MOLECULAR AND CELLULAR BIOLOGY, Aug. 1995, p. 4403–4409 Vol. 15, No. 8
0270-7306/95/$04.00+0

The Phosphotyrosine Interaction Domain of Shc Binds an LXNPXY Motif on the Epidermal Growth Factor Receptor

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Received 27 March 1995/Returned for modification 28 April 1995/Accepted 22 May 1995

Shc is an SH2 domain protein that is tyrosine phosphorylated in cells stimulated with a variety of growth factors and cytokines. Once phosphorylated, Shc binds the Grb2-Sos complex, leading to Ras activation. Shc can interact with tyrosine-phosphorylated proteins by binding to phosphotyrosine in the context of an NPXpY motif, where pY is a phosphotyrosine. This is an unusual binding site for an SH2 domain protein whose binding specificity is usually controlled by residues carboxy terminal, not amino terminal, to the phosphotyrosine. Recently we identified a second region in Shc, named the phosphotyrosine interaction (PI) domain, and we have found it to be present in a variety of other cellular proteins. In this study we used a dephosphorylation protection assay, competition analysis with phosphotyrosine-containing synthetic peptides, and epidermal growth factor receptor (EGFR) mutants to determine the binding sites of the PI domain of Shc on the EGFR. We demonstrate that the PI domain of Shc binds the LXNPXpY motif that encompasses Y-1148 of the activated EGFR. We conclude that the PI domain imparts to Shc its ability to bind the NPXpY motif.

After activation by a ligand, receptor-linked protein tyrosine kinases undergo autophosphorylation, establishing several binding sites for proteins with SH2 domains. The binding of SH2 domain-containing proteins to the autophosphorylated growth factor receptors then permits a variety of signaling cascades to be activated. The immediate sequences carboxy terminal to the phosphotyrosine are the primary determinant of specificity for SH2 domain binding (references 10, 27, and 36 and references therein). One of the SH2 domain proteins that bind to activated growth factor receptors is Grb2. The preferential binding site for the Grb2 SH2 domain is the sequence pY-L/I/V-N (3, 29, 35, 38), where pY is a phosphotyrosine. Grb2 is bound via its two SH3 domains to the Ras guanine nucleotide releasing factor, Sos (7, 9, 14, 22, 34). The binding of Grb2 to activated growth factor receptors brings the Sos protein to the membrane, where it acts to convert Ras from an inactive GDP-bound state to an active GTP-bound state (1, 32). Activated Ras initiates a kinase cascade that relays signal from the cell surface to the nucleus.

Grb2 can also bind to other tyrosine-phosphorylated proteins in the cell that display the pY-L/I/V-N motif. One of these proteins is the SH2 domain protein, Shc, that becomes tyrosine phosphorylated after cell activation with a large number of cytokines and growth factors (11, 28, 31). She can bind a number of tyrosine-phosphorylated growth factor receptors, including epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and TrkA (25, 28, 30, 37, 41). It also binds tyrosine-phosphorylated middle T antigen (MTAg) (8, 12). However, unlike most SH2 domain proteins, whose binding sites depend on phosphotyrosine and residues lying carboxy terminal to it, Shc binds an NPXpY motif on several of these tyrosine-phosphorylated proteins (8, 12, 25, 30, 41). Thus, Shc selects its tyrosine-phosphorylated binding site on the basis of residues amino terminal to phosphotyrosine, suggesting that mechanisms other than SH2 domain-mediated interactions might be involved.

In agreement with this theory, recent studies from our laboratory have identified a second region in Shc that can bind tyrosine-phosphorylated growth factor receptors (5). This domain encompasses amino acids 46 to 209 of the 52-kDa form of Shc. A similar region of Shc has also been identified by Kavanaugh and Williams as binding an unidentified 145-kDa protein in a phosphotyrosine-dependent fashion (19). We have termed this region the phosphotyrosine interaction domain (PI domain), while others have referred to it as the phosphotyrosine binding domain (19). We have recently identified several other proteins that also appear to contain a PI domain (6). In this paper we demonstrate that the Shc PI domain binds an NPXpY site that surrounds tyrosine 1148 on the EGFR. We also identify an important role for residues amino terminal to the NPXpY motif in mediating PI domain binding. We propose that Shc binding to the EGFR is mediated mainly via binding of the PI domain to Y-1148 and that it may be further stabilized by binding of the SH2 domain to Y-1173.

MATERIALS AND METHODS

Cell lines, immunoprecipitation, and immunoblotting. Cell lines overexpressing the human EGFR or the mutant EGFRs used in this study were the previously characterized HER14, Y1148F, and Y1173F (16). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. Prior to stimulation, the cells were starved for 24 h in 0.1% CS–DMEM. Cells were then stimulated with EGF (275 ng/ml) for 3 min at room temperature, washed with ice-cold phosphate-buffered saline, and lysed in lysis buffer {50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] [pH 7.5], 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 25 mM NaF, 50 μM $ZnCl_2$, 250 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 50 μg of trypsin inhibitor from soybeans per ml, 50 μg of pepstatin per ml}. Lysate protein content was normalized as described previously (3). Cell lysis, immunoprecipitation, and immunoblotting using ^{125}I-protein A (ICN) were performed as previously described (23). Immunoblotting using horseradish peroxidase-protein A (Kirkegaard and Perry) was performed with chemiluminescence reagents from NEN/Dupont or Boehringer Mannheim.

Antibodies. Anti-EGFR antibodies used in this study were mAb108, a mouse monoclonal antibody directed against domain III of the extracellular domain (4), and RK2, a rabbit antibody directed against a C-terminal peptide (residues 984 to 996) (20). RK2 and mAb108 were used for immunoblotting and immunopre-

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cipitation experiments, respectively. Polyclonal antiphosphotyrosine and anti-glutathione *S*-transferase (GST) antibodies were raised in rabbits against alanine-glycine-phosphotyrosine polymers coupled to keyhole limpet hemocyanin (17) and purified GST, respectively. Rabbit polyclonal anti-Shc antibodies were generated against the GST-Shc SH2 domain (human, residues 366 to 473 [28]) or purchased from Transduction Laboratories.

Peptide synthesis. A 9-fluorenylmethoxycarbonyl (Fmoc)-based strategy for peptide synthesis was used in conjunction with standard side chain-protecting groups as described previously (2). Fmoc-L-tyrosine (PO_3H_2)-OH (Anaspec, Inc.) was used for incorporation of phosphotyrosine. Peptides were purified by ether precipitation and preparative reverse-phase high-pressure liquid chromatography (HPLC). Analytical HPLC demonstrated that the products were purified to homogeneity; mass spectroscopy was performed to confirm the accuracy of synthesis.

Fusion proteins. GST fusion proteins of Shc residues 1 to 209 (Shc 1-209), Shc 46-209, the Shc SH2 domain, and the Grb7 SH2 domain were previously described (5, 40). Histidine-tagged Shc 1-209 (Shc His-1-209) was prepared by cutting the insert from pGEX2T encoding SHC 1-209 with *Eco*RI and ligating it into *Eco*RI-digested pET 28c (Novagen). Histidine-tagged proteins were expressed and purified on nickel beads according to the manufacturer's instructions. GST fusion proteins were purified on glutathione-agarose as previously described (3).

Peptide and fusion protein competition assays. For the competition experiments, the EGFR was immunoprecipitated from lysates of A431 cells starved for 48 h in 0.1% fetal bovine serum and prepared without tyrosine phosphatase inhibitors. The immunoprecipitates were washed four times with lysis buffer and subjected to in vitro autophosphorylation in the presence of 0.2 mM ATP and 10 mM $MnCl_2$ for 5 min at room temperature. After washing, the autophosphorylated EGFR was divided into equal aliquots (approximately 0.5 pmol) and incubated with 20 pmol of either Shc PI domain-GST (Shc 1-209) or Shc SH2-GST (human, residues 366 to 473 [28]) in the absence or presence of 50 pmol of the various phosphopeptides in a volume of 100 μl for 1 h at 4°C. After three washes with lysis buffer, the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose and then immunoblotted with anti-GST antibodies and analyzed by chemiluminescence as described above. To determine phosphopeptide concentrations necessary for half-maximal inhibition of PI or SH2 domain binding (ID_{50}s) competition experiments were performed in the presence of increasing amounts of phosphopeptides from 0 to 10 nmol. Immunoblotting was performed with ^{125}I-protein A, and the amount of bound protein was then quantified by using a PhosphorImager. One hundred percent binding was defined as the amount of protein bound in the absence of competing phosphopeptide.

Similar methods were used to determine the ability of peptides to inhibit the binding of full-length Shc to EGFR. After in vitro autophosphorylation of the EGFR as described above, full-length Shc (from 293 cells) was added to the receptor with or without various peptides. Incubations and analysis were then undertaken as described above, except that blotting was performed with anti-Shc antibodies. In another set of experiments, PI or SH2 domain fusion proteins were used instead of peptides to inhibit the binding of full-length Shc to the EGFR.

EGFR dephosphorylation protection assays. Identification of the tyrosine phosphorylation site to which the PI domain of Shc binds was performed by using a modified version of the dephosphorylation protection assay developed by Rotin et al. (33). Briefly, EGFR was immunoprecipitated from A431 cell lysate (cells starved for 48 h and lysed without tyrosine phosphatase inhibitors) and phosphorylated in vitro with 10 μCi of [γ-^{32}P]ATP (per assay), 2 μM unlabelled ATP, and 10 mM $MnCl_2$. The phosphorylated receptor was washed (four times) with ice-cold 20 mM HEPES, pH 7.5, and then dephosphorylated for 30 min at room temperature with A431 cell lysates (without tyrosine phosphatase inhibitors) in the presence of 0, 0.5, 1, or 2 μM Shc 1-209–GST; 0, 0.5, 1, or 2 μM Shc 46-209–GST; 0, 0.5, 1, or 2 μM Shc His-1-209; or 50 μM Shc SH2-GST fusion protein (human, residues 366 to 473 [28]). Proteins were then separated by SDS–8% PAGE, and the bands corresponding to the EGFR were excised. The gel pieces were swollen in 10% methanol, dried in a speed vacuum, and digested with 5 μg of trypsin in 1 ml of 50 mM ammonium bicarbonate overnight at 37°C. After filtration, the samples were dried again and digested for a second time with 5 μg of trypsin in 100 μl of 50 mM ammonium bicarbonate overnight at 37°C. The tryptic fragments were purified by HPLC using a C18 reverse-phase column with 0.1% trifluoroacetic acid in H_2O (buffer A) or in acetonitrile (buffer B), at a flow rate of 1 ml/min. The following gradient was used: 0 to 10 min, 0% buffer B; 10 to 50 min, linear gradient to 40% buffer B; and 50 to 51 min, increase to 60% buffer B. The assignment of tryptic fragments separated by HPLC to the autophosphorylation sites of the EGFR was described earlier (13, 24, 33, 43).

Blotting analysis with GST fusion proteins (far-Western blot). The eluted GST fusion proteins (3) were diluted in Tris-buffered saline–5% bovine serum albumin including 5 mM dithiothreitol. The nitrocellulose filters were incubated with the fusion protein solutions for 1 h at room temperature and then washed and immunoblotted with anti-GST antibodies according to the above-mentioned protocols.

RESULTS

The Shc PI domain prevents dephosphorylation of the EGFR. To determine the site on EGFR that interacts with the Shc PI domain, we utilized the dephosphorylation protection assay (33). This assay has been previously used to map the sites of SH2 domain interaction, as binding of an SH2 domain can protect a phosphotyrosine residue from dephosphorylation by cellular tyrosine phosphatases (3, 33). In our initial identification of the PI domain we first identified amino acids 1 to 209 of the 52-kDa form of Shc as a region that is able to bind the EGFR. This was subsequently narrowed down to a minimum binding domain consisting of residues 46 to 209 (5). We found that either GST or histidine-tagged Shc 1-209 and Shc 46-209 can protect the EGFR from dephosphorylation at the same tyrosine autophosphorylation site (Fig. 1 and data not shown). However, the Shc PI and SH2 domains protected different autophosphorylation sites on the EGFR, with the PI domain protecting phosphorylated Y-1148 (pY-1148) and the SH2 domain protecting pY-1173 from dephosphorylation (Fig. 1B). The PI domain was effective at preventing dephosphorylation at concentrations lower than that required for the Shc SH2 domain and approximately equivalent to that required for protection of Y-1068 by the Grb2 SH2 domain (3). While the PI domain primarily protected Y-1148 at a concentration of 0.5 μM (Fig. 1B), we found that the PI domain could also partially protect both Y-1173 and Y-1086 at 2 μM (data not shown). We were not able to analyze the protection profile of full-length GST-Shc protein, since it was rapidly degraded by proteolysis during bacterial expression.

Peptide competition analysis confirms pY-1148 as the binding site for the Shc PI domain. To further analyze the binding sites for the Shc PI domain, we studied the ability of synthetic phosphopeptides to compete for the binding of the Shc SH2 and PI domains to EGFR. We used synthetic phosphopeptides corresponding to the EGFR autophosphorylation sites that had variable numbers of residues carboxy terminal and amino terminal to the phosphotyrosine moiety (Table 1) (3). The peptide nomenclature is based on the number of residues lying carboxy terminal or amino terminal to the phosphorylated tyrosine. Thus, pY1148-7Y4 contains phosphotyrosine at Y-1148 with seven residues amino terminal and four residues carboxy terminal to it. As had been demonstrated previously, peptides containing phosphotyrosine at Y-1173 were able to compete for the binding of the Shc SH2 domain to the EGFR at submicromolar concentrations (Table 1). In contrast, the peptide pY1148-7Y4, but not the other EGFR peptides, was able to efficiently compete for the binding of the PI domain (Table 1 and Fig. 2A). A peptide containing the Shc binding site on the TrkA receptor (Y-490) with 10 residues amino terminal to the phosphotyrosine was also able to compete for binding of the PI domain to the EGFR (Table 1).

Both TrkA pY-490 and EGFR pY-1148 peptides contain the NPXpY motif. Accordingly, we tested various peptides containing mutations in this motif (Table 1 and Fig. 2C). Changing the phosphotyrosine to tyrosine in the EGFR Y1148-7Y4 peptide severely impaired the ability of the peptide to compete for PI domain binding. Altering asparagine to alanine (pY1148-7Y4 NA) limited the ability of the peptide to compete, whereas changing the proline to alanine (pY1148-7Y4 PA) had only a minor effect. Unlike the pY1148-7Y4 peptide, which contains seven residues amino terminal to the phosphotyrosine, the pY1148-4Y10 peptide containing four residues amino terminal to the phosphotyrosine could not compete for PI domain binding (Fig. 2A and B). This analysis indicated that a peptide containing only NPX amino terminal to the phosphotyrosine

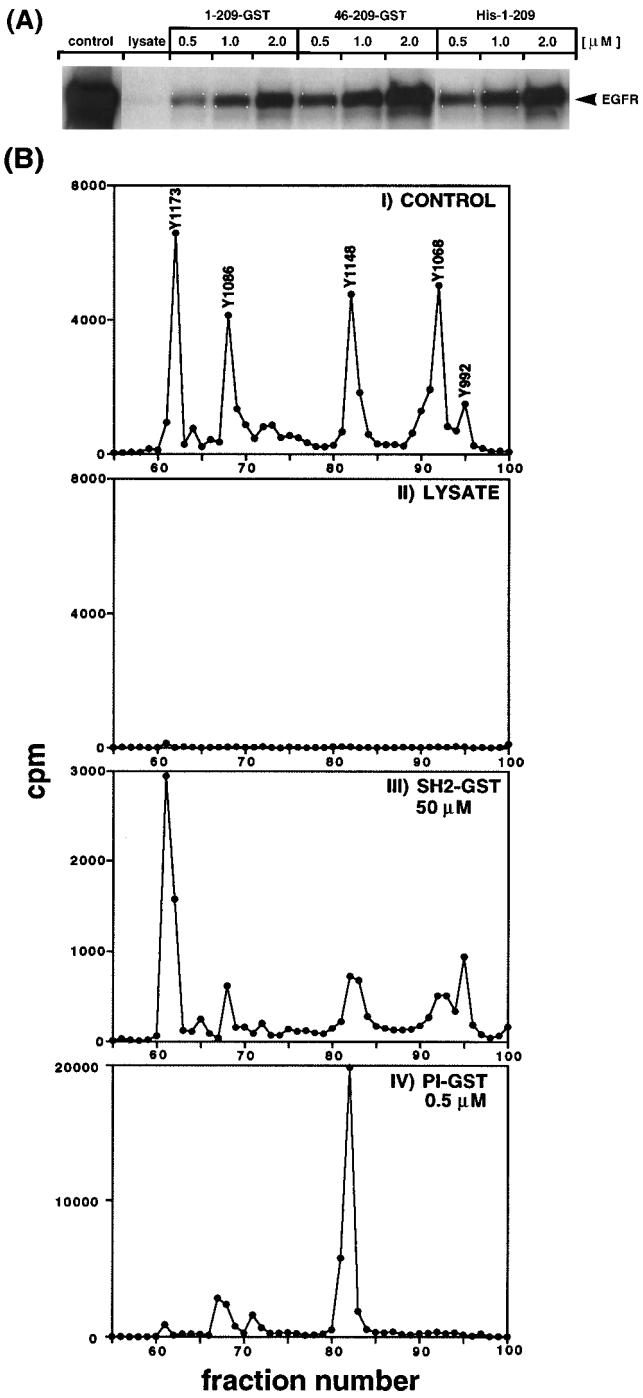

FIG. 1. Dephosphorylation protection assay using the PI and SH2 domains of Shc. The dephosphorylation protection assay was performed as described in Materials and Methods. (A) The Shc PI domain protects the EGFR from dephosphorylation. The autophosphorylated EGFR was protected from dephosphorylation with increasing concentrations of GST fusion proteins containing Shc amino acids 1 to 209 of the 52-kDa form of Shc (1-209-GST) or amino acids 46 to 209 of the 52-kDa form of Shc (46-209-GST). Also shown is protection by a histidine-tagged form of Shc 1-209 (His-1-209). Control, the autophosphorylated receptor without dephosphorylation; lysate, dephosphorylation in the absence of GST or histidine-tagged proteins. (B) Dephosphorylation of Y-1148 and Y-1173 is protected against by the PI and SH2 domains of Shc, respectively. Reverse-phase chromatography profiles of the control (I), lysate (II), 50 μM Shc SH2-GST plus lysate (III), and 0.5 μM Shc PI domain-GST (Shc 1-209) plus lysate (IV) are shown. The following gradient was used: 0 to 10 min, 0% acetonitrile; 10 to 50 min, linear gradient to 40% acetonitrile; and 50 to 51 min, increase to 60% acetonitrile.

TABLE 1. ID_{50}s for phosphopeptide inhibition of Shc PI domain-GST and Shc SH2-GST binding to the EGFR[a]

Phosphopeptide	Sequence	ID_{50} (μM)
PI domain-GST		
pY992-4Y10	DADE**pY**LIPQQGFFSK	>100
pY1068-4Y9	PVPE**pY**INQSVPKRK	>100
pY1086-4Y11	QNPV**pY**HNQPLNPAPSK	>100
pY1173-4Y10	ENAE**pY**LRVAPQSSEK	>100
pY992-8Y4	DDVVDADE**pY**LIPQ	>100
pY1086-8Y3	AGSVQNPV**pY**HNQ	30
pY1173-8Y4	GSTAENAE**pY**LRVA	30
pY1148-4Y10	DNPD**pY**QQDFFPKEAK	>100
pY1148-7Y4	ISLDNPD**pY**QQDF	0.04 ± 0.01
pY1148-7Y4 NA	ISLDAPD**pY**QQDF	2.46 ± 0.25
pY1148-7Y4 PA	ISLDNAD**pY**QQDF	0.08 ± 0.02
pY1148-7Y4 LG	ISGDNPD**pY**QQDF	9.18 ± 3.37
Y1148-7Y4 noPY	ISLDNPD**Y**QQDF	90 ± 10
TrkA 490-10Y7	LQGHIIENPQ**pY**FSDACVH	0.08 ± 0.007
SH2-GST		
pY1173-4Y10	ENAE**pY**LRVAPQSSEK	0.4
pY1173-8Y4	GSTAENAE**pY**LRVA	0.4

[a] The determination of ID_{50}s is described in Materials and Methods and in the legend to Fig. 2. All phosphopeptides are derived from the sequence of the human EGFR, except TrkA 490-10Y7.

was not sufficient for high-affinity binding to the PI domain (Table 1). This suggests that residues further amino terminal to the NPXY motif were required for high-affinity interaction between tyrosyl-phosphorylated peptides and the PI domain. Our attention focused on a hydrophobic residue 5 amino acids amino terminal to the phosphotyrosine that has been found in Shc binding sites from several proteins (8). In the case of the EGFR Y-1148, this residue is a leucine, while in TrkA Y-490 it is an isoleucine (Table 1). Indeed, mutation of the leucine to glycine in the pY-1148 peptide (pY1148-7Y4 LG) severely impaired the ability of the peptide to inhibit PI domain binding to EGFR (Fig. 2C). This phosphopeptide competition analysis confirmed the results obtained from the dephosphorylation protection assay and pointed to an LXNPXpY motif as the binding site on the EGFR for the Shc PI domain.

Mutations of Y-1148 limit PI domain binding. We then utilized GST fusion proteins of Shc PI and SH2 domains as probes in far-Western blot analysis of EGFR with individual point mutations in the autophosphorylation sites (Fig. 3). The EGFR mutants were immunoprecipitated from cell lysates, subjected to SDS-PAGE, and transferred to nitrocellulose. Blotting solutions containing the Shc PI (Shc 1-209–GST) domain or Shc SH2–GST were then used to probe the blots and visualized with anti-GST antibodies. In agreement with the results presented above, mutations of Y-1148 to phenylalanine inhibited PI domain binding while mutation of Y-1173 to phenylalanine impaired SH2 domain binding.

Peptides containing pY-1148 and the Shc PI domain can compete with full-length Shc for binding to EGFR. To determine the role of the PI domain in binding Shc to the EGFR, we tested the ability of the phosphopeptides to block the binding of full-length Shc to EGFR in vitro (Fig. 4). Lysates containing Shc proteins were mixed with activated EGFR in the presence or absence of phosphopeptides, and the amount of Shc bound to the EGFR was measured by using anti-Shc antibodies. This analysis indicates that peptides that blocked PI domain binding could also compete for full-length Shc binding to EGFR. Similarly nonphosphorylated, Y1148-7Y4 NA, pY1148-7Y4 LG, and the pY-1173 peptides were not able to compete for Shc

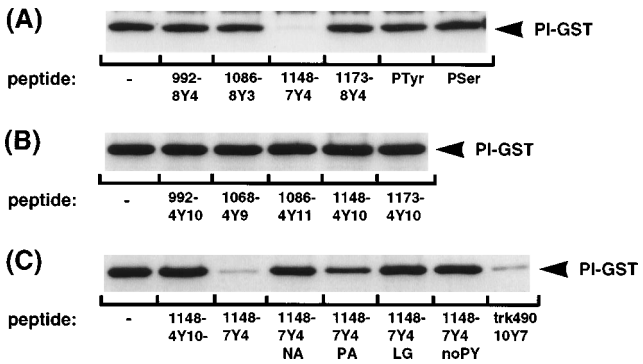

FIG. 2. Inhibition of Shc PI domain binding with synthetic phosphopeptides. EGFR was immunoprecipitated from starved A431 cells, autophosphorylated in vitro, and incubated with Shc PI domain-GST (0.2 μM) in the absence or presence of the indicated phosphopeptide (0.5 μM), phosphotyrosine (PTyr; 0.5 μM), or phosphoserine (PSer; 0.5 μM). Samples without addition (−) serve as a control for each panel. After washing, samples were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GST antibodies. (A) The ability of peptides with seven or eight residues amino terminal to the phosphotyrosine to inhibit the binding of the Shc PI domain. The phosphopeptide designation 992-8Y4 indicates that the competing peptide is composed of the sequence of the Y-992 autophosphorylation site with eight residues amino terminal and four residues carboxy terminal to the phosphotyrosine. (B) The inability of peptides with four residues amino terminal to the phosphotyrosine to inhibit the binding of the Shc PI domain. (C) The effect of alterations of the Y-1148 phosphopeptide on the ability of the peptide to inhibit binding of the PI domain to the EGFR. The NA peptide has the asparagine in the minus three position changed to alanine, the PA peptide has the proline in the minus two position altered to alanine, and the LG peptide has the leucine in the minus five position converted to glycine, while in the noPY peptide, the tyrosine is not phosphorylated. The upper parts of the nitrocellulose filters were immunoblotted with antiphosphotyrosine antibodies to confirm that equal amounts of phosphorylated EGFR were present in all lanes (data not shown). The band seen above the PI domain in some parts of the figure represents the immunoglobulin heavy chain.

binding to the EGFR. The pY1148-7Y4 PA peptide was able to inhibit Shc binding but to a lesser extent than the wild-type pY1148 peptide. We then examined the abilities of the Shc PI and SH2 domains to compete with full-length Shc for binding

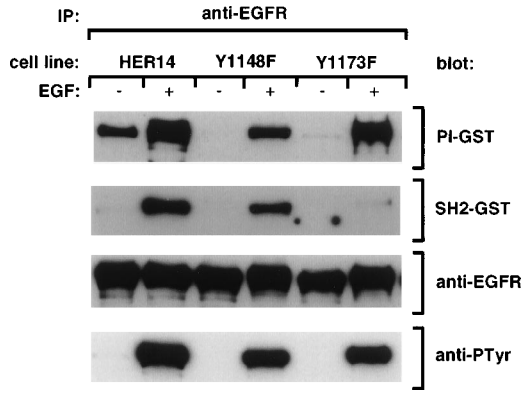

FIG. 3. Blotting of wild-type, Y1148F, and Y1173F EGFRs using Shc PI domain-GST or Shc SH2-GST. Cells were incubated in the absence (−) or presence (+) of epidermal growth factor (EGF) for 3 min at room temperature. Lysates were subjected to immunoprecipitation with anti-EGFR antibodies (MAb108) as described in Materials and Methods. Samples were loaded on four separate SDS gels and transferred to nitrocellulose. For the upper two panels, far-Western blotting was performed with the indicated fusion proteins at 0.1 μg/ml (Shc PI domain-GST [Shc 1-209]) and 5 μg/ml (Shc SH2-GST). For the lower two panels, immunoblotting was performed with anti-EGFR and antiphosphotyrosine (anti-PTyr) antibodies. Horseradish peroxidase-protein A and chemiluminescence were used for detection as described in Materials and Methods. Exposure times for the panels, from top to bottom, were as follows: 15 s, 3 min, 10 s, and 30 s.

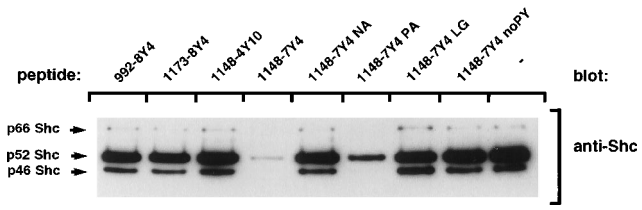

FIG. 4. Inhibition of binding of Shc to EGFR with synthetic phosphopeptides. EGFR was immunoprecipitated from starved A431 cells and autophosphorylated in vitro, and 100 μg of 293 lysates (as a source of Shc) was added in the absence or presence of the indicated phosphopeptide (1 μM). After binding and washing, samples were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Shc antibodies. The upper part of the nitrocellulose filter was immunoblotted with antiphosphotyrosine antibodies to confirm that equal amounts of phosphorylated EGFR were present in all lanes (data not shown).

to EGFR in a similar assay. The Shc PI domain, but not the SH2 domain of Shc or Grb7, was able to compete in vitro with full-length Shc for binding to the EGFR (Fig. 5).

DISCUSSION

In this study we sought to delineate the binding site on EGFR for the PI domain of Shc. Several independent pieces of evidence indicate that this domain binds with high affinity to residues encompassing the Y-1148 tyrosine autophosphorylation site of the EGFR. First, the Shc PI domain protects pY-1148 from dephosphorylation in the phosphatase protection assay. Second, peptides containing pY-1148 and the sequences amino terminal to it were able to compete with high affinity for the binding of the Shc PI domain to EGFR, whereas nonphosphorylated peptides or phosphorylated peptides containing other EGFR autophosphorylation sites were not. In support of this conclusion we have obtained similar data demonstrating that the PI domain of Shc binds the NPXpY site encompassing tyrosine 490 of TrkA (11a).

We further examined the residues surrounding Y-1148 for their importance in defining the specificity of PI domain binding. Synthetic peptides with only 4 amino acids amino terminal to pY-1148 could not compete with the PI domain for binding to the EGFR. However, peptides that contained seven residues amino terminal to phosphotyrosine Y-1148 competed effi-

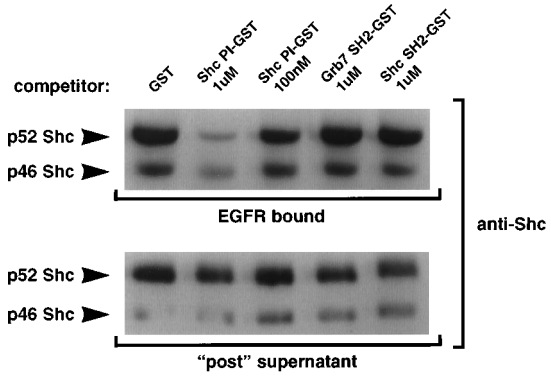

FIG. 5. Inhibition of binding of Shc to EGFR by the PI domain. Autophosphorylated EGFR was incubated with 293 lysates (as a source of Shc) in the presence of no fusion protein, 1 μM Shc 1-209–GST (Shc PI-GST), 100 nM Shc 1-209–GST, 1 μM Grb7 SH2-GST, and 1 μM Shc SH2-GST. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and detected with anti-Shc antibodies (upper panel). The lower panel demonstrates that equal amounts of Shc were recovered from the postbinding supernatants.

TABLE 2. Alignment of Shc binding sites[a]

Site	Sequence
Targets for the Shc PI domain	
EGFR position 1148	SLDNPDYQQDF
c-erbB-3 position 1309	AFDNPDYWHSR
v-erbB position 1154	NLDNPDYQQDF
TrkA position 490	IIENPQYFSDA
MTAg position 250	LLSNPTYSVMR
Predicted targets for the Shc PI domain	
TrkB	VIENPQYFGIT
TrkC	VIENPQYFRQG

[a] Described in references 8, 12, 21, 25, and 30 and this report.

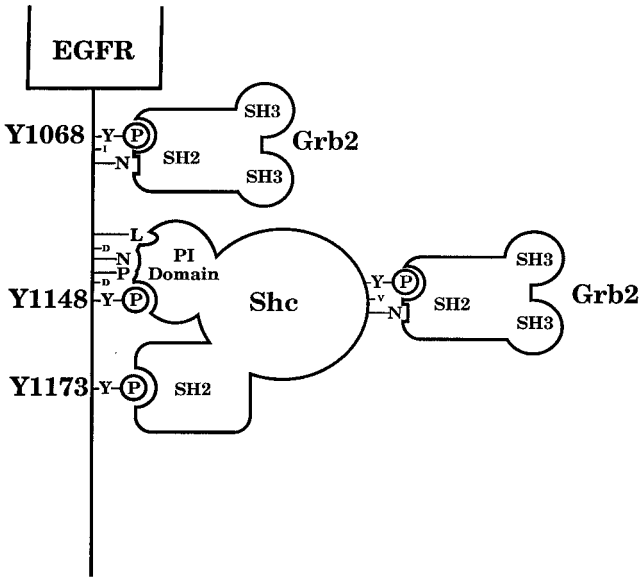

FIG. 6. Schematic diagram of a model for the interaction of Shc with the EGFR. The model suggests that a cooperative interaction between the PI and SH2 domains may be important in mediating Shc interaction with the EGFR. The PI domain of Shc binds to pY-1148, while the SH2 domain of Shc might stabilize the complex by binding to pY-1173. The Shc phosphorylation site at Y-317 supports Grb2 binding, as does pY-1068 on the EGFR via an asparagine residue at the plus two position. This creates redundant pathways for Ras activation. P, phosphorylation of tyrosines on the EGFR and Shc. Amino acids adjacent to the phosphorylation sites indicate the positions of residues important for the PI or SH2 domain binding. The residues carboxy terminal to pY-1173 that support Shc SH2 domain binding are uncertain at present.

ciently. Changing specific residues amino terminal to Y-1148 added further information as to the residues crucial for binding to the Shc PI domain. The initial alterations focused on asparagine 1145 and proline 1146, which formed part of the consensus NPXpY that has been shown to be the binding site for full-length Shc in TrkA, ErbB3, and MTAg (Table 2). Mutation of asparagine 1145 to alanine severely limited the binding ability of the pY1148-7Y4 peptide, but conversion of proline 1146 to alanine had a smaller effect. We also identified an important role for leucine 1143 of the EGFR in PI domain binding, confirming the analysis of Campbell et al., who identified a conserved hydrophobic residue in the minus five positions of Shc binding sites on MTAg and autophosphorylated growth factor receptors (8). In contrast to the case with SH2 domains, we find that the PI domain binding specificity is based on residues lying amino terminal to the phosphotyrosine. Clearly, altering the phosphotyrosine of the Y-1148 peptide to tyrosine severely limited the ability of the peptide to bind to the PI domain. Nonetheless, an affinity could be detected for the nonphosphorylated peptide, suggesting that the residues amino terminal to the phosphotyrosine provide significant binding energy for the interaction.

We find that the Shc SH2 domain interacts at Y-1173 (3) and the PI domain interacts at Y-1148 of the EGFR. However, the exact role of the SH2 domain versus the PI domain in mediating binding of Shc to EGFR in the context of living cells is probably more complicated. This issue has been examined by several different groups. Soler et al. demonstrated that mutation of both Y-1148 and Y-1173 was required to reduce association of Shc with EGFR (39). The same result was also obtained by Okabayashi and coworkers (26). However, in contrast to our results, these workers mapped the major binding site for the Shc SH2 domain to pY-1148 of the EGFR. Similar studies have also been carried out with the v-erbB protein, the product of the oncogene derived from the chicken EGFR (21). These results indicate that pY-1154 (equivalent to pY-1148 in human EGFR) is important for the association of Shc and chicken EGFR.

Our data indicate that both Shc SH2 and PI domains can interact with EGFR. However, the affinity of the PI domain is much higher than the affinity of the SH2 domain. The in vitro results presented in this report indicate that the PI domain may play the more important role. We have shown that a pY-1148 phosphopeptide can efficiently block the binding of full-length Shc to EGFR while a pY-1173 phosphopeptide cannot. Moreover, the PI domain can inhibit the binding of Shc to full-length EGFR, whereas the SH2 domain cannot. However, both our data (3a) and the data of others listed above indicate that in living cells both Y-1173 and Y-1148 need to be mutated in order to significantly reduce the binding of Shc to EGFR.

It has repeatedly been difficult to ascertain specific sites of binding to the EGFR for individual SH2 domain proteins. For example, while phospholipase Cγ binding can be attributed to one autophosphorylation site on the platelet-derived growth factor receptor, there are at least three sites on the EGFR that can support this binding (18, 33, 42). Furthermore, a specific binding site for Ras GAP on the EGFR cannot be specifically determined (3, 39). One explanation for this difficulty is that the close proximity of the autophosphorylation sites on the EGFR leads to locally high concentrations of SH2 and PI domain binding sites. The cooperative effect of the phosphotyrosines may allow EGFR to bind target molecules even when the primary site of interaction has been eliminated by mutagenesis. While this mechanism allows the EGFR to be more effective in binding target molecules, it makes it difficult to assign the specific roles of individual phosphotyrosines in binding target proteins. Despite these limitations, we can speculate as to the mechanism of interaction between Shc and EGFR in living cells. One explanation, depicted in Fig. 6, is that both the SH2 and PI domains can interact with the EGFR in vivo, with the PI domain binding to Y-1148 and the SH2 domain interacting at Y-1173. This would lead to a cooperative interaction supporting high-affinity interactions between Shc and the EGFR. Support for this explanation comes from our work that suggests that SH2 domain mutations can impair interactions between Shc and EGFR (5). In vitro we may detect a lesser role for the SH2 domain because the Shc SH2 domain may not attain the proper conformation when expressed in bacteria. Another explanation is that the Shc PI domain is more promiscuous when binding to the EGFR in vivo. Although the main binding site for the Shc PI domain is Y-1148, this domain might also be able to bind to Y-1173 or Y-1086, depending on

the local concentration of autophosphorylation sites. Inspection of these autophosphorylation sites does demonstrate similar residues amino terminal to the phosphotyrosines in Y-1148, Y-1173, and Y-1086 (Table 1). We also demonstrate in Fig. 3 that there is residual binding of the Shc PI domain to the EGFR Y1148F mutant. Thus, further experimentation will be necessary to determine the relative role of the SH2 domain versus the PI domain in mediating Shc interactions with the EGFR in living cells.

This uncertainty should not distract from the major finding of this paper, i.e., the delineation of the binding site for the Shc PI domain as an NPXpY motif. We also have strong evidence that the PI domain mediates the binding of Shc to TrkA (11a) and that it is involved in the interaction of Shc with MTAg, ErbB2, and ErbB3 (Table 2). It is possible that residues in addition to LXNPXpY are also important for PI domain binding. We have not formally tested all residues amino terminal to the phosphotyrosine but rather have focused on the residues that are conserved in the known Shc binding sites (Table 2). We did not detect a role for residues carboxy terminal to the phosphotyrosine, but we cannot yet exclude a role for residues in the minus 1 or minus 4 position. The finding that the Shc PI domain binds the sequence LXNPXpY suggests that other PI domains (6) may be involved in the binding of phosphotyrosine-containing peptides with specificities based on sequences amino terminal to the phosphotyrosine. Our results are somewhat in agreement with those reported by Gustafson et al. (15), who identified an NPXpY motif encompassing Y-960 on an insulin receptor that can bind the amino terminus of Shc and IRS1. However, the sequence surrounding the insulin receptor Y-960 does not contain a hydrophobic residue at the minus five position, and there is no evidence that Shc binds to the insulin receptor in living cells. Furthermore, the domain in IRS1 that binds the NPXpY motif on the insulin receptor is not similar to PI domains as we have defined them (6). This suggests that two possibly related structural domains can interact with the NPXpY motif on proteins involved in signaling pathways controlled by tyrosine kinases.

ACKNOWLEDGMENTS

We thank Valsan Mandiyan for the Shc 1-209 and Shc 46-209 constructs in pGEX-2T, Jane McGlade and Tony Pawson for human GST-Shc SH2, and Ron Beavis for mass spectroscopy analysis of the peptides.

This work was supported by grants from Sugen and the Lucille P. Markey Charitable Trust. B.M. is a Lucille P. Markey and Kaplan Cancer Center Scholar.

ADDENDUM IN PROOF

While the manuscript was in preparation, the binding of the Shc amino terminus to an NPXY motif was also reported elsewhere (P. van der Geer, S. Wiley, V. K.-M. Lai, J. P. Olivier, G. D. Gish, R. Stephens, D. Kaplan, S. Shoelson, and T. Pawson, Curr. Biol. **5:**404–412, 1995).

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