

Evidence for a Requirement for Both Phospholipid and Phosphotyrosine Binding via the Shc Phosphotyrosine-Binding Domain In Vivo

KODIMANGALAM S. RAVICHANDRAN,^{1*} MING-MING ZHOU,² JOANNE C. PRATT,³
JOHN E. HARLAN,² SCOTT F. WALK,¹ STEPHEN W. FESIK,² AND STEVEN J. BURAKOFF³

Beirne Carter Center for Immunology Research and Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908¹; Pharmaceutical Research Division, Abbott Laboratories, Abbott Park, Illinois 60064²; and Division of Pediatric Oncology, Dana-Farber Cancer Institute, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115³

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The adapter protein Shc is a critical component of mitogenic signaling pathways initiated by a number of receptors. Shc can directly bind to several tyrosine-phosphorylated receptors through its phosphotyrosine-binding (PTB) domain, and a role for the PTB domain in phosphotyrosine-mediated signaling has been well documented. The structure of the Shc PTB domain demonstrated a striking homology to the structures of pleckstrin homology domains, which suggested acidic phospholipids as a second ligand for the Shc PTB domain. Here we demonstrate that Shc binding via its PTB domain to acidic phospholipids is as critical as binding to phosphotyrosine for leading to Shc phosphorylation. Through structure-based, targeted mutagenesis of the Shc PTB domain, we first identified the residues within the PTB domain critical for phospholipid binding in vitro. In vivo, the PTB domain was essential for localization of Shc to the membrane, as mutant Shc proteins that failed to interact with phospholipids in vitro also failed to localize to the membrane. We also observed that PTB domain-dependent targeting to the membrane preceded the PTB domain's interaction with the tyrosine-phosphorylated receptor and that both events were essential for tyrosine phosphorylation of Shc following receptor activation. Thus, Shc, through its interaction with two different ligands, is able to accomplish both membrane localization and binding to the activated receptor via a single PTB domain.

The adapter protein Shc is a critical regulator of downstream signaling events that lead to such diverse biological processes as neuronal differentiation (20, 34, 35, 55), lymphocyte proliferation (31), cell survival mediated by cytokines and integrins (11, 64), and cellular transformation via polyoma virus middle T antigen (3, 7). Shc mediates these effects, at least in part, through the activation of Ras proteins by a variety of receptors, including growth factor receptors (38, 40, 46, 47, 53), antigen receptors on B and T cells (44, 51), cytokine receptors (5, 39, 42), and G protein-coupled receptors (29, 59). Recently, a role for Shc in leading to Myc activation and prevention of apoptosis during interleukin-3 (IL-3) signaling has also been reported (13, 14).

The p52 isoform of Shc is composed of an N-terminal phosphotyrosine-binding (PTB) domain, a central collagen homology (CH) region (CH1), and a C-terminal SH2 domain but no apparent catalytic domain (2, 23, 38, 63). Following activation of many receptors, Shc is tyrosine phosphorylated (within the CH domain) and subsequently interacts with Grb2 (47, 48, 61). Grb2, in turn, binds to a Ras guanine nucleotide exchange factor, mSos (36). The Shc-Grb2-mSos complex becomes localized to the membrane through the interaction of Shc with the activated, tyrosine-phosphorylated receptor, thereby leading to Ras activation (32, 52). Recently, a negative influence on mitogen-activated protein kinase activation through the second CH domain (CH2), present only in the p66 isoform of Shc,

has been recognized. However, the mechanism(s) of regulation via the CH2 domain is currently unknown (33).

The Shc-receptor interaction can be mediated via either the SH2 or the PTB domain. Although both domains bind phosphotyrosine-containing sequences, the specificities of recognition differ (67). PTB domain binding is determined by residues that are N terminal to the phosphotyrosine, while the binding preference of the SH2 domain is determined by residues C terminal to the phosphotyrosine (1, 22, 54, 58, 62, 67). The Shc SH2 domain binds to the receptors for epidermal growth factor (EGF) (38), platelet-derived growth factor (46, 66), and the T-cell receptor (44), while the PTB domain binds to the receptors for EGF (2, 63), nerve growth factor (63), IL-2 (43), and IL-3 (39). Structures of the Shc PTB and Shc SH2 domains bound to their respective phosphopeptides have been determined (68, 69) and have revealed the molecular basis for the differences in phosphopeptide specificities observed for the SH2 and PTB domains.

One of the interesting features of the structure of the Shc PTB domain is its remarkable similarity to the structures of the pleckstrin homology (PH) domains, despite the absence of amino acid sequence homology (69). PH domains have been shown to bind acidic phospholipids in vitro and have been implicated to play a role in membrane localization of proteins (9, 15, 16, 18). Because of its structural analogy to PH domains, we had examined the Shc PTB domain and showed that it can also interact with acidic phospholipids such as phosphatidylinositol 4-phosphate (PtdIns-4P) and PtdIns 4,5-bisphosphate [PtdIns(4,5)P₂] in vitro (69). However, the in vivo functional significance of the relatively low-affinity phospholipid interactions (50 to 150 μ M) of the Shc PTB domain (69) and many other PH domains (49), as well as the

* Corresponding author. Mailing address: Beirne Carter Center, MR4-Rm 4012F, University of Virginia, Charlottesville, VA 22908. Phone: (804) 243-6093. Fax: (804) 924-1221. E-mail: kr4h@virginia.edu.

contribution of phospholipid binding to the membrane localization of PH domain-containing proteins, has been debated (27). In this report, we investigate the significance of phospholipid binding to the Shc PTB domain both *in vitro* and *in vivo* using a structure-based mutagenesis approach. We observe a requirement for both phosphotyrosine and phospholipid binding via the PTB domain, both of which appear necessary for tyrosine phosphorylation of Shc following receptor activation.

MATERIALS AND METHODS

Cells. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. The BaF/Bo3 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 10% WEHI-conditioned medium (as a source for IL-3), 2 mM L-glutamine, penicillin, streptomycin, and 2×10^{-5} M 2-mercaptoethanol.

Plasmids. Plasmids encoding glutathione *S*-transferase (GST)-tagged wild-type and mutant PTB domains (corresponding to amino acids 17 to 207) for bacterial expression were generated with pGEX2TK (Pharmacia), and the fusion proteins were expressed as described previously (69). The His-tagged wild-type and mutant PTB domains for nuclear magnetic resonance (NMR) studies were cloned into pET15b (Novagen). The His tag was cleaved off before NMR structural studies and before we performed lipid-binding assays (see below). For eukaryotic expression, GST-tagged full-length (FL) wild-type Shc (FL-Shc wt), FL Shc with an R175Q mutation, (FL-Shc R175Q), the PTB domain only (Shc PTB), and the CH and SH2 domains (CH-SH2) were generated by PCR and subcloning of DNA fragments into the pEBG vector, as described previously (39, 56). Site-directed mutagenesis was performed by a two-step PCR as described previously (69). All constructs were sequenced, and the presence of appropriate mutations was confirmed.

Antibodies. Antibodies specific for Shc and GST were purchased from Transduction Labs (Lexington, Kentucky) and Santa Cruz Biotechnology (Santa Cruz, Calif.), respectively. Antiphosphotyrosine antibodies, the horseradish peroxidase-labeled antiphosphotyrosine antibody RC20H, and monoclonal antibody 4G10 were purchased from Transduction Labs and Upstate Biotechnology Inc. (Lake Placid, N.Y.), respectively.

NMR studies. Uniformly ^{15}N -labeled wild-type and mutant proteins of the Shc PTB domain were expressed in bacteria and purified as described previously (69). For NMR titrations of phosphopeptide and phospholipid, protein samples were at concentrations of 0.3 to 0.5 mM in a solution containing 50 mM Tris- d_11 -HCl (pH 6.5), 50 mM NaCl, and 5 mM dithiothreitol- d_{10} in 90% H_2O -10% $^2\text{H}_2\text{O}$. The NMR spectra were acquired at 25°C on a Bruker model AMX-500 or AMX-600 spectrometer. Two-dimensional ^1H , ^{15}N heteronuclear single quantum coherence spectra were acquired with 128 and 1,024 complex points in w_1 and w_2 , respectively. Stock solutions of the TrkA phosphopeptide and GIP2 for titration studies were prepared in $\text{Me}_2\text{SO}-\text{d}_6$ - H_2O and H_2O , respectively. NMR titrations of TrkA phosphopeptide or GIP2 were performed with wild-type Shc PTB (0.5 mM) and Shc PTB with a triple mutation (0.3 mM) by sequential addition of TrkA phosphopeptide or GIP2 out to a maximum of 2.0 molar equivalents.

Transfections, immunoprecipitations, and immunoblotting. Transient transfections into COS cells were performed with DEAE-dextran and chloroquine as described previously (39) with 1 μg each of the DNAs indicated in the figures. Approximately 24 h posttransfection, the cells were starved overnight without serum (about 16 h) and harvested and the proteins were analyzed as described below. For separation of membrane and cytosolic fractions, transfected COS cells were trypsinized and resuspended in 300 μl of hypotonic buffer (20 mM HEPES [pH 7.2], 2 mM EDTA, 10 mM NaF, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 0.4 mM NaVO_3 , 4 mM phenylmethylsulfonyl fluoride) and subjected to two rounds of snap freeze-thaw in a liquid nitrogen-37°C water bath. Lysis of cells was confirmed by trypan blue exclusion. Cells were spun at $14,000 \times g$ to pellet membrane components. The supernatant was saved as the cytosolic fraction. The membrane pellet was subjected to two more washes in hypotonic solution to remove cytosolic contamination. Detergent-containing lysis buffer (hypotonic buffer with the addition of 0.5% Triton X-100 and 150 mM NaCl) was added to membrane pellets, which were incubated for 15 min at 4°C and centrifuged at $14,000 \times g$ to pellet the cytoskeletal components. This supernatant (membrane fraction) and the cytosolic fraction (to which detergent-containing lysis buffer had been added) were precipitated with glutathione-Sepharose beads and analyzed by anti-Shc or anti-GST immunoblotting.

BaF/Bo3 cells (10^7) were washed once with RPMI 1640 and incubated for 10 min with 20 μg of DNA encoding GST-Shc or GST alone (vector control) in RPMI 1640-10% FBS. Cells were electroporated at 300 V and 800 μF , allowed to recover for 10 min, and transferred to fresh media containing 10% WEHI supernatant (as a source of IL-3)-10% FBS in RPMI 1640. After 3 h, cells were washed and cultured in 10% FBS-RPMI 1640 for 18 h. Cells were then harvested and stimulated with 10 U of recombinant IL-3 per ml for 5 min at 37°C. The cells were lysed, and the GST-tagged proteins were precipitated with glutathione-Sepharose beads and analyzed by antiphosphotyrosine or anti-Shc immunoblotting.

Phospholipid-binding assays. The binding of Shc PTB domain to phospholipids was determined with unilamellar vesicles in a centrifugation assay (69). Organic solvent was evaporated from the lipid solutions under nitrogen, buffer was added, and the lipid suspensions were subjected to five rounds of rapid freeze-thaw cycles, followed by extrusion through 0.1- μm -pore-size polycarbonate membranes (Costar, Cambridge, Mass.). Wild-type and mutant Shc PTB domains were incubated with PtdIns-4P or PtdIns(4,5)P2 (5% [wt/wt]) in unilamellar vesicles containing 1,2-distearoyl(dibromo)-*sn*-glycero-3-phosphocholine (BrPC) in 50 mM bis-Tris (pH 6.5)-100 mM NaCl as described previously (69). Protein (25 μl at 0.5 mg/ml) and lipid vesicles (25 μl at 10 mg/ml, total lipid concentration) were added to centrifuge tubes, incubated for 5 min, and then subjected to centrifugation at $100,000 \times g$ for 30 min in a Beckman model TL-100 ultracentrifuge. The protein concentrations of the supernatants were then determined by the BCA protein assay (Pierce, Rockford, Ill.). Shc PTB domain binding to BrPC vesicles alone [without PtdIns-4P or PtdIns(4,5)P2] was used to determine background binding.

RESULTS

Identification of Shc PTB domain residues critical for phospholipid binding. We had previously demonstrated that the Shc PTB domain specifically interacts with PtdIns(4,5)P2 and that it does not bind to phosphatidylserine, phosphatidylethanolamine, phosphatidic acid, or PtdIns (69). Shc PTB domain binding to PtdIns(4,5)P2 was stereospecific, since it did not interact with the related phospholipid PtdIns(3,4)P2 (41a). To elucidate the phospholipid binding sites on the Shc PTB domain, we analyzed the ^1H and ^{15}N amide chemical shifts of the ^{15}N -labeled Shc PTB domain induced upon binding to GIP2, an analog of the phospholipid head group of PtdIns(4,5)P2. ^1H , ^{15}N correlation spectra of the Shc PTB domain bound to GIP2 revealed that the chemical shift changes were localized to specific regions within the protein (Fig. 1). Specifically, prominent changes in chemical shifts were observed for one side of the β -sandwich that contains the positively charged residues R112, K116, and K139 (Fig. 1). This part of the protein corresponds to analogous regions in PH domains that bind to acidic phospholipids (9, 16, 18). Additional chemical shift changes were detected around the previously determined binding pocket for phosphotyrosine (Fig. 1) (69). Based on analogy of the Shc PTB domain to the PH domains (9, 16, 18), more than one of these positively charged residues may be important for contacting the negatively charged phospholipid head groups.

To identify the phospholipid contact residues, we mutated specific positively charged amino acids located in the region of the PTB domain shown to bind to GIP2 by the NMR experiments. Our mutagenesis strategy was targeted toward obtaining PTB domains that would have a specific defect only in phospholipid binding (without having an effect on phosphotyrosine binding) to distinguish the importance of binding to these two ligands *in vivo*. The R112, K116, and K139 residues were chosen for mutagenesis, since these residues are distal to the binding pocket for phosphotyrosine and had no detectable chemical shifts during phosphopeptide titrations (69). Moreover, the side chains of these three positively charged residues are solvent exposed, suggesting that mutations of these amino acids are less likely to cause major overall structural changes in the PTB domain. R112, K116, and K139 were mutated individually or in combination, and the mutant PTB domains were tested for their ability *in vitro* to interact with phosphoproteins and phospholipids in comparison to that of the wild-type PTB domain.

Analysis of different mutants suggested that individual mutation of R112, K116, or K139 had little effect on phospholipid binding while double mutations of R112Q-K139 and R112Q-K116 had only a partial effect on PtdIns(4,5)P2 binding (data not shown). However, a triple mutation (R112Q-K116A-K139A) of the PTB domain nearly abolished the ability of this

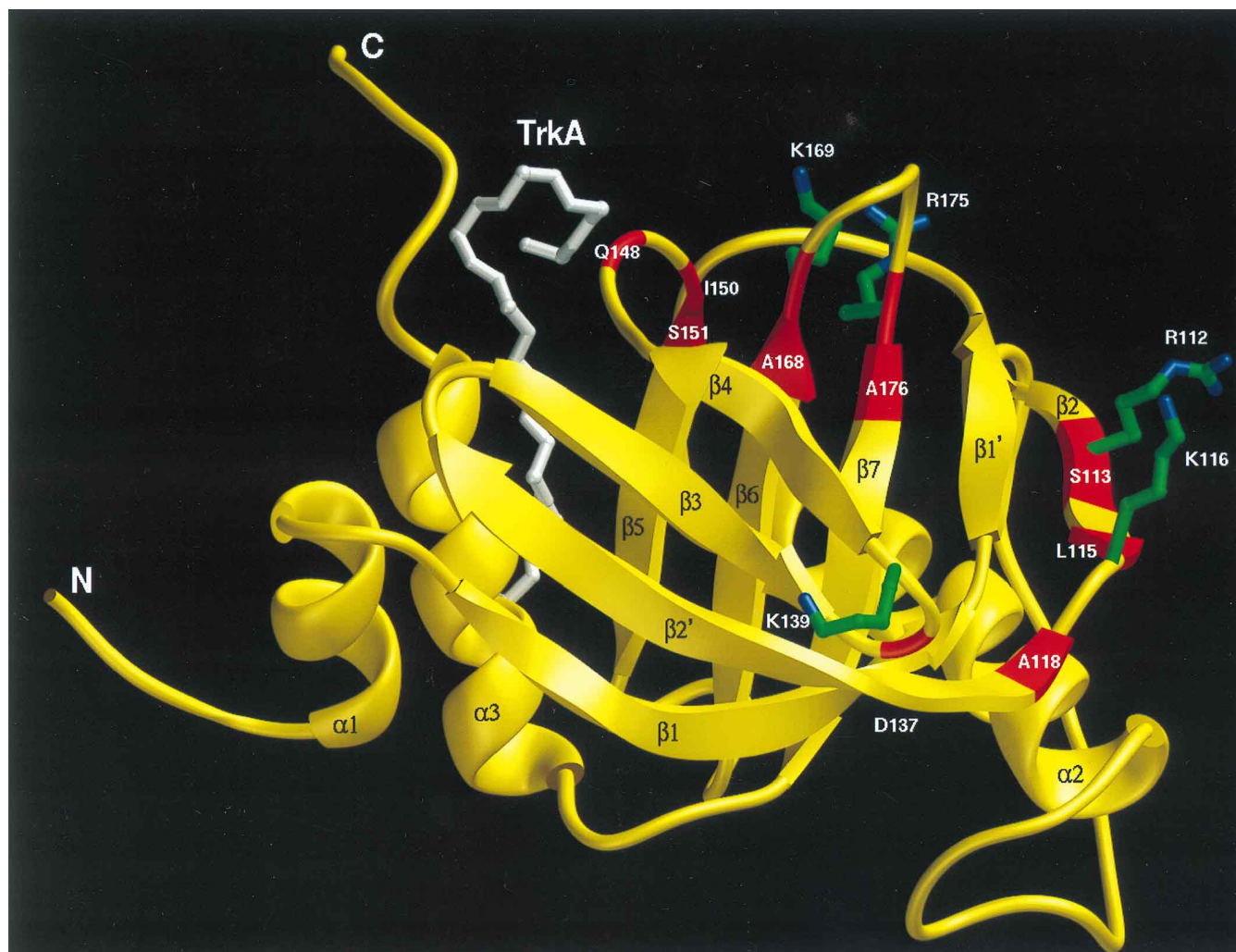


FIG. 1. Ribbon diagram of the Shc PTB domain (complexed to the TrkA phosphopeptide) depicting the chemical shift changes induced by the addition of GIP2. Amino acid residues whose backbone amide nitrogen and proton resonances displayed significant chemical shift changes as a function of GIP2 concentration during the titration are shown in red. Side chains of positively charged arginine and lysine residues in the GIP2 binding site are color-coded by atom type.

protein to interact with PtdIns(4,5)P₂ compared to that of the wild-type protein (Fig. 2A). The finding that all of these three positively charged residues need to be mutated to obtain a lipid-binding-defective mutant protein is consistent with studies of other PH domains, which indicated that several positively charged amino acids make contacts with the phosphate oxygens of the phospholipid head groups (9, 16, 18). Comparison of the heteronuclear single quantum coherence spectra of the wild-type and triple-mutation PTB domains showed that the triple mutation did not affect the folding of this protein (Fig. 2C), thus ruling out structural anomalies as the cause for decreased binding of the triple-mutant PTB domain to phospholipids.

Several lines of evidence suggested that the R112, K116, and K139 residues contribute to phospholipid binding but not phosphotyrosine binding. Mutation of R112, K116, or K139, either singly or in combination, had no detectable effect on binding of Shc PTB to SHIP, a 145-kDa tyrosine-phosphorylated protein previously demonstrated to interact with the Shc PTB domain (Fig. 2B) (23, 45). However, mutation of R175 (within the binding pocket for phosphotyrosine) to glutamine or even to a lysine severely compromised its ability to interact

with SHIP. Furthermore, during TrkA phosphopeptide titration, both the triple-mutant and the wild-type proteins undergo slow exchange on the NMR time scale between the free and complexed forms. This finding is indicative of tight binding, and the binding affinity of the protein with the triple mutant for the tyrosine-phosphorylated peptide is comparable to that of the wild-type protein (~50 nM). This result is illustrated by the indole NH signals of two specific tryptophan residues (W24 and W38) shown in Fig. 2C (inset). Thus, the triple-mutant PTB domain had a specific defect only in phospholipid binding.

We had previously observed competition between phosphopeptide and acidic phospholipids in binding to the Shc PTB domain, suggesting an overlap between regions that interact with phosphotyrosine and phospholipids (69). Consistent with this possibility, we noted some chemical shift changes in the binding pocket for phosphotyrosine in the PTB domain upon binding to GIP2 (Fig. 1). To identify the contribution of residues within the binding pocket for phosphotyrosine to phospholipid binding, we mutated and tested several residues within this region. Mutation of R175 abolishes binding to tyrosine-phosphorylated proteins (Fig. 2B) (39, 62, 65, 69). However, the PTB domain with the R175Q mutation bound

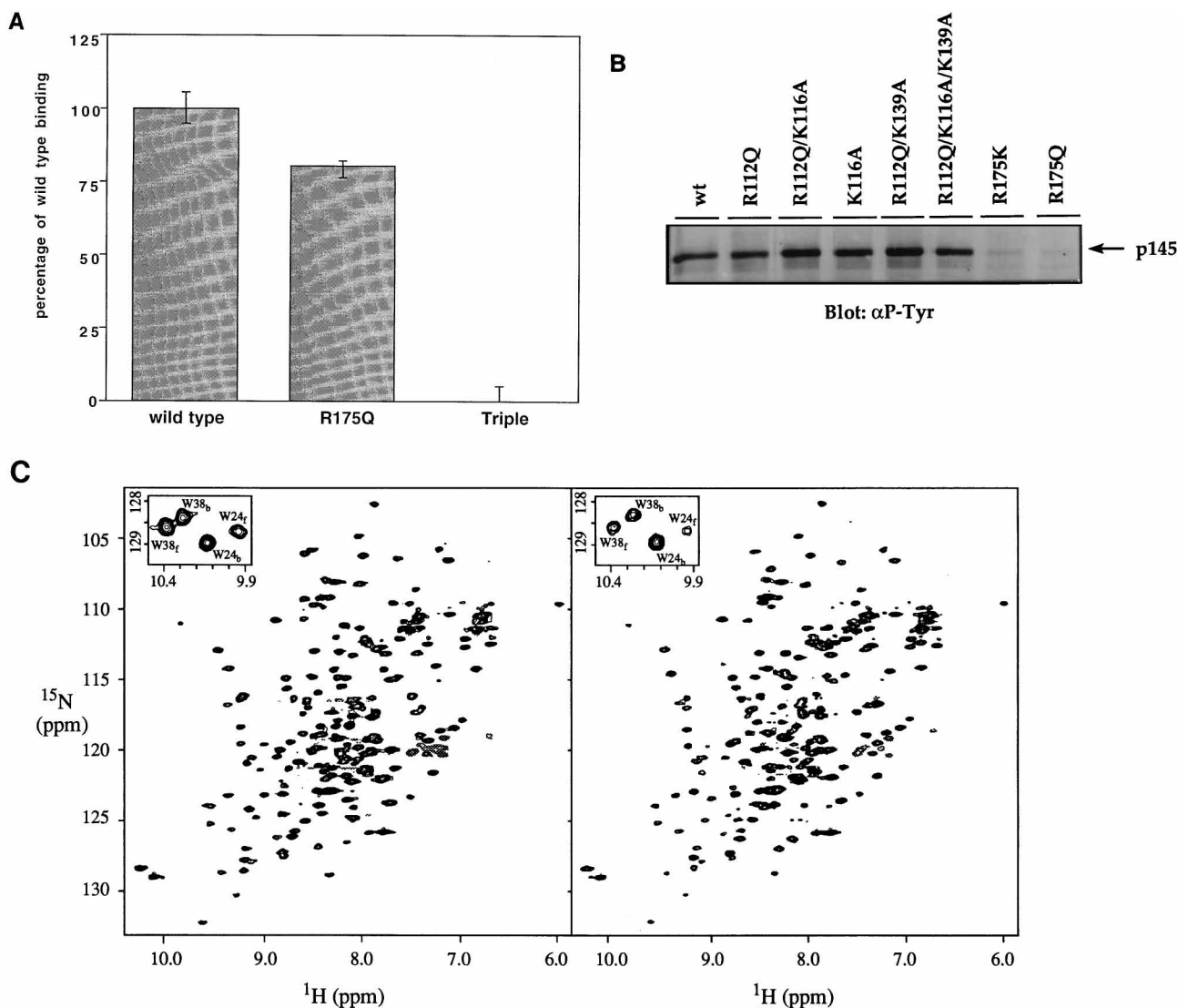
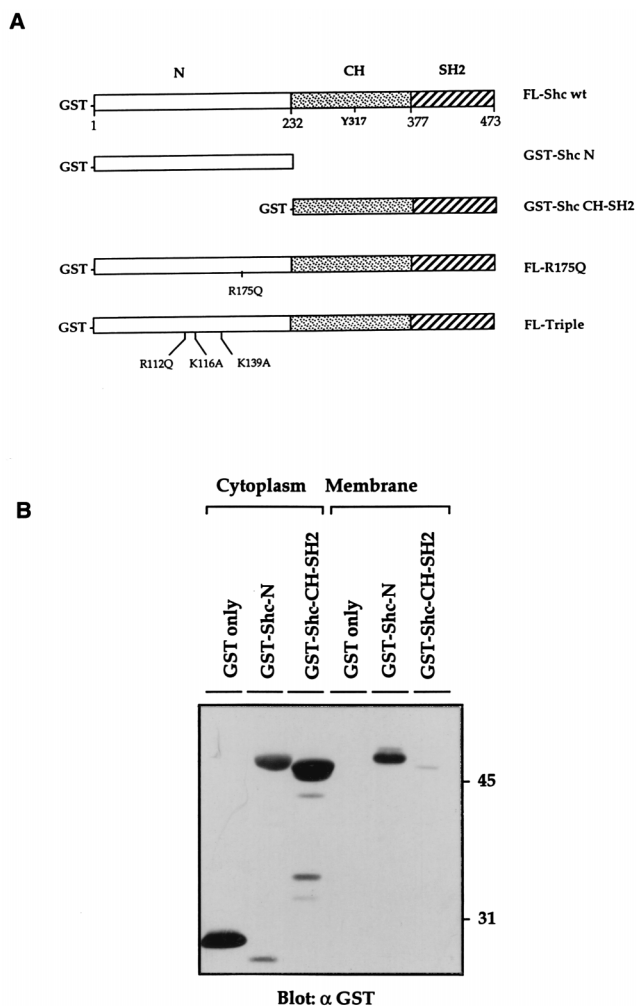


FIG. 2. Analyses of wild-type and mutant Shc PTB domains for phospholipid and phosphotyrosine binding. (A) Levels of binding of the wild-type, R175Q, and triple-mutant (R112Q-K116A-K139A) Shc PTB domains to PtdIns(4,5)P₂ were determined as described in Materials and Methods. Binding of the wild-type protein was considered 100%. (B) Interactions of wild-type (wt) and mutant PTB domains with phosphotyrosine-containing proteins. Individual or combined site-directed mutagenesis resulting in the R112Q, K116A, K139A, and R175Q mutations was performed, GST fusion proteins (expressed in *Escherichia coli* and bound to glutathione-Sepharose beads; 2 μ g each) were incubated with K562 lysates (2×10^7 cell equivalents), and the proteins bound to the beads were analyzed by anti-phosphotyrosine (α P-Tyr) immunoblotting. Similar results have been obtained with activated T-cell lysates where SHIP is inducibly phosphorylated and has been shown to interact with the Shc PTB domain (26). Immunoblotting with anti-GST antibody revealed that the same level of fusion protein was present in all lanes (data not shown). (C) Comparison of the ¹H, ¹⁵N spectra of the wild-type (left) and triple-mutant (right) Shc PTB domain proteins (0.3 mM) in the free forms. The insets show the spectra of the indole signals of two tryptophan residues (W24 and W38) in the free and phosphopeptide-bound forms during the TrkA-peptide titration with a protein-peptide molar ratio of 1:0.5. Intensities of the tryptophan peaks are consistent with the difference between the concentrations of the wild-type (0.5 mM) and triple-mutant (0.3 mM) Shc PTB domains. ¹⁵N, ¹H signals undergo slow exchange on the NMR time scale between the free and complexed forms of the proteins, indicating that the protein with the triple mutation binds to the TrkA peptide with tight binding affinity comparable to that of the wild-type Shc PTB domain (~50 nM).

PtdIns(4,5)P₂ nearly as well as that of the wild-type protein (80%) (Fig. 2A), thereby providing a mutant defective predominantly in phosphotyrosine binding. Mutations of a number of other residues (M66, R67, S159, and K169), previously determined to contribute to phosphotyrosine binding (69), also did not have any effect on PtdIns(4,5)P₂ binding (data not shown). Thus, although the regions of Shc PTB interaction with phosphotyrosine and phospholipid may overlap, the residues critical for binding to the two ligands are distinct. However, it is important to note that in vitro, the binding of phosphopeptides to the Shc PTB domain inhibits its interaction with phospholipids and that, most likely, the two ligands can-

not interact simultaneously (69). The competition of the peptides for phospholipid binding is probably due to steric hindrance and is likely to have a functional significance (see below). The proteins with the R175Q mutant and the triple mutant, with specific defects in binding to phosphotyrosine and phospholipid, respectively, were used in subsequent analyses in vivo.

Role of the PTB domain in membrane localization. The in vivo significance of the Shc PTB domain binding to acidic phospholipids was next investigated. Some amount of Shc protein (about 5%) is basally associated with the membrane fraction of resting T cells (data not shown) and BaF cells (see



below in Fig. 4A). A recent report also indicated localization of Shc to the membrane in NIH 3T3 cells (30). Although this report indicated localization of Shc to the endoplasmic reticulum membranes, our immunofluorescence studies have revealed the presence of Shc in several membranes, including the plasma membrane (data not shown). Thus, in the studies detailed below we have focused on the total cellular membranes. First, we determined the contribution of individual Shc domains in membrane localization by transiently expressing either the PTB domain alone or the CH and SH2 domains of Shc (expressed as fusion proteins with GST) in COS cells (Fig. 3A and B). Membrane and cytosolic fractions were prepared and analyzed for the presence of the expressed proteins by anti-GST immunoblotting (Fig. 3B). A significant amount of the Shc PTB domain was found associated with the membrane, while very little, if any, of the CH-SH2 domain was found in the membrane fractions. The vector alone, which expressed the GST tag, was not localized in the membrane. The GST-tag was not responsible for the observed effects, since studies of phospholipid binding with either an untagged or a GST-tagged PTB domain gave similar results (data not shown) and all of the NMR studies were performed with untagged proteins. The data above suggested that the PTB domain of Shc is necessary and sufficient for membrane localization.

We next determined the effects of mutations within the PTB

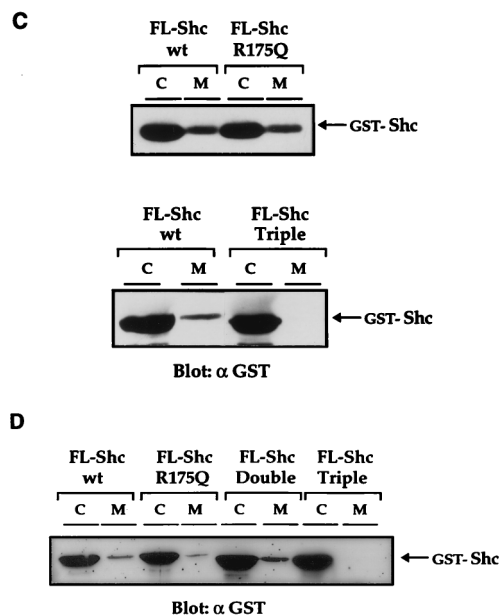


FIG. 3. Membrane localizations of wild-type and mutant Shc proteins. (A) Schematic diagrams of different Shc proteins. (B) The Shc PTB domain is sufficient for membrane localization. GST-Shc-N, GST-Shc-CH-SH2, and the vector expressing GST alone were expressed in COS cells, and the presence of these proteins in the cytosolic and membrane fractions was analyzed by anti-GST (α GST) immunoblotting as described in Materials and Methods. (C) FL Shc with the triple mutation fails to localize to the membrane. FL-Shc wt, FL-Shc R175Q, and FL-Shc Triple were transiently expressed in COS cells, and the presence of GST-Shc in cytosolic (C) and membrane (M) fractions was analyzed by anti-GST immunoblotting. The results of these 2 experiments are representative of at least 10 such experiments. (D) FL-Shc wt, FL-Shc R175Q, FL-Shc Double (R112Q-K139A), and FL-Shc Triple were transiently expressed in COS cells and analyzed for membrane localization as described above.

domain on the localization of FL Shc to the membrane. Comparison of membrane localization of FL-Shc R175Q, which is defective for phosphotyrosine binding, and FL-Shc Triple, which is defective for lipid binding, are shown in Fig. 3C (representative of at least 10 such experiments). The triple mutation completely abrogated localization of Shc to the membrane, while the R175Q mutation was found associated with the membrane at a level comparable to that of the wild-type protein. Shc proteins carrying a single mutation and double mutations of phospholipid-binding sites localized to the membranes in amounts comparable to that of wild-type Shc (data not shown), and this result correlated well with our data from the *in vitro* phospholipid binding assay. The data for the membrane localization of one of the double mutants (R112Q-K139A) are shown in Fig. 3D. Thus, the phospholipid-binding sites that we had identified by NMR-based mutagenesis appeared to be necessary for membrane localization of Shc *in vivo*. Since the R175Q mutant was found in the membrane fraction but did not bind to tyrosine-phosphorylated proteins (39, 69), we conclude that localization of this protein to the membrane was due to its intact lipid-binding sites and that basal membrane localization is independent of phosphotyrosine binding. Similar results were obtained with cells grown in 10% FBS or after serum starvation, thereby ruling out effects of serum components on basal localization of Shc proteins (data not shown).

Both phosphotyrosine- and phospholipid-binding sites on Shc PTB are required for Shc phosphorylation. Recently, a critical role for tyrosine phosphorylation of Shc following IL-3

stimulation has been demonstrated for both c-Fos and c-Myc activation (13). To determine if basal membrane localization is important in signaling via Shc, we examined the tyrosine phosphorylation of wild-type and mutant Shc proteins following IL-3 stimulation. This model system provides two important advantages. (i) The PTB domain of Shc is essential for its interaction with the IL-3 receptor β_c chain; i.e., mutation of a specific tyrosine (Y577) within β_c or R175 within the Shc PTB domain abolishes this interaction (39). (ii) Tyrosine phosphorylation of Shc upon IL-3 stimulation requires the binding of Shc to the tyrosine-phosphorylated β_c chain, and disruption of the Shc- β_c interaction results in failure of Shc phosphorylation (8, 19, 39, 50). Using this system, we could address the requirement for membrane localization of Shc by determining the IL-3-dependent phosphorylation of FL Shc with the triple mutation (which has intact phosphotyrosine binding but cannot localize to the membrane). Based on the recent correlation between Shc phosphorylation and c-Fos and c-Myc activation following IL-3 stimulation (13), we have used the tyrosine phosphorylation of Shc as an index for Shc-mediated signaling in the following experiments.

IL-3-dependent BaF/Bo3 cells were transiently transfected with DNAs expressing FL wild-type Shc, FL-Shc R175Q, or FL-Shc Triple. Subcellular fractionation of transfected BaF cells revealed that there was a significant decrease in membrane localization of FL-Shc Triple compared to that of FL wild-type Shc (Fig. 4B). The FL-Shc R175Q was localized to the membrane in amounts comparable to FL-Shc wt (data not shown). These results were in agreement with the data obtained with COS cells (Fig. 3C). The transfected BaF cells were then stimulated with IL-3, and the phosphorylation states of the expressed proteins were analyzed by antiphosphotyrosine immunoblotting (Fig. 4C). While the FL-Shc wt protein was efficiently phosphorylated upon IL-3 stimulation, phosphorylation of FL-Shc R175Q and FL-Shc Triple was severely diminished. After protein levels were normalized, the decreases in phosphorylation of FL Shc with the R175Q and triple mutations (averages of three independent experiments) were about 99 and 85%, respectively (Fig. 4C). Although we had shown earlier that the triple mutation (R112Q-K116A-K139A) does not affect phosphotyrosine binding, we wanted to rule out that the decrease in IL-3-dependent tyrosine phosphorylation of FL-Shc Triple was not due to a decrease in binding to β_c . Due to the low level of receptors expressed in BaF cells, we were unable to directly demonstrate coprecipitation of β_c either with FL-Shc wt or FL-Shc Triple. However, in COS cell lysates, Shc with the triple mutation was able to bind to tyrosine-phosphorylated β_c at a level equivalent to that of the wild-type protein (Fig. 4D). This suggested that the protein with the triple mutation has the potential to interact with phosphorylated β_c and that the decrease in its phosphorylation is most likely due to its failure to localize to the membrane. The failure of the R175Q mutant protein to be phosphorylated was not unexpected based on previous studies showing a requirement for phosphotyrosine-dependent binding of the PTB domain to the β_c chain. These data suggested that both localization to the membrane and interaction with the phosphorylated receptor are required for efficient Shc phosphorylation.

To determine if there would be changes in the levels of Shc proteins prior to and after IL-3 activation, we transiently transfected BaF/Bo3 cells with FL-Shc wt, FL-Shc R175Q, or FL-Shc Triple and examined the localizations of the transfected proteins in the cytosolic and membrane fractions (Fig. 4E). Consistent with earlier results, the wild-type and the R175Q proteins were found in the membrane fractions basally but there was no detectable increase in the levels of either the wild

type or the R175Q mutant protein after receptor activation. FL-Shc Triple was not detected in the membrane fractions, irrespective of the activation status of the cells. Although we were surprised to find a lack of an increase in the levels of FL-Shc wt in the membrane fractions after receptor activation, phospholipid-dependent recruitment of Shc during receptor activation remains a possibility (see Discussion).

DISCUSSION

The PTB domain of Shc has been ascribed an important role in signaling via several receptors (60). Structural studies of the PTB domain demonstrated a striking homology to PH domains and suggested acidic phospholipids as a second ligand for the Shc PTB domain (69). An interesting possibility was that binding to one or both ligands through the PTB domain may contribute to recruitment of Shc and influence Shc-mediated signaling. In this report, we have identified the residues within the Shc PTB domain that are important for phospholipid binding and phosphotyrosine binding through the use of NMR and targeted mutagenesis. Although the contact regions on Shc PTB for the two ligands overlapped and binding of phosphopeptides inhibited the binding of the phospholipids (69), the critical residues required for binding to phosphotyrosine and phospholipid were different. Specifically, R112, K116, and K139 residues were critical for phospholipid binding (but not phosphotyrosine binding), while R175 was critical for phosphotyrosine binding (but not phospholipid binding). In vivo, the Shc PTB domain mediated basal localization of Shc to the membrane and Shc with the triple mutation (R112Q-K116A-K139A) failed to localize to the membrane, consistent with its failure to interact with PtdIns(4,5)P₂ in vitro. During IL-3 signaling, both FL-Shc R175Q and FL-Shc Triple had markedly decreased tyrosine phosphorylation, suggesting that both membrane localization and phosphotyrosine binding via the PTB domain are necessary for efficient Shc phosphorylation.

While phosphotyrosine binding via the Shc PTB domain and binding Shc to activated, tyrosine-phosphorylated receptors have been well recognized, to our knowledge, this is the first demonstration that membrane localization, prior to receptor activation, also plays a role in leading to Shc phosphorylation. The failure of the Shc protein with the triple mutation to bind to phospholipids in vitro correlated with its inability to efficiently localize to the membranes. The failure to detect phosphorylation of the R175Q mutant protein, which is not affected in its ability to localize to the membrane, was most likely due to its inability to interact with the phosphorylated β_c receptor. Thus, both membrane localization and binding to the phosphorylated receptor appears necessary for signaling via Shc. Considered together, these findings suggested that recruitment of Shc to the activated receptor may occur in two steps: the initial phospholipid-mediated localization of Shc to the membrane and then interaction of Shc with the phosphorylated receptor. Although speculative, one potential advantage of such a two-step process may be that this provides Shc with partial access to the proximity of the receptor, thereby facilitating the rapid recruitment of Shc to activated receptors. This process may also explain the observation that Shc is one of the earliest tyrosine-phosphorylated proteins after activation of many receptors.

It has been demonstrated previously that phosphopeptides (that can bind to the Shc PTB domain) can compete with phospholipid binding in vitro. Since the affinity of Shc PTB binding to acidic phospholipids (50 to 150 μ M) (69) is weaker than its interaction with phosphopeptides (50 to 100 nM) (67), we propose a model whereby Shc may localize to the mem-

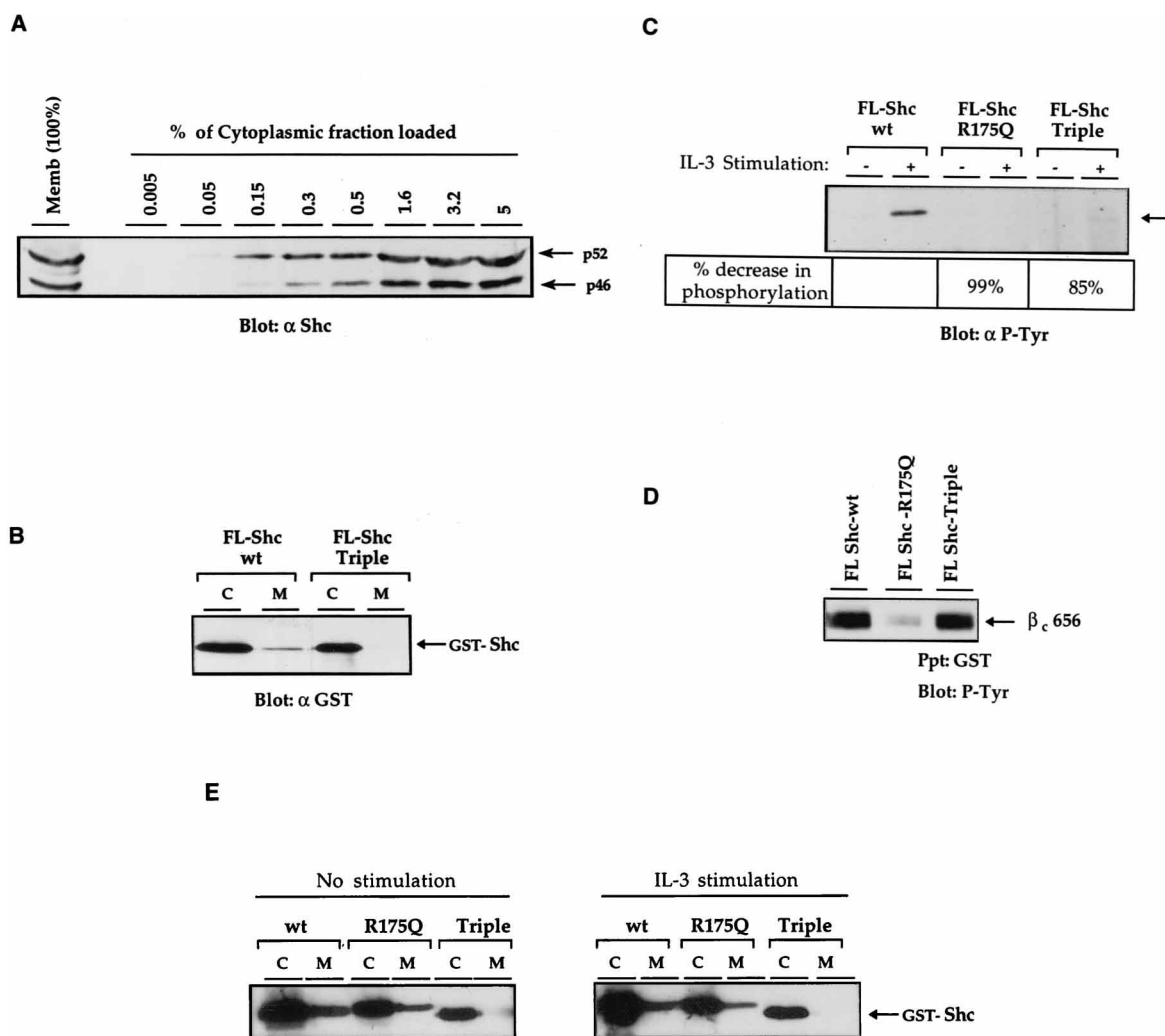


FIG. 4. IL-3-dependent tyrosine phosphorylation of wild-type (wt) and mutant Shc proteins. (A) Basal localization of endogenous Shc to the membrane fractions. Cytosolic and membrane fractions from 2×10^7 BaF/Bo3 cells were isolated as described in Materials and Methods and analyzed for the presence of Shc by anti-Shc (α Shc) immunoblotting (the migrations of p52 and p46 isoforms are indicated). The cytoplasmic fractions were run at various concentrations for comparison. Rough quantitation of the bands indicated that $\sim 2.5\%$ of Shc was in the membrane fractions in this experiment. (B) BaF/Bo3 cells were transiently transfected with FL-Shc wt or FL-Shc Triple, and the membrane and cytosolic fractions were separated, precipitated with glutathione-Sepharose beads, and analyzed for the presence of expressed Shc proteins by anti-GST immunoblotting. (C) FL-Shc wt, FL-Shc R175Q, and FL-Shc Triple were transiently expressed in BaF/Bo3 cells, and after IL-3 stimulation, the phosphorylation of expressed Shc proteins (precipitated with glutathione-Sepharose beads) was analyzed by antiphosphotyrosine (α P-Tyr) immunoblotting. The levels of transiently expressed Shc proteins were determined by anti-Shc immunoblotting. For three independent experiments, the phosphotyrosine signal was normalized for the level of Shc proteins expressed (by densitometry) and the average percent decreases in phosphorylation of FL-Shc R175Q and FL-Shc Triple compared to the level of phosphorylation of FL-Shc wt were estimated. (D) The binding of FL-Shc wt, FL-Shc R175Q, and FL-Shc Triple to tyrosine-phosphorylated β_c (expressed in COS cells along with JAK2) was analyzed by antiphosphotyrosine immunoblotting. Anti-GST immunoblotting revealed comparable levels of tagged Shc proteins in all lanes. Ppt, precipitate. (E) BaF/Bo3 cells were transiently transfected with FL-Shc wt, FL-Shc R175Q, or FL-Shc Triple, and the cytoplasmic (C) and membrane (M) fractions were isolated before or after IL-3 stimulation (10 U/ml for 5 min at 37°C). The levels of transiently expressed Shc present in the two fractions were determined by anti-GST immunoblotting. Although the wild-type protein was expressed at higher levels in this experiment, there was no detectable increase in the levels of either FL-Shc wt or FL-Shc R175Q in the membrane fractions after IL-3 stimulation. FL-Shc Triple was not present in the membrane fraction irrespective of the activation status. The results shown are representative of two independent experiments.

brane via its PTB domain in resting cells (a lower-affinity interaction) and then translocate to the receptor that becomes tyrosine phosphorylated upon cellular activation (a higher-affinity interaction). The data from experiments using proteins with the triple mutation and the R175Q mutation are consistent with such a model. However, it should be emphasized that our model is based on the data obtained with IL-3 signaling and that there may be other situations where Shc may be recruited to the receptor directly from the cytoplasm (e.g., in EGF signaling [see below]).

We did not observe a noticeable change in the levels of

membrane-bound Shc before and after IL-3 stimulation, which could be due to several, not mutually exclusive, reasons. (i) Since only a small fraction of Shc is tyrosine phosphorylated after receptor activation (~ 5 to 10%) (data not shown), it is possible that only a small fraction of Shc may be recruited to the activated receptor and that this amount is below our detection limit. (ii) If only a fraction of the receptors at the cell surface is involved in signaling, the detection of changes in the receptor-bound fraction of Shc may be further limited. (iii) During IL-2 receptor signaling, we found that only a small fraction of all the phosphorylated Shc is bound to activated

IL-2 receptor (43). It is conceivable that a "cycling" of Shc molecules occurs, such that membrane-bound Shc moves to the phosphorylated receptor and then "fall off" and is replaced by other Shc molecules. In this case, the steady-state levels of Shc protein in the membrane may not be significantly altered. Thus, although we did not observe a detectable change in the levels of membrane-associated Shc proteins, modulation of the levels of specific phospholipids during activation *in vivo*, leading to differences in recruitment and signaling via Shc, remains an attractive possibility.

Among the different phospholipids we have tested *in vitro*, the binding of the PTB domain was observed with PtdIns(4,5)P₂ (69). The binding of the Shc PTB domain to PtdIns(4,5)P₂ was stereospecific, since it did not interact with PtdIns(4,5)P₂ (41a). The Shc PTB domain also bound to PtdIns-4P, and the triple mutation of the PTB domain also affected PtdIns-4P binding (data not shown). The correlation between the *in vitro* binding and the membrane localization of wild-type and mutant Shc proteins suggested that the interaction with PtdIns(4,5)P₂ (and possibly PtdIns-4P) may contribute to the basal membrane localization of Shc *in vivo*. Although we make that correlation based on the available data, it is quite possible that phospholipids other than PtdIns(4,5)P₂ also mediate the localization of Shc to the membrane. Recently, we have also observed an interaction between the Shc PTB domain and PtdIns(3,4,5)P₃ *in vitro* (41a). Since PtdIns 3,4,5-triphosphate [PtdIns(3,4,5)P₃] is a phospholipid generated upon activation of many receptors, this circumstance may not contribute to the localization of Shc observed prior to receptor activation but may be important during receptor activation. The *in vivo* significance of Shc PTB domain binding to this phospholipid is being tested.

Recently, phosphorylation of specific residues within the CH domain of Shc has been implicated in leading to activation of different cellular pathways and interaction with unique proteins (13, 61). For example, tyrosine phosphorylation of Y317 has been ascribed to transcriptional activation of c-Fos while Y239 and Y240 have been implicated in leading to c-Myc activation and prevention of apoptosis (13). Although the tyrosine phosphorylation of the protein with the triple mutation is greatly reduced, we observed a small, yet detectable, phosphorylation of the protein with the triple mutation during IL-3 signaling (5 to 15% of the wild-type level in different experiments). We are currently testing the possibility that membrane localization may affect phosphorylation of the Y317 residue differently from that of the Y239 and Y240 residues. It is also possible that different receptors may have different requirements for leading to Shc phosphorylation. We did observe that tyrosine phosphorylation of Shc in response to EGF is unaffected in our triple mutant (data not shown). However, this result is not surprising, since despite the very tight binding of the Shc PTB domain to the phosphorylated EGF receptor, deletion of either the PTB or the SH2 domain of Shc or deletion of the EGF receptor cytoplasmic tail does not seem to affect Shc phosphorylation in response to EGF (data not shown and references 12, 48, and 65). It has been shown that Shc phosphorylation after activation of G protein-coupled receptors occurs via a PtdIns(3,4,5)P₃-dependent mechanism (29, 57, 59). Thus, there may be situations where receptor-mediated activation increases PtdIns(3,4,5)P₃, which may in turn increase membrane-associated Shc. In the case of G protein-coupled receptors, certain levels of PtdIns(3,4,5)P₃ may need to be attained before Shc phosphorylation occurs. Given that Shc is recruited and phosphorylated in many different receptor systems, it is conceivable that requirements for membrane lo-

calization may be influenced by both cell type and the nature of the receptor.

The *in vivo* functional significance of acidic phospholipid binding by many PH domains and the structurally analogous PTB domain has been unclear. Recently, Lemmon et al. (27) argued that the low-affinity phospholipid binding of many PH domains, including the Shc PTB domain, may be irrelevant *in vivo*. We suggest two conceptually important conclusions from the data presented here. (i) The Shc PTB domain is capable of recognizing both phospholipids and phosphotyrosine-containing proteins, and the interaction with phospholipids, although of lower affinity, is critical for signaling via Shc *in vivo*. By extension, PH domains of other signaling proteins, which also bind acidic phospholipids with relatively low affinity, may also play a role in membrane targeting. In this regard, the recent data with the PH domains of Akt and Sos support this notion (4, 10, 17, 24). (ii) The PTB domain of Shc provides a prototype for a hybrid domain that can bind two different ligands *in vitro* and mediate two functions that can be distinguished *in vivo*. Many signaling proteins possess a PH domain in conjunction with an SH2 domain, possibly for separately attaining phospholipid binding and mediating interaction with a tyrosine-phosphorylated receptor. The PTB domain of Shc is capable of accomplishing both membrane localization and receptor binding through a single domain. Although some SH2 domains have been shown to bind phospholipids, this binding has been proposed as a mechanism for desensitization rather than activation, and the details of this interaction have not yet been established (41).

Our data also extend the original definition of adapter proteins (such as Shc), in that adapters mediate not only protein-protein interactions but also protein-lipid interactions. Interestingly, the PTB domain of Shc, perhaps due to its versatility, is highly conserved among Shc genes from drosophilas to humans (25, 37). Recently, a 145-kDa tyrosine-phosphorylated protein that associates with Shc upon stimulation of several hematopoietic receptors has been identified as an SH2 domain-containing 5'-inositol phosphatase (6, 21, 28). The interaction of Shc with a phospholipid-phosphatase, together with the data presented here, strongly suggests that Shc participates in both phosphotyrosine and phospholipid-dependent intracellular signaling events.

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