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Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites

(tyrosine phosphorylation/signal transduction)

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ABSTRACT **The Shc adaptor protein contains two phosphotyrosine [Tyr(*P*)] binding modules—an N-terminal Tyr(*P*) binding (PTB) domain and a C-terminal Src homology 2 (SH2) domain. We have compared the ability of the Shc PTB domain to bind the receptors for nerve growth factor and insulin, both of which contain juxtamembrane Asn-Pro-Xaa-Tyr(*P*) motifs implicated in PTB binding. The Shc PTB domain binds with high affinity to a phosphopeptide corresponding to the nerve growth factor receptor Tyr-490 autophosphorylation site. Analysis of individual residues within this motif indicates that the Asn at position −3 [with respect to Tyr(*P*)], in addition to Tyr(*P*), is critical for PTB binding, while the Pro at position −2 plays a less significant role. A hydrophobic amino acid 5 residues N-terminal to the Tyr(*P*) is also essential for high-affinity binding. In contrast, the Shc PTB domain does not bind stably to the Asn-Pro-Xaa-Tyr(*P*) site at Tyr-960 in the activated insulin receptor, which has a polar residue (Ser) at position −5. Substitution of this Ser at position −5 with Ile markedly increased binding of the insulin receptor Tyr-960 phosphopeptide to the PTB domain. These results suggest that while the Shc PTB domain recognizes a core sequence of Asn-Pro-Xaa-Tyr(*P*), its binding affinity is modulated by more N-terminal residues in the ligand, which therefore contribute to the specificity of PTB–receptor interactions. An analysis of residues in the Shc PTB domain required for binding to Tyr(*P*) sites identified a specific and evolutionarily conserved Arg (Arg-175) that is uniquely important for ligand binding and is potentially involved in Tyr(*P*) recognition.**

Shc is a member of a group of cytoplasmic signaling proteins we have collectively termed adaptor proteins (1). Shc proteins become phosphorylated on tyrosine after stimulation with a wide variety of growth factors and cytokines (2–10). Phosphorylation of mammalian Shc on Tyr-317 creates a binding site for the SH2 domain of Grb2, which is in turn associated with the Ras guanine nucleotide exchange factor Sos (11). The interaction of Shc with the Grb2–Sos complex may provide a mechanism for Ras activation (3, 12–17).

Shc contains an N-terminal phosphotyrosine [Tyr(*P*)] binding (PTB) domain, a central Gly/Pro-rich region that contains the principal Tyr phosphorylation site at Tyr-317, and an SH2 domain at its C terminus. The Shc SH2 domain recognizes Tyr(*P*) in the context of C-terminal residues and binds preferentially to phosphopeptides with the sequence pY-E/L/I/Y-X-L/I/M, where pY is Tyr(*P*) (18). The PTB domain, which is highly conserved in Shc-related proteins, was recently identified as a sequence of ≈160 amino acids that bind specific Tyr(*P*)-containing proteins (19–22). Both the sequence and binding properties of the Shc PTB domain appear quite different from those of known SH2 domains.

Shc binds to activated growth factor receptors through the recognition of phosphorylated Tyr residues that in many cases lie within the sequence NPXpY (23, 24). The Shc PTB domain binds to the activated nerve growth factor receptor (NGFR) and the activated epidermal growth factor receptor (EGFR), which possess such NPXpY motifs, and to a 145-kDa protein that becomes phosphorylated on Tyr in platelet-derived growth factor-stimulated cells (19–21, 25). The NGFR binds Shc through a single juxtamembrane autophosphorylation site, Tyr-490, that lies within an NPXY element (23, 26). A mutant NGFR with Phe in place of Tyr-490 fails to interact with Shc *in vivo* or with the Shc PTB domain *in vitro* (19, 23).

These results suggest that the Shc PTB domain recognizes Tyr(*P*) residues within the sequence NPXpY and, therefore, differs from SH2 domains in the sense that it recognizes specific residues N terminal to the Tyr(*P*). Consistent with this view, the Shc PTB domain recognizes specific phosphopeptides with such motifs *in vitro* (25, 27–30). Here we have investigated the residues in both the PTB domain and its ligands that control complex formation.

MATERIALS AND METHODS

Cell Lines, Antisera, and Fusion Proteins. CHO cells expressing wild-type (wt) insulin receptors (IR) (31) were grown in F-12 medium containing 25 mM Hepes (pH 7.4) and 10% (vol/vol) fetal bovine serum. NIH 3T3 cells expressing wt and Phe-490 mutant NGFRs (23) were grown in Dulbecco–Vogt's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. NIH 3T3 cells overexpressing the human EGFR (32) were grown in DMEM containing 10% calf serum and G418 (400 μg/ml). The anti-IR monoclonal antibody 51 was obtained from I. Goldfine (33, 34). Polyclonal anti-NGFR and anti-Shc sera have been described (6, 35). The anti-Tyr(*P*) monoclonal antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). The glutathione *S*-transferase (GST)–Shc PTB fusion protein used for receptor binding experiments is identical to GST–ShcB as described (19). The GST–dShc PTB fusion protein has been described (22).

Immunoprecipitations and PTB Binding Assays. Cells were grown to confluence and starved 16 hr in serum-free medium. CHO cells expressing the IR were stimulated with 100 nM

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Abbreviations: Tyr(*P*) or pY, phosphotyrosine; PTB, Tyr(*P*) binding; NGFR, nerve growth factor receptor; EGFR, epidermal growth factor receptor; wt, wild type; IR, insulin receptor; GST, glutathione *S*-transferase; DER, *Drosophila* EGFR homologue.

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insulin for 5 min at 37°C. NIH 3T3 cells expressing NGFRs were stimulated with NGF (50 ng/ml) for 5 min at 37°C, and NIH 3T3 cells expressing the human EGFR were stimulated with EGF (100 ng/ml) for 5 min at 37°C. Cell lysates were made, and immunoprecipitations and PTB binding assays (in the absence or presence of 2 or 5 μM competing phosphopeptide) were performed as described (19).

Surface Plasmon Resonance Analysis of Phosphopeptides Interacting with the Shc PTB Domain. Peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry with direct incorporation of Tyr(*P*) as the N^{α}-fluorenylmethoxycarbonyl-*O*-dimethylphosphono-L-tyrosine derivative. Cleavage of the peptide from the resin and deprotection was achieved through an 8-hr incubation at 4°C in trifluoroacetic acid containing 2 M bromotrimethyl silane and a scavenger mixture composed of thioanisole, *m*-cresol, and 1,2-ethanedithiol (1.0:0.5:0.1, percent by volume). The product was precipitated with ice-cold *t*-butyl ethyl ether and collected by centrifugation. After desalting of the crude material, pure phosphopeptide was isolated by using reverse-phase HPLC. The authenticity of the phosphopeptide was confirmed by amino acid analysis and mass spectroscopy. Surface plasmon resonance analysis was carried out using a Biacore apparatus (Pharmacia Biosensor) as described (36). The peptide LSLLSNPTpYSVMRSK was immobilized to a biosensor chip through injection of 0.5 mM phosphopeptide, in 50 mM Hepes, pH 7.5/2 M NaCl, across the chip surface previously activated by procedures outlined by the manufacturer. Typically in surface plasmon resonance experiments, a signal of 1500 resonance units was obtained from injection of GST–Shc PTB in the absence of competing peptide.

Expression of Torso–*Drosophila* EGFR Homologue (DER) fusion protein in Transgenic Flies. Transgenic flies expressing the activated Torso–DER chimeric protein, containing the cytoplasmic domain of DER, under the control of the heat shock promoter were obtained from E. Hafen (Universität Zürich, Zurich). Protein expression was induced by growing the flies at 37°C for 45 min after which they were allowed to recover at room temperature for 2.5 hr. Lysates were made as described (22).

RESULTS

Characterization of the Core PTB Binding Motif. To investigate the contribution of the Asn and Pro residues within the consensus PTB binding site to phosphopeptide recognition, we changed these residues to Ala in a phosphopeptide based on the sequence around Tyr-490, the Shc-binding site in the NGFR. The wt and mutant peptides were tested for their ability to compete with NGFRs, present in lysates of NGF-stimulated cells, for binding to a GST fusion protein containing the Shc PTB domain (Fig. 1). The wt phosphopeptide (HIIENPQpYFSD) competed efficiently for binding. Changing the Asn at position −3 [relative to the Tyr(*P*)] to Ala completely abolished detectable binding to the PTB domain, as measured in the competition assay, whereas changing the Pro at position −2 to Ala induced a significant but more modest reduction in the affinity of the PTB–peptide interaction. To determine the contribution of the different residues in the NPXpY motif more precisely, various concentrations of wt or mutant phosphopeptides were tested for their ability to inhibit binding of the Shc PTB domain to a Tyr(*P*)-containing peptide, from polyoma middle tumor antigen (19, 24, 37), immobilized on a Biacore chip (Table 1). This analysis confirmed that the Asn residue at position −3 is essential for binding to the PTB domain *in vitro*, while the Pro at position −2 is less important. These findings are in agreement with those of others (28, 29).

PTB Recognition Sites Contain Residues Outside the NPXpY Motif That Regulate Binding Specificity. Our previous

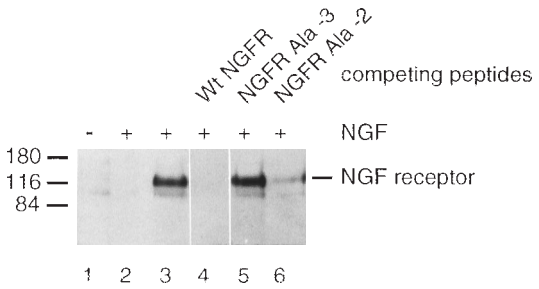

FIG. 1. Asn present within the NPXpY motif is essential for binding to the PTB domain. GST (lane 2) and GST Shc PTB (lanes 1 and 3–6) fusion proteins bound to glutathione-agarose were incubated with NGFRs present in lysates from control (lane 1) and NGF-stimulated (lanes 2–6) cells in the absence (lane 1–3) and presence of wt (lane 4) and mutant (lanes 5 and 6) competing Tyr(*P*)-containing peptides, based on the sequence around Tyr-490, the Shc PTB binding site in the NGFR. Bound proteins were examined by anti-Tyr(*P*) immunoblot analysis. Competing peptides: wt NGFR, HIIENPQpYFSD (lane 4); NGFR Ala −3 mutant, HIIE**A**PQpYFSD (lane 5); NGFR Pro −2 mutant, HIIEN**A**QpYFSD (lane 6). Amino acid substitutions introduced in these peptides are shown in boldface type.

results have suggested that an NPXpY motif may be necessary but is not sufficient for stable PTB binding (19). To pursue the identification of residues that might modulate Shc PTB binding, we have employed two receptors, those for NGF and insulin, both of which have juxtamembrane NPXY autophosphorylation sites (Fig. 2*A*) but have markedly different abilities to interact with Shc. Shc coprecipitated with the wt NGFR from NGF-stimulated cells but not with a mutant receptor with a Phe replacing Tyr-490 (Fig. 2*B*). Two additional Tyr(*P*)-containing proteins of unknown identity also coprecipitated with Shc from lysates of control and NGF-stimulated NIH 3T3 cells expressing either wt or Phe-490 NGFRs (Fig. 2*B*, lanes 1, 2, 5, and 6). Consistent with previously published data, the NGFR bound to the Shc PTB domain *in vitro* in a fashion that was dependent on phosphorylation of the NGFR at Tyr-490 (Fig. 2*C*). In contrast to the NGFR, the activated IR failed to bind stably to Shc *in vivo* or *in vitro* (7, 38), despite the presence of an NPXpY autophosphorylation site at Tyr-960 (39). Consistent with this finding, no tyrosine-phosphorylated IR was bound to the Shc PTB domain *in vitro* (Fig. 2*C*).

It is possible that Shc fails to bind the IR because the NPXpY site is not properly exposed for interaction with the PTB domain. We therefore tested the ability of a Tyr(*P*)-

Table 1. Peptide binding to the Shc PTB domain

Tyr(*P*)-containing peptide	IC_{50}, nM
HIIENPQpYFSD	175
HIIE**A**PQpYFSD	80,000
HIIEN**A**QpYFSD	2,500
H**A**IENPQpYFSD	20
HI**A**ENPQpYFSD	250
H**AA**ENPQpYFSD	475
H**AS**ENPQpYFSD	15,000
YASSNPEpYLSA	7,000
YA**I**SNPEpYLSA	90

Binding was measured by competition with a polyoma virus middle tumor antigen phosphopeptide (LSLLSNPTpYSVMRSK). Surface plasmon resonance technology was used to evaluate the ability of phosphopeptides derived from sequences around Tyr-490 in the NGFR (HIIENPQpYFSD) and Tyr-960 in the IR (YASSNPEpYLSA) to bind to the Shc PTB domain. Amino acid substitutions introduced into these peptides are shown in boldface type. Peptide concentrations that inhibited binding of a GST–Shc PTB fusion protein to the middle tumor antigen phosphopeptide, immobilized on a Biacore chip, by 50% (IC_{50}) are listed.

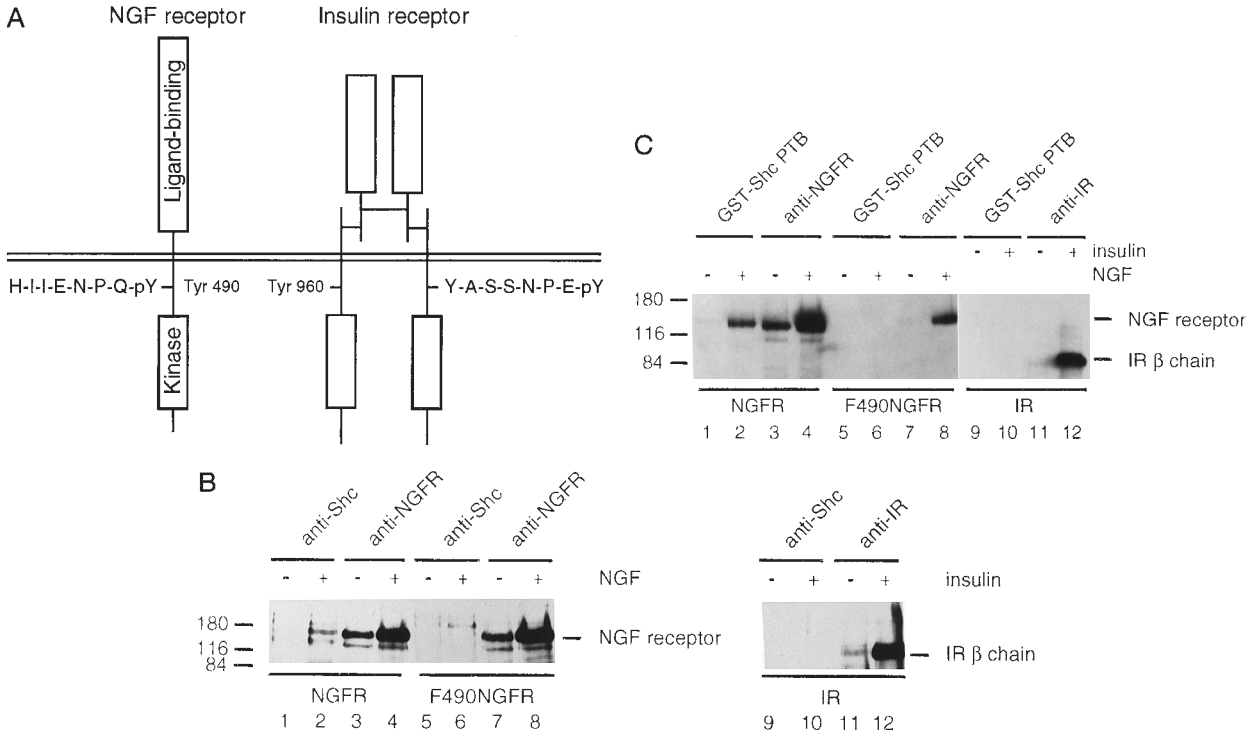

FIG. 2. Shc PTB domain does not stably bind to the NPXpY motif in the IR. (*A*) Both the NGFR and the IR contain an autophosphorylation site within an NPXpY motif in the juxtamembrane domain. In both receptors, the tyrosine residues within these motifs become phosphorylated upon receptor activation, but in contrast to the NGFR, the IR does not stably associate with Shc. (*B*) Anti-Shc immunoprecipitates (lanes 1, 2, 5, 6, 9, and 10) from control (lanes 1, 5, and 9) and growth factor-stimulated (lanes 2, 6, and 10) NIH 3T3 fibroblasts expressing wt (lanes 1 and 2; NGFR) or Phe-490 mutant (lanes 5 and 6; F490NGFR) NGFRs or CHO cells expressing wt IRs (lanes 9 and 10; IR) were examined by anti-Tyr(*P*) immunoblot analysis. Anti-NGFR (lanes 3, 4, 7, and 8) and anti-IR (lanes 11 and 12) immunoprecipitates from control (lanes 3, 7, and 11) and growth factor-stimulated (lanes 4, 8, and 12) cells were analyzed in parallel. (*C*) wt (lanes 1 and 2) and Phe-490 mutant (5 and 6) NGFRs present in lysates from control (lanes 1 and 5) and NGF-stimulated (lanes 2 and 6) cells, and IRs present in lysates from control (lane 9) and insulin-stimulated (lane 10) cells were incubated with GST–Shc PTB fusion proteins bound to glutathione-agarose. Bound proteins were examined by anti-Tyr(*P*) immunoblot analysis. Anti-NGFR immunoprecipitates (lanes 3, 4, 7, and 8) and anti-IR immunoprecipitates (lanes 11 and 12) from control (lanes 3, 7, and 11) and growth factor-stimulated (lanes 4, 8, and 12) cells were analyzed in parallel.

containing peptide based on the sequence around Tyr-960 in the IR to bind the Shc PTB domain (assayed by its ability to compete with the NGFR for binding to the PTB domain). The resulting data show that in contrast to the NGFR phosphopeptide, the IR peptide did not bind to the Shc PTB domain (Fig. 3, lanes 2 and 6, and Table 1). These results indicate that a phosphopeptide based on the IR Tyr-960 autophosphorylation site, which extends 7 residues N-terminal to the Tyr(*P*), has a very low affinity for the Shc PTB domain. Thus the inability of the IR to associate stably with Shc *in vivo* reflects the intrinsically low affinity of the Tyr-960 autophosphorylation site for the Shc PTB domain.

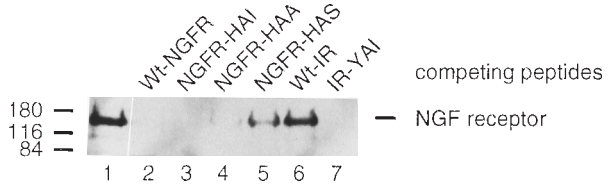

FIG. 3. Aliphatic residue 5 amino acids N-terminal to the Tyr(*P*) is an important determinant for Shc PTB binding. GST–Shc PTB domain fusion proteins bound to glutathione-agarose were incubated with activated NGFRs present in lysates of NGF-stimulated cells in the absence (lane 1) or presence (lanes 2–7) of 2 μM competing wt and mutant Tyr(*P*)-containing peptides based on the sequence around Tyr-490, the Shc PTB binding site in the NGFR (lanes 2–5) or Tyr-960, an autophosphorylation site present within an NPXpY motif in the IR (lanes 6 and 7). Bound proteins were examined by anti-Tyr(*P*) immunoblot analysis. wt NGFR peptide (lane 2), HIIENPQpYFSD; Ala −6 NGFR peptide (NGFR-HAI), H**A**IENPQpYFSD; Ala −6/Ala −5 NGFR peptide (NGFR-HAA), H**AA**ENPQpYFSD; Ala −6/Ser −5 NGFR peptide (NGFR-HAS), H**AS**ENPQpYFSD; wt IR peptide, YASSNPEpYLSA; Ile −5 IR peptide (IR-YAI), YA**I**SNPEpYLSA. Amino acid substitutions introduced in these peptides are shown in boldface type.

A common feature of high-affinity Shc-binding sites with NPXpY motifs is the presence of large aliphatic residues 5 and 6 residues N-terminal to the Tyr(*P*) (positions −5 and −6) (19, 21, 24, 27, 29). The NGFR Tyr-490 site, for example, has Ile at both residues −5 and −6. However, the IR Tyr-960 site has Ser in place of a hydrophobic residue at position −5 (Table 1). To test the possibility that the nature of residues −5 and −6 is important for Shc PTB binding, several substitutions at these positions were made in the NGFR peptide, and these mutant peptides were tested for binding to the Shc PTB domain by using the competition assay described above. Changing the Ile at position −6 to Ala had no inhibitory effect on binding to the PTB domain (Fig. 3). Changing both residues −5 and −6 from Ile to Ala slightly reduced binding to the PTB domain (Fig. 3). Strikingly, changing the Ile residue at position −5 to Ser in addition to changing the Ile at position −6 to Ala, as found at the IR Tyr-960 site, abolished binding of the NGFR phosphopeptide to the Shc PTB domain (Fig. 3). These results were quantitated by using surface plasmon resonance (Table 1). These data implicate the aliphatic residue at position −5 as being important for binding to the Shc PTB domain. This model suggests that the affinity of the IR Tyr-960 autophosphorylation site for the Shc PTB domain might be increased by incorporation of a more hydrophobic residue at position −5. To test this prediction, residue −5 of the IR peptide was

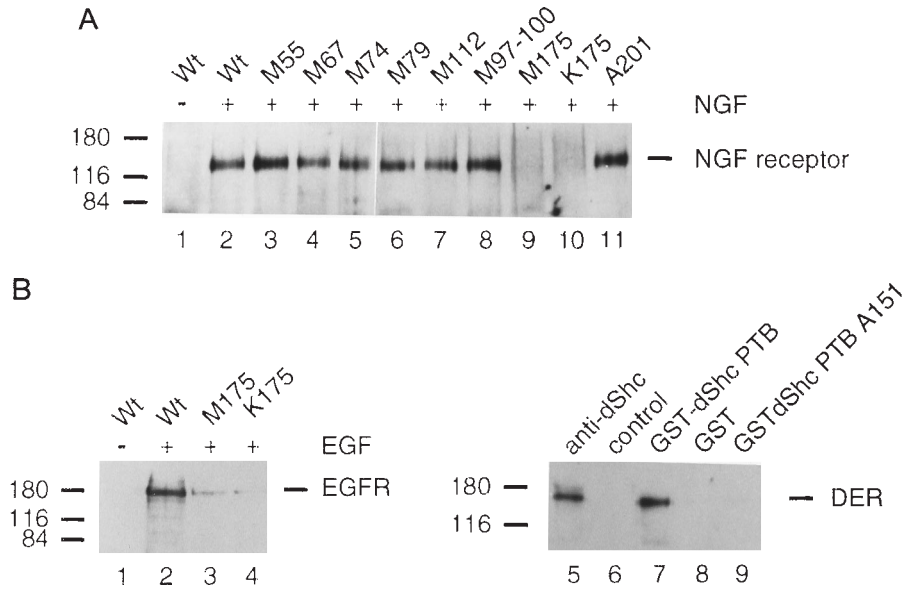

FIG. 4. Requirement for an Arg residue at position 175 in the human Shc PTB domain has been conserved in evolution. (*A*) GST fusion proteins containing wt (lanes 1 and 2) or mutant (lanes 3–11) Shc PTB domains were incubated with NGFRs present in lysates of control (lane 1) and NGF-stimulated (lanes 2–11) cells. Bound proteins were analyzed on an immunoblot with anti-Tyr(*P*). (*B*) Immobilized GST fusion proteins containing wt (lanes 1 and 2) or Met-175 (lane 3) and Lys-175 (lane 4) mutant Shc PTB domains were incubated with lysates from control (lane 1) or EGF-stimulated cells expressing EGFR (lanes 2–4). Immobilized GST (lane 8) and GST fusion proteins containing wt (lane 7) or Ala-151 (lane 9) mutant dShc PTB domain were incubated with fly lysates containing an activated Torso–DER chimeric receptor. Bound proteins were examined by anti-Tyr(*P*) blot analysis. An anti-Shc (lane 5) and a normal rabbit serum immunoprecipitate (lane 6) from the same fly lysates are shown as controls.

changed from Ser to Ile. This mutant IR peptide bound 75-fold more strongly to the PTB domain than its wt counterpart (Fig. 3 and Table 1). These data indicate that Shc PTB binding is modulated by residues outside the NPXpY core binding site.

Identification of a Conserved Arg Residue in the Shc PTB Domain That Is Required for Binding to Tyr(*P*) Sites. The identities of residues within the PTB domain that are important for ligand recognition and the mechanism by which the PTB domain binds Tyr(*P*)-containing sites are unknown. The SH2 Tyr(*P*)-binding pocket is composed of basic residues that form hydrogen bonds with the phosphate oxygens or make amino–aromatic interactions with the Tyr ring (40, 41). One invariant Arg of SH2 domains is absolutely essential for Tyr(*P*) binding. (42, 43). To investigate whether basic residues that are conserved in the PTB domains of different Shc family members might also be important for binding Tyr(*P*) sites, all conserved Arg residues in the Shc PTB domain, starting at Arg-55 of $p52^{shc}$ (6), were individually substituted with Met or Ala. Three consecutive Arg residues and the following Lys located at residues 97–100 were changed in combination. GST fusion proteins containing mutant PTB domains were then tested for their ability to bind to the activated NGFR (Fig. 4). Of 10 Arg residues tested, only substitution of Arg-175 had a marked effect on the affinity of the Shc PTB domain for the activated NGFR (Fig. 4*A*). Fusion proteins were expressed at similar levels (results not shown). Substitution of Arg-175 with Met or Lys severely impaired PTB binding to both the NGFR and the EGFR (Fig. 4), indicating that a positive charge in the context of an Arg residue is required at this position. The PTB domain of the *Drosophila* Shc (dShc) protein contains an Arg at residue 151, which is homologous to Arg-175 in human Shc. As was shown (22), the wt dShc PTB domain binds stably *in vitro* to the activated DER, which contains a VDNPEpY site in its C-terminal tail. In contrast, a mutant dShc PTB domain with Arg-151 replaced with Ala was unable to bind efficiently to the activated DER *in vitro* (Fig. 4*B*), suggesting that the requirement for this Arg residue in Tyr(*P*) binding has been conserved in evolution.

DISCUSSION

Identification and Characterization of a Core PTB Binding Motif. The binding of Shc to activated growth factor receptors and cytoplasmic phosphoproteins appears to be an important step in stimulating mitogenic and differentiation pathways. Recent work suggests that the PTB domain, located at the Shc N terminus, plays a significant role in these interactions. Shc frequently associates with proteins that contain NPXpY phosphorylation sites, and phosphopeptides containing the NPXpY sequence can compete for binding of activated growth factor receptors such as the NGFR and EGFR to the Shc PTB domain (19, 21, 25, 27, 29). We have shown (23) that binding of the Shc PTB domain to the NGFR requires autophosphorylation of the receptor at Tyr-490. Here we report that a phosphopeptide modeled on this site binds with high affinity to the Shc PTB domain.

The Tyr-490 NGFR phosphopeptide has been used to analyze the contribution of different residues within the NPXpY motif to PTB binding. The Asn at position −3 appears to be critical for high-affinity binding. The Pro at position −2 is less important for binding *in vitro* although its presence increases the affinity of the interaction approximately 10-fold. This may be important *in vivo*, consistent with the observation that the Pro at position −2 is conserved in several known Shc-binding sites. These findings are in general agreement with those of others that used distinct phosphopeptides or a degenerate phosphopeptide library to analyze the PTB binding site (28, 29, 44).

PTB Recognition Sites Contain Residues Outside the NPXpY Site That Regulate Binding. The activated IR, which contains an NPXpY autophosphorylation site, does not bind stably to Shc *in vivo* or *in vitro* (7, 38). Shc, however, is variably phosphorylated in response to insulin, and the Shc PTB domain has been shown to interact with Tyr-960 in the IR by using the two-hybrid method in yeast (44). Shc and the IR may, therefore, engage in a low-affinity interaction but are unable to form a stable complex *in vivo*. These observations suggest that residues outside the NPXpY core PTB binding site affect

receptor–PTB interactions. A comparison of several high-affinity Shc PTB binding sites shows that they possess hydrophobic residues at positions −5 and −6 (Ile in the case of the NGFR), while the IR Tyr-960 site has Ser at position −5. Experiments using NGF and IR phosphopeptides with substitutions at positions −5 and −6 indicate that residue −5 indeed has a critical effect on Shc PTB binding. Ala residues at positions −5 and −6 of the NGFR peptide are tolerated (Table 1). However, the presence of Ser at position −5, as found in the IR, disrupts high-affinity binding of the NGFR peptide to the Shc PTB domain (Table 1). Conversely, the substitution of the Ser at position −5 in the IR phosphopeptide with Ile strikingly increases binding affinity. These data indicate that the Shc PTB domain recognizes the NPXpY sequence and also show that residues N-terminal to the core NPXpY motif are important in determining PTB binding specificity, in a fashion reminiscent of the residues C-terminal to Tyr(*P*) in SH2-binding sites.

Ligand Recognition by the Shc PTB Domain. Several Arg residues that are conserved in SH2 domains have been shown to be directly involved in Tyr(*P*) binding (42, 43, 45). We have initiated a characterization of the PTB domain by mutagenesis of Arg residues that are conserved in the PTB domains of different members of the Shc family. This analysis has defined a specific Arg residue near the C terminus of the PTB domain that is important for its interaction with activated growth factor receptors. This Arg is conserved in dShc and is also essential for binding of the dShc PTB domain to activated DER. In SH2 domains, Tyr(*P*) recognition occurs through a direct ion pair between the phosphate moiety and an invariant Arg (45). It is possible that Arg-175 in the Shc PTB domain plays a similar role, in forming part of a Tyr(*P*)-recognition pocket.

Despite the possible similarity in Tyr(*P*) recognition, the general structures of PTB and SH2 domains, and their bound ligands, are likely to be quite different. The low affinity of mutant NGFR phosphopeptides with substitutions of the Asn at position −3 or Pro at position −2 for the Shc PTB domain is consistent with the notion that a reverse-turn secondary conformation is an important structural element for ligand recognition. The magnitude of the effects of the individual Asn and Pro substitutions on binding affinity parallels the known abilities of the relevant amino acids to form a type I β-turn (46). NMR studies of peptides corresponding to the NPXY internalization signal in the low-density lipoprotein receptor and the NPXY autophosphorylation site in the IR have shown that these molecules in solution adopt a type I β-turn with the Asn in position i and the Tyr at position $i + 3$ (47). This is quite different from SH2 peptide ligands, which adopt an extended conformation when bound to the SH2 domain (40, 41, 48, 49).

In addition to inferring that the Tyr(*P*) present at position $i + 3$ of a NPXpY type I β-turn characterizes a core motif for PTB binding, our results show that hydrophobic residues N-terminal to this motif play a role in augmenting binding affinity for the Shc PTB domain. These results suggest that the Shc PTB domain has a hydrophobic region that can interact favorably with the Ile at position −5 but is repelled by more polar residues such as Ser.

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