binding to all bona fide PTB domains, although it differs significantly in conformation at the end of the augmenting strand (where the NPXY motif is present in other PTB domain ligands and a VLAA sequence is present in this case). The PH- or PTB-like subdomain in these FERM domains thus closely resembles PTB domains in the way in which peptide ligands are recognized. Binding of FERM domains to these ligands may be regulated by phosphoinositides. Phosphoinositide binding to the FERM domain has been proposed to reverse an autoinhibitory head-to-tail interaction in the FERM protein that masks the PTB-like peptide binding site observed crystallographically in the F3 subdomain (*110*, *111*). This mode of allosteric regulation of FERM domains could represent an alternative (to phosphorylation) for control of interactions mediated by their PTB-like subdomains.

Functions of PTB domains in RTK signaling. Just as they are

more diverse in sequence than SH2 domains, PTB domains will also be more diverse in function. For the PTB domains of Shc, IRS-1, and FRS2, phosphotyrosine-dependent interaction with a sequence in the juxtamembrane region of an activated RTK provides a clear and strong parallel with the functions discussed earlier for SH2 domains. Such phosphotyrosine-dependent recruitment to an activated receptor allows Shc to become tyrosine phosphorylated, and thus to recruit (through SH2 domain-mediated interactions) Grb2, SOS, and Gab1 into a signaling complex, as discussed above (see Fig. 3). The PTB domain-containing docking proteins (IRS1, IRS2, and FRS2) all contain a membrane-association domain at their N-terminus (a PH domain in IRS proteins, a myristoylation site in FRS2), which may be followed by a PTB domain. The PTB domain specifies the interaction of the docking protein with activated receptor at the membrane (insulin receptor or IL-4R for the IRS proteins; NGFR or FGFR for FRS2). The large C-terminal region of the docking protein then becomes multiply phosphorylated, and serves as a "platform" for the recruitment of multiple downstream signaling molecules. PTB domain-containing docking proteins can be viewed as providing a "surrogate receptor-tail" for the insulin, FGF, IL-4, and other receptors. It is also possible that PTB domains play a unique role in signaling by being able to couple to several different receptors that may be activated in different ways. The multiple different modes of ligand binding to the FRS2 PTB domain (both p-Tyr dependent and independent) provides a mechanistic illustration of this possibility. It is intriguing to note that PTB domain-containing docking proteins can augment receptor signaling under some conditions, as outlined for IRS proteins and FRS2, yet play a role in signal attenuation under other conditions. Indeed p-Tyr-dependent (PTB domain-mediated) recruitment of the docking protein $p56^{Dok2}$ (but not $p62^{Dok1}$) reduces EGF-stimulated MAP kinase activation (*112*). The PTB domains of Dok2 and Shc, proteins that appear to have opposing effects on MAP kinase activation, compete for the same p-Tyr-containing binding site on the EGF receptor. This may provide a mechanism for limiting MAP kinase activation in cells under certain states of stimulation. Studies of FRS2 have also identified a unique pathway for negative feedback of receptor signaling (*113*). FRS2 is phosphorylated by MAP kinase in a manner that appears to reduce its tyrosine phosphorylation, and thus limits the extent of signaling through receptors that utilize this docking protein.

Interactions mediated by the PTB domain of RGS12 (regulator of G protein signaling-12) may also have a key role in controlling inhibition of the N-type calcium channel that occurs when the $GABA_B$ receptor is activated in sensory neurons (*114*). The N-type calcium channel was found to become tyrosine phosphorylated by a Src-like kinase after stimulation of neurons with GABA. RGS12 then associates with the channel in a PTB- and p-Tyr-dependent manner, with significant effects on the rate of desensitization of the channel.

Given the focus of this review on signaling pathways that involve tyrosine phosphorylation, we will only consider very briefly the functions of PTB domain interactions that are not known to be regulated by this modification. The reader is directed to recent reviews (*88*, *98*, *99*) for discussion of what is understood concerning other interactions, beyond what was outlined above. However, one set of phosphotyrosine-independent PTB domain interactions that are important in cellular signaling are those involving the cytoplasmic domains of β integrins (*115*). The PTB domains from Numb (a negative regulator of Notch signaling), Dab1, Dab2, EPS8 (a regulator of Rac signaling), Tensin, Talin, and the docking protein Dok1, all bind to NPXY or NPXY-like motifs (nonphosphorylated) in the cytoplasmic tails of β integrins, and do so with detectably different (but significantly overlapping) specificities (*115*). Given that phosphorylation has been shown either to promote or to inhibit NPXY peptide binding to PTB domains (depending on the particular PTB domain), it was proposed that phosphorylation of β-integrin cytoplasmic tails could function as a "switch" to control which PTB domain-containing proteins bind to which integrins in the cell under different conditions of stimulation (*115*). Thus, although these PTB domain interactions are not "phosphotyrosine-dependent" in the normal sense, there are hints that these PTB domain (and FERM domain) interactions may nonetheless be regulated by tyrosine phosphorylation. Determination of whether this intriguing hypothesis is correct awaits further experimentation.

Multidomain Cooperation

Most proteins with SH2 or PTB domains also contain one or several additional protein modules that direct either protein-protein, or protein-phospholipid interactions. This multi-domain organization of signaling proteins is likely to influence their binding and interactions with proteins of the cell membrane and elsewhere. Membrane recruitment will be facilitated by the simultaneous binding of multiple, different domains to their distinct targets at the cell membrane (Fig. 5). Ras-GAP, for example, contains two SH2 domains that bind to activated RTKs, as well as a PH domain and C2 domain, both of which interact with the membrane surface. Similarly, the tyrosine kinase Btk contains PH, SH3, and SH2 domains in its N-terminus. Simultaneous binding of the PH domain to $PtdIns(3,4,5)P_3$ and the SH2 domain to p-Tyr sites in membrane receptors should result in stronger binding of Btk to the cell membrane. Similar considerations also apply for membrane association of the multidomain signaling proteins Shc, SLP76, Vav, PLC-γ, and PI3K; scaffold proteins and enzymes whose targets are located at the cell membrane. Thus, the cooperation of multiple binding events at the cell membrane, involving multiple domains, will increase the efficiency of enzymatic reactions that take place at the interface of the cell membrane with the cytoplasm (Fig. 5).

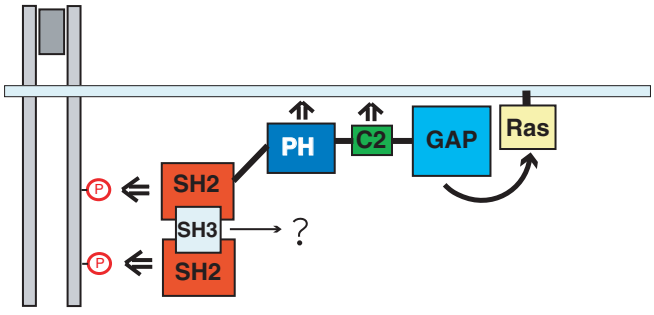

Fig. 5. Multidomain cooperation. Efficient targeting of Ras-GAP to the cell membrane is mediated by interactions between its two SH2 domains, PH domain, C2 domain, and perhaps also SH3 domain with tyrosine-phosphorylated receptors and other membrane components. The cooperation of multiple binding events at the cell membrane will position the GAP domain of Ras-GAP in proximity to Ras, which results in stimulation of GTPase activity.

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