The adaptor protein Shc has been implicated in Ras signaling via association with many tyrosine-phosphorylated receptors, including growth factor receptors, antigen receptors on T and B cells, and cytokine receptors. Shc could interact with the activated receptors through the carboxy-terminal Src homology 2 (SH2) domain or the structurally unrelated amino-terminal phosphotyrosine binding (PTB) domain. Using NMR and surface plasmon resonance techniques, we have measured the binding affinities of the SH2 and the PTB domains of Shc to a series of phosphotyrosine-containing peptides derived from known Shc binding sites. Tyrosine-phosphorylated peptides derived from Trk (pY490), polyoma virus middle T-antigen (pY250), ErbB3 (pY1309), and epidermal growth factor receptor (pY1086, pY1148, and pY1114) that contain NP

The SH2 domain of Shc binds to the carboxy-terminal domain of activated growth factor receptors such as epidermal growth factor, TrkA, and HER2/neu (8). The amino acid sequence of the Shc SH2 domain for phosphopeptides derived from the T-cell receptor C chain of the T-cell receptor bind preferentially to the PTB domain of Shc with K_d values of 0.02–5.3 μM. The binding affinities of these peptides to the Shc SH2 domain were in the range of 220-1290 μM. In contrast, tyrosine-phosphorylated peptides from epidermal growth factor receptor (pY992, pY1173) and the ε chain of the T-cell receptor bind preferentially to the SH2 domain with K_d = 50–130 μM versus the PTB domain (K_d > 680 μM). From these studies, the relative contribution of the individual domains of Shc for binding to the phosphotyrosine-containing portions of these proteins was determined. In addition, our data indicate that the high affinity binding of the PTB domain to the NPXPY-containing peptides results from a very high association rate and a rapid dissociation rate, which is similar to previous results observed for the SH2-phosphopeptide complexes.

The PTB domain has been shown to bind tyrosine-phosphorylated proteins of approximately 145 kDa (p145) in growth factor-stimulated cells (7, 17), the insulin receptor (9, 10), and autophosphorylated growth factor receptors such as epidermal growth factor receptor (TrkA), and HER2/neu (8). The amino acid sequence of the PTB domain is very different from these of SH2 domains except for the YLVR sequence, which is somewhat similar to the FLVR signature motif found in SH2 domains. However, mutation of the Arg residue to Leu in this motif did not affect binding of Shc PTB domain to the phosphorylated proteins (7). Furthermore, unlike SH2 domains, which recognize peptide sequences COOH-terminal to the phosphotyrosine residue, the PTB domain of Shc binds to amino acids NH2-terminal to the phosphotyrosine, preferentially with peptides containing an NPXPY sequence (18, 19). Thus, the PTB domain of Shc represents a new class of protein modules that bind to phosphotyrosines.

Despite its significant role in signal transduction, much less is known about the PTB domain as compared with the SH2 domains. Information regarding its ability to bind the tyrosine-phosphorylated proteins via the NPXPY motif has been derived from qualitative gel assays (7, 9) or inferred from peptide competition experiments using glutathione S-transferase (GST) fusion proteins, which can overestimate the binding affinity due to dimerization of GST (20). Thus far, no direct affinity measurements of the PTB domain to NPXPY-containing peptides have been reported. Furthermore, no direct comparison of the binding affinities between the Shc SH2 and PTB domains leads to tyrosine phosphorylation of Shc, the binding of Grb2 to the phosphorylated Shc protein, and an interaction between Grb2 and the Ras guanine nucleotide exchange factor, mSOS. The recruitment of the Shc-Grb2-mSOS complex to the membrane, through the interaction of Shc with activated receptors, leads to Ras activation. Shc contains two domains capable of interacting with tyrosine-phosphorylated receptors: the carboxy-terminal SH2 domain and the recently described amino-terminal phosphotyrosine-binding (PTB) domain (7–9). The Shc SH2 domain was found to recognize peptide sequences containing pY(I/E/Y/L)X(I/L/M) as determined using a degenerate phosphotyrosine-containing peptide library. This is consistent with the observed binding affinities of the Shc SH2 domain for phosphopeptides derived from the T-cell receptor ε chain (12) and epidermal growth factor receptor (13) as well as with the NMR structure of the Shc SH2 domain (12), which contains a phosphotyrosine and Tyr(P) hydrophobic binding pocket. However, the SH2 domain structure cannot be used to readily explain the ability of Shc to bind to peptide sequences containing an NPXPY motif such as that found in TrkA and polyoma virus middle T-antigen (14–16).

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¶ The abbreviations used are: EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; HSQC, heteronuclear single quantum coherence; PAGE, polyacrylamide gel electrophoresis; PTB, phosphotyrosine binding; SH2, Src homology 2; Fmoc, N-(9-fluorenylethoxycarbonyl); SPR, surface plasmon resonance; MT, polyoma virus middle T-antigen; cRU, corrected resonance units.
domains to the known Shc binding sites has been made. Here, we describe the binding affinities of a series of phosphotyrosine-containing peptides for the PTB and SH2 domains of Shc as measured by NMR and surface plasmon resonance. These phosphopeptides were derived from known Shc binding sites on TrkA, ErbB2, ErbB3, mouse polyoma virus middle T-antigen, the z chain of the T-cell receptor, and the epidermal growth factor receptor. From these studies, the relative contribution of the individual domains of Shc for binding to the phosphotyrosine-containing portions of these proteins has been determined.

MATERIALS AND METHODS

Protein and Peptide Preparation—The Shc SH2 domain, corresponding to residues 370–473 of the full Shc protein, was subcloned into the pET20b plasmid (Novagen) and expressed in E. coli BL21(DE3) cells. The recombinant protein contained an additional Leu-Glu-His6 sequence at the COOH terminus to aid in protein purification. The NH2-terminal Shc PTB domain (amino acids 17–207) was subcloned into the bacterial expression vector pET15b, which introduces a His tag followed by a thrombin cleavage site at the NH2 terminus of the recombinant protein. This protein was expressed in E. coli BL21(DE3) cells. E. coli cells were co-transformed with TRNA plasmid Pom61etetR). A GST-Shc PTB fusion protein encoding amino acids 17–207 was also generated by polymerase chain reaction and cloned in pGex2T (Pharmacia Biotech Inc.) for protein expression in E. coli. The bacteria were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C, and the fusion proteins were purified using glutathione-Sepharose beads.

Uniformly 15N-labeled proteins were prepared from bacteria grown at 37 °C in a minimal medium containing [15N]NH4Cl (Isotech). Both His-tagged versions of the Shc SH2 and PTB domains were purified by affinity chromatography on a nickel-IDA column (Invitrogen), followed by ion exchange chromatography. In the case of the Shc SH2 domain, an anion exchange column was used for purification in 20 mM sodium phosphate buffer (pH 7.8), whereas a cation exchange column was chosen for the Shc PTB domain in 20 mM HEPES (pH 7.5), containing 50 mM NaCl and 5 mM β-mercaptoethanol. Before ion exchange chromatography, the NH2-terminal His tag of the PTB domain was cleaved by treating with thrombin in 20 mM Tris/HCl (pH 8.0) containing 50 mM NaCl, 2.5 mM CaCl2, and 5 mM β-mercaptoethanol.

All of the tyrosine-phosphorylated peptides were prepared on an ABI (Applied Biosystems, Inc.) 340A peptide synthesizer using Fmoc/HBTU chemistry. Phosphorysine was incorporated using the reagent Fmoc-Tyr(PO2H)2 with HBTU/HOAc activation and extended coupling times for the tyrosine and all subsequent residues. The peptides were cleaved from the resins using 80% trifluoroacetic acid, 5% phenol, 5% isopropanol, 5% water, 2.5% ethanedithiol, 2.5% triisopropylsilane for 2.5 h at room temperature. After precipitation with methly t-butyl ether, the peptides were further purified by reverse-phase high performance liquid chromatography, and their composition was confirmed by mass spectrometric analysis. An unphosphorylated peptide TrkA Y490 (H11ENPYQFSDA) was purchased from PeptidoGenic Research & Co. (Livermore, CA).

NMR Titrations—Protein samples of the Shc PTB and SH2 domains (nonfusion proteins) used in the NMR experiments were prepared at a concentration of 0.3–1.0 mM in 50 mM Tris-d1/HCl (pH 6.5), 50 mM NaCl, and 5 mM DTT-d5 in 90% H2O, 10% D2O. The NMR spectra were acquired at 25 °C on a Bruker AMX-500 or AMX-600 spectrometer. Two-dimensional 1H-15N heteronuclear single quantum correlation (HSQC) spectra were acquired with 128 and 1024 complex points in δn and δH respectively. Stock solutions of peptides for the titration studies were prepared in MeSO-D2/H2O.

The binding constants were obtained by fitting the recorded chemical shift as a function of increasing peptide concentrations using a simple bimolecular equilibrium binding equation,

$$
\Delta \delta_{\text{obs}} = \delta_{\text{Shc}} + \delta_{\text{peptide}} - \delta_{\text{Shc} + \text{peptide}}
$$

where $\Delta \delta_{\text{obs}}$ is the observed chemical shift change for NMR titrations, $\delta_{\text{Shc}}$ is the chemical shift of the free protein, and $\delta_{\text{peptide}}$ is the difference in chemical shift between free and fully complexed protein. Molar fractions ($x_{\text{Shc}})$ were calculated from a dissociation constant, $K_d$, using known initial concentrations of protein and peptide. A least squares analysis was then performed by systematic variation of $K_d$, $x_{\text{Shc}}$, and $x_{\text{peptide}}$.

Surface Plasmon Resonance (SPR) Analysis—The concepts and operation of SPR in the study of protein-ligand interactions have been described extensively elsewhere (21, 22). SPR measurements were performed on the BIAcore 2000 (Pharmacia). The phosphopeptides were immobilized to the biosensor chip (CM5) via an amine coupling of the amino terminus of the peptide to carboxyl groups in the dextran matrix of the chip. Immobilizations were performed at 25 °C with a flow rate of 5–10 μl/min and an exposure time of 0.5–2 min. Because of the high negative charge of these peptides, it was necessary to present them to the matrix in a buffer containing an unusually high salt concentration (6 mM HEPES, 2.1 mM NaCl, 0.03% surfactant P20, pH 7.4) as well as a high peptide concentration (1 mg/ml). To remove unbound peptide and stabilize the matrix, 10 30-s pulses of 5 mM guanidine hydrochloride (pH 4.5) were applied to the chip. The machine was then primed with running buffer containing 10 mM HEPES, 150 mM NaCl, 20 mM DTT, 0.05% surfactant P20, pH 7.4. Serial dilutions of Shc PTB (0.01–10 μM) in the running buffer were injected at 10 μl/min for 10–60 min. Each injection was followed by a regeneration step of two 30-s pulses of guanidine hydrochloride (pH 4.5). Since both on- and off-rates in the PTB domain-phosphopeptide interactions were very rapid, the dissociation constants ($K_d$) were determined via Scatchard-type analysis using the binding responses at equilibrium with several concentrations of the protein (20).

Phosphopeptide Inhibition of GST-Shc PTB Binding to p145—GST-Shc (amino acids 17–207) fusion protein (2 μg) bound to glutathione-agarose beads was incubated with different phosphopeptides (25 μg) for 30 min on ice. The beads were then incubated with K562 cell lysates (15 × 106 cell equivalents). Bcr-Abl-transformed K562 cells were used that expressed a constitutive phosphorylation of Shc and p145. Lysis of cells and the precipitation with fusion proteins were performed as described previously (17). The proteins bound to the beads were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by anti-phosphotyrosine immunoblotting using ECL (Amersham Corp.). The bands were quantitated by densitometry and compared with the lane corresponding to the samples that did not contain any phosphotyrosine-containing peptide to obtain the percent inhibition caused by the different phosphopeptides.

RESULTS

Binding of Phosphopeptides to the Shc SH2 Domain—In order to measure the binding affinities of the tyrosine-phosphorylated peptides to the Shc SH2 domain, 15N/1H chemical shift changes of the residues located in phosphopeptide binding pockets in the Shc SH2 domain were monitored as a function of increasing concentration of the phosphopeptides. These NMR signals in the 15N/1H-HSQC spectra of the protein were in fast exchange on the NMR time scale and shifted upon the addition of each of the phosphopeptides as illustrated in Fig. 1A for residue Arg β5 of the Shc SH2 domain. From an analysis of the chemical shift changes as a function of increasing concentration of phosphopeptide, the binding affinities were measured (Table I). Phosphopeptides derived from the z chain of the T-cell receptor and the EGFR (yp1173) bind more tightly to the Shc SH2 domain compared with the other tyrosine-phosphorylated peptides examined in this study. Both of these peptides contain a hydrophobic residue at the Tyr(P) 1 position, which is consistent with the specificity for binding to the Shc SH2 domain determined on the basis of a degenerate phosphopeptide library (11) as well as analysis of NMR structure of Shc SH2 domain-phosphopeptide complex (12). Although it has been suggested that the Shc SH2 domain binds to peptides containing an NpXpY sequence and recognizes amino acid residues NH2-terminal to the phosphotyrosine such as in NPXpY sequences (23), peptides containing this sequence bind only weakly to the Shc SH2 domain (Table I). Furthermore, the unphosphorylated peptide does not exhibit any detectable binding affinity to the Shc SH2 domain.

In a previous study (13), the binding affinity of an EGFR 1173 phosphopeptide to the Shc SH2 domain was measured using surface plasmon resonance. A Kd of 65 nm was reported (13), which is 3 orders of magnitude different from the value that we obtained (60 μM) for the same interaction. The fact that we were able to follow the 15N/1H signals of the SH2 domain during the NMR titration strongly indicates that the binding affinity is not in the nanomolar range. It should be noted that
Binding of Phosphopeptides to the Shc SH2 and PTB Domains

A GST-Sh2 fusion protein was used to measure the dissociation constants in the previous study. It has been recently shown that due to the possible dimerization of the GST fusion proteins, binding affinities could be greatly overestimated (20), which could explain the differences in the measured affinities.

Binding of Phosphopeptides to the Shc PTB Domain—The three phosphopeptides that exhibit the tightest binding to the Shc SH2 domain all bind relatively weakly to the NH2-terminal domain of Shc (Table I). The binding affinities of these phosphopeptides to the PTB domain were able to be measured using NMR by following the chemical shift changes of the indole NH of Trp24 and Trp38 (Fig. 1B) or several well resolved amide signals of the protein that were also shifted as a function of increasing concentration of phosphopeptide. However, the NXPXY-containing phosphopeptides were found to bind much more tightly to the NH2-terminal domain of Shc. Upon the addition of these phosphopeptides, the 15N/H signals of several residues of the free protein decreased in intensity, and new signals appeared that corresponded to the peptide-bound form of the Shc PTB domain (Fig. 2). Thus, the 15N/H signals undergo slow exchange on the NMR timescale between the free and complexed forms of the protein, which is indicative of tight binding. Since the chemical shift changes could not be followed upon the addition of phosphopeptide, the binding affinities could not be measured by NMR. Instead, for these phosphopeptides, binding to the Shc PTB domain was examined by surface plasmon resonance using nonfusion protein of Shc PTB domain. Fig. 3A illustrates a typical SPR sensogram for binding of the PTB domain to middle T-antigen peptide (pY250). The rapid response in the association and dissociation phases of the response curves indicates that the binding of the PTB domain to the phosphopeptide occurs at an extremely fast association rate ($K_{on}$) as well as a rapid dissociation rate ($K_{off}$). These rates are similar to those observed previously for high affinity SH2 domain-phosphopeptide complexes (24). Qualitative measurement of the $K_{on}$ and $K_{off}$ rates in this case proved to be difficult due to a poorly exchanging boundary layer forming between the bulk buffer and analytical surface (22, 24). However, the dissociation constant ($K_d$) can be determined via Scatchard analysis using the directly measured response at equilibrium with several protein concentrations. The Scatchard plot of cRU versus cRU/[PTB] is linear (Fig. 3B), indicating the binding of the PTB domain follows a bimolecular process.

The dissociation constants measured by SPR and NMR are reported in Table I. All of the phosphorylated peptides containing the NXPXY sequence bind much more tightly to the PTB domain of Shc compared with the SH2 domain. Thus, the NXPXY sequences such as those found in EGFR, ErbB2, ErbB3, TrkA, and polyoma virus middle T-antigen are recognized by the ShcPTB domain rather than the SH2 domain. The three phosphopeptides that do not contain an NXPXY sequence bind much more weakly to the PTB domain, suggesting that the amino acid residues NH2-terminal to the phosphorylated tyrosine are critical for interacting with the Shc PTB domain. The unphosphorylated peptide does not exhibit any binding affinity for the PTB domain of Shc.

Phosphopeptide Inhibition of GST-Shc PTB Association with p145—It has recently been demonstrated that the PTB domain of Shc interacts with a ≈145-kDa protein (p145) upon platelet-derived growth factor or T-cell receptor stimulation (7, 17). To determine whether the different peptides that bind to Shc PTB can inhibit the PTB-p145 interaction, GST-Shc PTB fusion proteins bound to glutathione-Sepharose beads were incubated with different peptides and then mixed with K562 cell lysates (which contain phosphorylated p145). Then, the precipitation of p145 by the PTB domain was analyzed by antiphosphotyrosine immunoblotting. Quantitation of the bands by densitometry revealed that the ability of the peptides to compete with the p145 for binding to PTB correlates well with the binding affinity determined by NMR titration and surface plasmon resonance. TrkA pY1490 and ErbB3 pY1309 peptides, which have the highest affinity, completely inhibited the PTB-p145 interaction, while EGFR pY992 and pY1173, which have the lowest affinity, failed to inhibit this interaction. The immunoblot for inhibition with some of the peptides is shown in Fig. 4, and the percent inhibition for various peptides is given in Table I. These data indicate that the NXPXY motif may be relevant for binding of the Shc PTB domain to p145 in vivo.

**DISCUSSION**

In this study we have compared the binding affinities of the PTB and SH2 domains of Shc for a number of phosphorylated peptides derived from proteins that are known to interact with Shc. The nerve growth factor receptor (TrkA), ErbB2, ErbB3, and polymer virus middle T-antigen all have only one binding site for Shc, and all contain an NXPXY sequence motif. The Shc PTB domain binds to these NXPXY-containing peptides 3 orders of magnitude more strongly compared with the SH2 domain. Moreover, the measured binding affinities of the peptides are in good agreement with their ability to inhibit the interaction between the tyrosine-phosphorylated protein p145 and the nerve growth factor receptor (TrkA), ErbB2, ErbB3, and polymer virus middle T-antigen.
and the Shc PTB domain in growth factor-stimulated cell lysates.

In contrast to SH2 domains which mainly recognize amino acid residues COOH-terminal to the phosphotyrosine, the PTB domain of Shc binds to amino acid residues NH2-terminal to the phosphotyrosine. In particular, residues Asn and Pro at Tyr(P)23 and Tyr(P)22 positions, respectively, are essential for binding to the Shc PTB domain. Peptides or proteins that lack either the Asn or Pro residue markedly decrease the binding to the PTB domain. This is consistent with recent studies of the association of Shc with ErbB3 (2), polyoma virus middle T-antigen (25, 26), and the insulin receptor (9) in which mutations of either Asn (Tyr(P)23) or Pro (Tyr(P)22) abolish the binding to Shc. Mutations of the Tyr(P)13 residue have much less of an effect on the binding to Shc (9). Indeed, as shown in Table I, peptides containing a Gln, Thr, Asp, Glu, and Val in this position can all bind to Shc PTB domain. These results are also consistent with the preferential binding of NPXpY sequences determined using a degenerate library of phosphotyrosine-containing peptides (18) as well as with recent studies using alanine substitution (19).

Peptides that do not contain an NPXpY motif, such as the phosphopeptide derived from the z chain of the T-cell receptor, or two sites on the EGFR (pY992 and pY1173) preferentially bind to the Shc SH2 domain. These peptides contain a hydrophobic residue at the Tyr(P)13 position, which is important for binding to this SH2 domain (10–12). Other parts of the EGFR (pY1148, pY1086, and pY1114) bind tightly to the PTB domain of Shc. Thus, it appears that both the PTB and SH2 domains of Shc can bind to different portions of the EGFR. Considering that the EGFR pY1114 and pY1086 sites are not the major autophosphorylation sites and that the binding affinities of the

### Table I

| Protein  | Tyr(P) sites | Tyr(P) Peptides          | She SH2 Kd | She PTB Kd | Inhibition of Shc PTB to p145 interaction |
|----------|--------------|--------------------------|------------|------------|------------------------------------------|
| Human TrkA, pY490 | HIIENPQYFSDA | 280 | 0.053 | 100 |
| Mouse polyoma MT, pY250 | SLLSNPQPSVMRS | 320 | 0.140 | 100 |
| Human ErbB3, pY1309 | SAIDNPyWHSLRF | 230 | 0.020 | 100 |
| Human ErbB2, pY1248 | AENENPQYGLDLV | 1290 | ND | ND |
| Human EGFR, pY1086 | GSVQNPQYNNQPLN | 220 | 5.30 | ND |
| Human EGFR, pY1148 | SLDDNPyQDQDDF | 1000 | 0.200 | ND |
| Human EGFR, pY1114 | TAVGNNPyLNTVQ | 290 | 0.140 | ND |
| Human EGFR, pY992 | VADASPQYLLQQ | 130 | 680 | 0 |
| Human EGFR, pY1173 | TANAGPQXLRA | 60 | 770 | 0 |
| Human TCR CD3 z chain, pY141 | GHDGLPYQGLSTATK | 50 | >10000 | 0 |
| Human TrkA, Y490 | HIIENPQYFSDA | ND | >20000 | ND |
| Human TCR CD3 z chain, Y141 | GHDGLPYQGLSTATK | >10000 | ND | ND |

* NMR titrations were done in 50 mM Tris/HCl buffer (pH 6.5) containing 5 mM DTT at 25 °C.
* SPR measurements were performed in 10 mM HEPES, 150 mM NaCl, 20 mM DTT, 0.05% surfactant P20 (pH 7.4) at 25 °C.
* Cell lysates were precipitated with GST-Shc PTB in the presence of peptides and immunoblotted with antibody to phosphotyrosine. The p145 band was quantitated by densitometry and the percent inhibition was determined compared with the no peptide lane.
* ND, not determined.
Binding of Phosphopeptides to the Shc SH2 and PTB Domains

Shc PTB domain to tyrosine-phosphorylated peptides is generally higher than those of the Shc SH2 domain, it appears that EGFR, pY1148 may be the primary binding site for Shc. This observation is in agreement with Okabayashi et al. (23) but disagrees with Schlessinger and co-workers (13) in which they report the major and minor binding sites, respectively, for Shc on the EGFR. This apparent discrepancy can be explained by the fact that only the SH2 domain of Shc was used in the work of Schlessinger and co-workers (12).

The binding specificity and lack of sequence homology to SH2 domains suggest that the PTB domain of Shc is a new protein module capable of binding tyrosine-phosphorylated proteins. Due to the differential use of translational sites and alternative splicing, Shc has three isoforms (46, 52, and 66 kDa), which differ in the extent of their amino-terminal sequences (1). However, the functional difference of these three isoforms is not clear. Although it has been reported that the minimal binding region of Shc PTB domain comprises amino acid residues 46–209, which was concluded largely based on studies using GST fusion proteins, the apparent binding affinity of the GST-Shc(46–209) to the tyrosine-phosphorylated proteins is substantially lower than that of the GST-Shc(1–209) (7, 8). Furthermore, in in vivo studies only the 52- and 66-kDa proteins of Shc bind to the tyrosine-phosphorylated middle T-antigen via the NPxY motif (16, 27). In fact, in an NMR titration experiment the non-fusion protein of Shc(46–207) showed no detectable binding to middle T-antigen peptide (pY250) at peptide concentrations up to 4 mM. Therefore, it is tempting to speculate that the 45-kDa isoform of Shc may be a PTB activity-deficient protein in cells. The three-dimensional structure of the NH2-terminal PTB domain of Shc complexed to a phosphotyrosine-containing peptide (in progress) will reveal the overall fold of this new protein module and delineate the important molecular interactions that stabilize the PTB-phosphopeptide complex.

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Binding Affinities of Tyrosine-phosphorylated Peptides to the COOH-terminal SH2 and NH-terminal Phosphotyrosine Binding Domains of Shc
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