A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides

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Background: Signal transduction by growth factor receptor protein-tyrosine kinases is generally initiated by autophosphorylation on tyrosine residues following ligand binding. Phosphotyrosines within activated receptors form binding sites for the Src homology 2 (SH2) domains of cytoplasmic signalling proteins. One such protein, Shc, is tyrosine phosphorylated in response to a large number of growth factors and cytokines. Phosphorylation of Shc on tyrosine residue Y317 allows binding to the SH2 domain of Grb2, and hence stimulation of the Ras pathway. Shc is therefore implicated as an adaptor protein able to couple normal and oncogenic protein-tyrosine kinases to Ras activation. Shc itself contains an SH2 domain at its carboxyl terminus, but the function of the amino-terminal half of the protein is unknown.

Results: We have found that the Shc amino-terminal region binds to a number of tyrosine-phosphorylated proteins in v-*src*-transformed cells. This domain also bound directly to the activated epidermal growth factor (EGF) receptor. A phosphotyrosine (pY)-containing peptide modeled after the Shc-binding site in polyoma middle T antigen (LLSNPTpYSVMRSK) was able to compete efficiently with the activated EGF receptor for binding to the Shc amino terminus. This competition was dependent on phosphorylation of the tyrosine residue within the peptide, and was abrogated by deletion of the leucine residue at position −5. The Shc amino-terminal domain also bound to the autophosphorylated nerve growth factor receptor (Trk), but bound significantly less well to a mutant receptor in which tyrosine Y490 in the receptor's Shc-binding site had been substituted by phenylalanine.

Conclusion: These data implicate the amino-terminal region of Shc in binding to activated receptors and other tyrosine-phosphorylated proteins. Binding appears to be specific for phosphorylated tyrosine residues within the sequence NPXpY, which is conserved in many Shc-binding sites. The Shc amino-terminal region bears only very limited sequence identity to known SH2 domains, suggesting that it represents a new class of phosphotyrosine-binding modules. Consistent with this view, the amino-terminal Shc domain is highly conserved in a *Drosophila* Shc homologue. Binding of Shc to activated receptors through its amino terminus could leave the carboxy-terminal SH2 domain free for other interactions. In this way, Shc may function as an adaptor protein to bring two tyrosine-phosphorylated proteins together.

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Introduction

Many growth and differentiation factors bind to cell-surface receptors with protein-tyrosine kinase activity [1]. Such ligand binding results in dimerization and activation of the receptor, possibly as a result of receptor cross-phosphorylation [2,3]. A number of autophosphorylation sites in activated receptors, usually located outside of the catalytic domain, function as binding sites for the Src homology 2 (SH2) domains of cytoplasmic-signalling or adaptor proteins [4,5]. Thus, growth factors induce autophosphorylation of receptors at specific sites, and hence recruitment of specific SH2-containing molecules to the activated receptor.

SH2 domains were originally identified as functional signalling elements that are conserved among non-receptor protein-tyrosine kinases, and were later found to bind to phosphorylated tyrosine residues [6–9]. The specificity of these SH2-mediated interactions is apparently determined by the amino-acid sequence carboxy-terminal to the phosphorylated tyrosine residue [10–15]. A wide range of SH2-containing signalling and adaptor molecules with distinct biochemical and biological activities have been identified [4,5]. The SH2 domains of these signalling proteins control not only their association with activated receptors, but also their interactions with other intracellular phosphoproteins. These interactions regulate the subcellular localization, phosphorylation and enzymatic activity of intracellular signalling proteins, thereby stimulating biochemical pathways that elicit cellular responses to growth factor stimulation.

Shc was identified in a screen for human cDNAs encoding novel SH2 domain-containing proteins [16]. Shc proteins contain a carboxy-terminal SH2 domain, an

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amino-terminal domain and a central proline- and glycine-rich domain with some homology to α1 collagen. Anti-Shc antisera specifically recognize three proteins, of 46, 52 and 66 kD, in cell lysates, all of which are apparently generated by alternative translational initiation from the same Shc mRNA, or by alternative splicing [16]. These three Shc proteins differ from each other only by the extent of their amino-terminal sequences.

Shc proteins are phosphorylated on serine residues in resting cells and become phosphorylated on tyrosine residues in response to a large number of growth factors and cytokines [17–23]. Shc is also tyrosine-phosphorylated in cells transformed by oncogenic tyrosine kinases such as v-Src, v-Fps and Bcr–Abl [24,25]. The principal site of Shc phosphorylation is on tyrosine residue Y317, within the sequence YVNV, which forms a high-affinity binding site for the SH2 domain of the adaptor protein Grb2 [23,25,26]. Grb2 has a single SH2 domain located between two SH3 domains [27–29], which can associate with the Ras guanine nucleotide exchange factors, mSos1 and mSos2 [30–34]. Sos proteins, in turn, can activate Ras by catalyzing the exchange of GTP for GDP bound to Ras. Shc overexpression induces fibroblast transformation and Ras-dependent neurite outgrowth in PC12 cells; in addition, tyrosine-phosphorylation of Shc appears to be important for Ras activation following stimulation by growth factors [23,26,35]. The ability of tyrosine-phosphorylated Shc to bind to Grb2 and Sos provides a mechanism by which it might stimulate the Ras/mitogen-activated protein (MAP) kinase pathway.

The carboxy-terminal SH2 domain of Shc can bind to phosphotyrosine (pY)-containing proteins. The binding specificity of the Shc SH2 domain — pY(I/E/Y)X(I/L/Y), where X is any amino acid — has been determined by binding studies with degenerate phosphotyrosine-containing peptides [13]. However, a number of preferred Shc-binding sites, such as those found in the nerve growth factor (NGF) receptor (Trk), polyoma middle T antigen, the epidermal growth factor (EGF) receptor and ErbB3, are characterized by the sequence NPXpY [36–40]. Furthermore, despite evidence that the amino-terminal region of Shc might be functionally important (P.G. Pelicci, personal communication), no biochemical activity has yet been ascribed to this Shc domain.

Results

An amino-terminal Shc domain is conserved in evolution

As an approach towards understanding the functions of Shc proteins in signal transduction, we have recently isolated cDNAs for mouse Shc and for a *Drosophila* Shc homologue (V.K-M.L, J.P.O. and T.P., unpublished observations). Comparison of the amino-acid sequences of human, mouse and *Drosophila* Shc proteins shows that the carboxy-terminal region, corresponding to the SH2 domain, has the highest degree of sequence conservation (64 % between human and *Drosophila*). The amino-terminal regions of mammalian and *Drosophila* Shc proteins are also strongly conserved, suggesting the presence of a second domain that might be important for protein–protein interactions (Fig. 1). In contrast, the central region separating the amino-terminal and SH2 domains is less well conserved.

Association of the amino-terminal Shc domain with phosphotyrosine-containing proteins and activated EGF receptors

To test the hypothesis that the conserved amino-terminal Shc domain is involved in the formation of protein complexes, we generated several expression vectors encoding glutathione S-transferase (GST) fusion proteins which included parts of the Shc amino terminus (Fig. 2a). GST–ShcA contains the first 108 amino acids of Shc, starting at the initiating methionine of the p52 isoform [16]; GST–ShcB and GST–ShcC contain Shc residues 1–225 and residues 1–307, respectively; GST–Shc$^+$

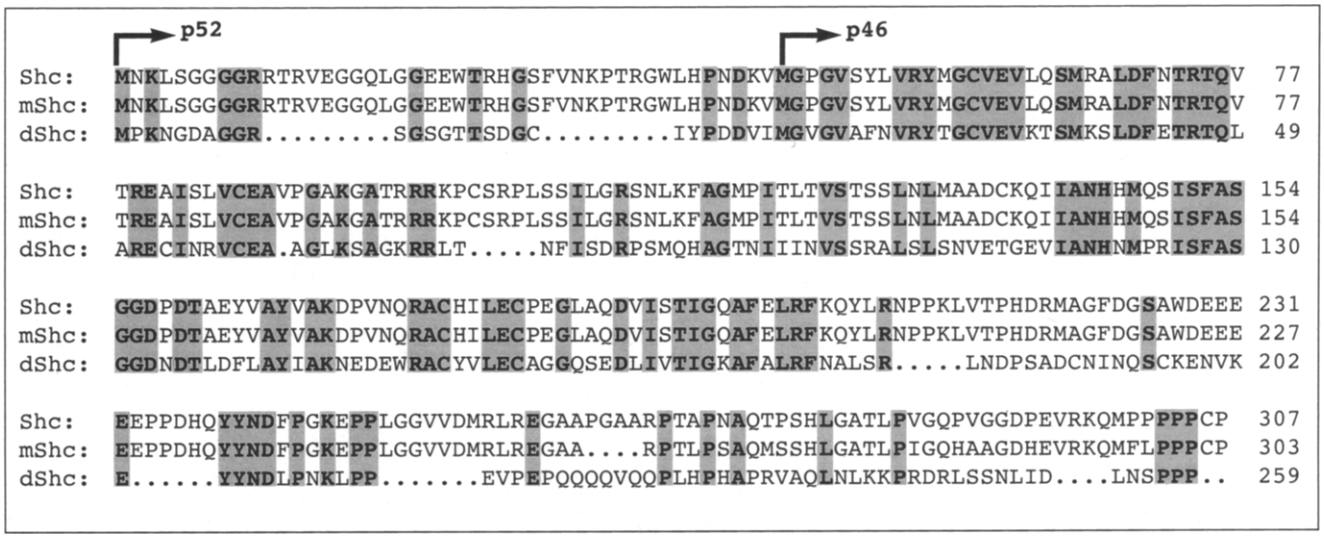

Fig. 1. Comparison of the amino-acid sequences of mammalian and *Drosophila* Shc homologues identifies a novel, conserved amino-terminal domain spanning residues 40–205. Residues identical in the three sequences are shaded.

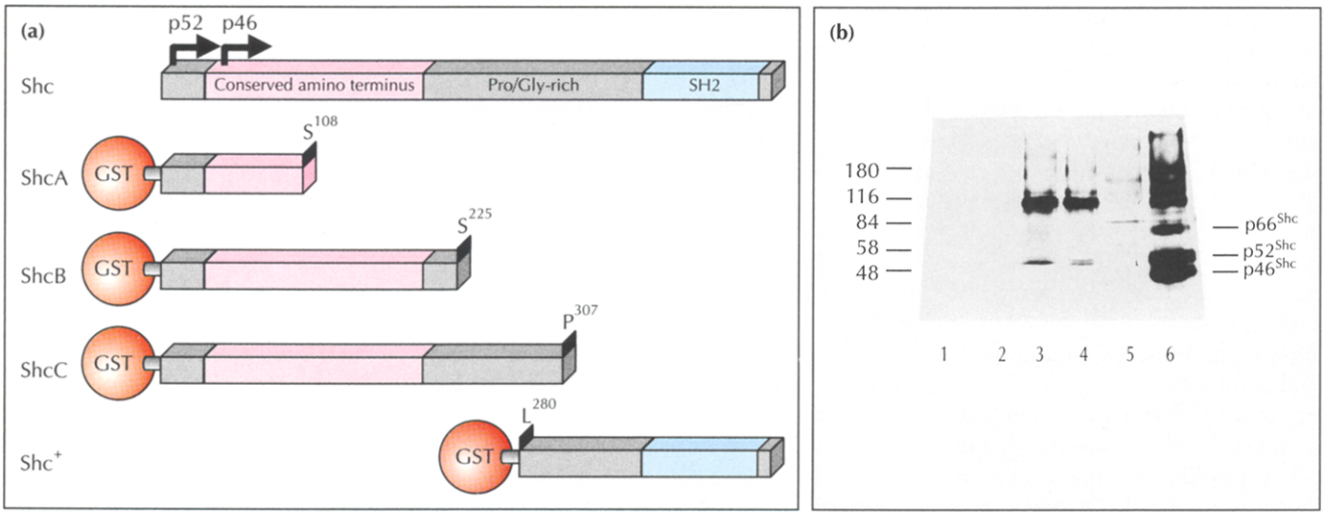

Fig. 2. Fusion proteins containing the amino terminus of Shc bind to phosphotyrosine-containing proteins in v-*src*-transformed cells. **(a)** The Shc amino terminus was expressed as a series of GST-fusion proteins: GST–ShcA contains $p52^{Shc}$ residues 1–108; GST–ShcB contains $p52^{Shc}$ residues 1–225; GST–ShcC contains $p52^{Shc}$ residues 1–307; GST–Shc^+ contains $p52^{Shc}$ residues 280–473, including the SH2 domain. **(b)** GST–Shc fusion proteins bind a number of phosphotyrosine-containing proteins in lysates of v-*src*-transformed Rat-2 fibroblasts (Rat-2 v-*src* cells). Approximately equal amounts of GST (lane 1), GST–ShcA (lane 2), GST–ShcB (lane 3), GST–ShcC (lane 4), and GST–Shc^+ (lane 5), bound to glutathione–Sepharose beads, were incubated with Triton X100 lysates from Rat-2 v-*src* cells. An anti-Shc immunoprecipitate (lane 6) is shown for comparison. Bound proteins were analyzed by anti-phosphotyrosine immunoblotting.

contains the carboxy-terminal 193 amino-acid residues of Shc, which include the SH2 domain, and was used as a control. These purified proteins were tested for their ability to bind proteins from v-*src*-transformed cells. Lysates from v-*src*-transformed Rat-2 cells were incubated with approximately equal amounts of GST, GST–ShcA, GST–ShcB, GST–ShcC or GST–Shc^+, bound to glutathione beads. Bound proteins were analyzed by immunoblotting with an anti-phosphotyrosine antiserum, and Shc immunoprecipitate was analyzed in parallel (Fig. 2b). GST–ShcB and GST–ShcC, containing the entire conserved amino-terminal region, bound a number of phosphotyrosine-containing proteins (Fig. 2b), whereas neither GST nor GST–ShcA showed significant binding activity. Although GST–Shc^+, containing the SH2 domain, also associated with tyrosine-phosphorylated proteins, these appeared to be different from those bound by the Shc amino terminus, based on their mobilities during SDS-polyacrylamide gel electrophoresis. The amino terminus and the SH2 domain appeared to bind complementary sets of phosphoproteins, together accounting for most of the phosphotyrosine-containing proteins present in the Shc immunoprecipitates (Fig. 2b).

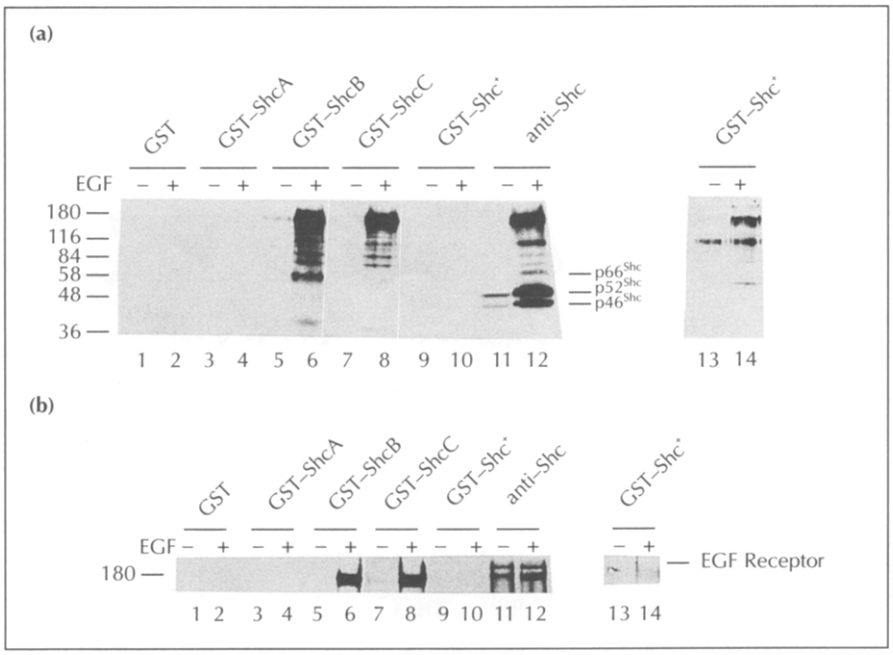

Fig. 3. The conserved Shc amino-terminal domain binds a number of phosphotyrosine-containing proteins, including the EGF receptor, in lysates of EGF-stimulated cells. **(a)** Triton X100 lysates from control (lanes 1, 3, 5, 7, 9, 11) and EGF-stimulated (lanes 2, 4, 6, 8, 10, 12) HER14 cells were incubated with approximately equal amounts of GST (lanes 1, 2), GST–ShcA (lanes 3, 4), GST–ShcB (lanes 5, 6), GST–ShcC (lanes 7, 8), and GST–Shc^+ (lanes 9, 10) bound to glutathione–Sepharose beads. Shc immunoprecipitates were analyzed in parallel (lanes 11, 12). Lanes 13 and 14 show a longer exposure of lanes 9 and 10. Bound proteins were analyzed by anti-phosphotyrosine immunoblotting. **(b)** The blot obtained in an experiment parallel to that shown in (a) was probed with an anti-EGF receptor antiserum.

To investigate whether binding to the Shc amino-terminal region could be induced by growth factor treatment, the GST–Shc fusion proteins were incubated with lysates of quiescent and EGF-stimulated NIH3T3 cells over-expressing EGF receptors (HER14 cells). Bound proteins were analyzed by anti-phosphotyrosine immunoblotting and Shc immunoprecipitates were analyzed in parallel. Little binding activity was exhibited by any of the Shc fusion proteins towards proteins present in lysates of unstimulated cells (Fig. 3a). However, the GST–ShcB, GST–ShcC and GST–Shc$^+$ fusion proteins all associated with a number of phosphotyrosine-containing proteins in lysates of EGF-stimulated cells, whereas none bound to GST or GST–ShcA. A phosphoprotein with a molecular weight of ~175 kD that bound to GST–ShcB, GST–ShcC and GST–Shc$^+$ in lysates of EGF-stimulated cells was also present in the parallel Shc immunoprecipitates. This protein was identified as the autophosphorylated EGF receptor by probing the blot from a parallel experiment with an anti-EGF receptor antiserum (Fig. 3b). No EGF receptor molecules were bound by the Shc amino-terminal or SH2 domains prior to EGF stimulation. These results suggest that the conserved Shc amino-terminal domain binds specifically to the EGF receptor in lysates from EGF-stimulated cells. In order to test whether the platelet-derived growth factor (PDGF) receptor could also associate with the Shc amino terminus following receptor activation, lysates of quiescent and PDGF-stimulated Rat-2 fibroblasts were incubated with GST–Shc fusion proteins: no binding of activated PDGF receptors to the Shc amino terminus was observed (data not shown).

The amino-terminal Shc domain binds directly to the autophosphorylated EGF receptor

To investigate whether the Shc amino-terminal region binds directly or indirectly to the activated EGF receptor,

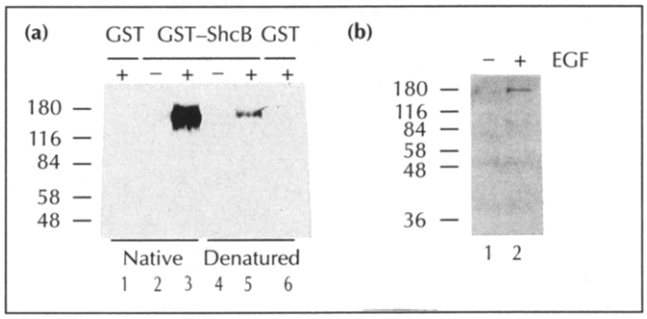

Fig. 4. The Shc amino-terminal domain binds directly to the EGF receptor. **(a)** Triton X100 lysates (lanes 1–3) and lysates boiled in the presence of SDS (lanes 4–6), from control (lanes 2 and 4) or EGF-stimulated (lanes 1, 3, 5, and 6) HER14 cells, were incubated with approximately equal amounts of GST (lanes 1 and 6) or GST–ShcB (lanes 2–5) bound to glutathione–Sepharose beads. Bound proteins were analyzed by anti-EGF receptor immunoblotting. **(b)** Anti-EGF receptor immunoprecipitates from control (lane 1) and EGF-stimulated (lane 2) HER14 cells were resolved by SDS–PAGE and transferred to a PVDF membrane. The membrane was incubated with a bacterial lysate containing GST–ShcB, and was subsequently probed for the presence of bound GST–ShcB with an anti-GST antiserum.

lysates were boiled in the presence of 0.5 % SDS to denature all proteins and thereby disrupt protein–protein interactions. Boiled lysates were diluted and incubated with the GST–ShcB fusion protein; the resulting complexes were separated and probed for the presence of the EGF receptor by immunoblotting (Fig. 4a). Activated EGF receptors present in boiled lysates of EGF-stimulated HER14 cells were still able to bind to the Shc amino-terminal region, although less efficiently compared to receptors from lysates containing Triton X100 as the only detergent. In an independent approach, EGF receptors were immunoprecipitated from control and EGF-stimulated HER14 cells, resolved by SDS-polyacrylamide gel

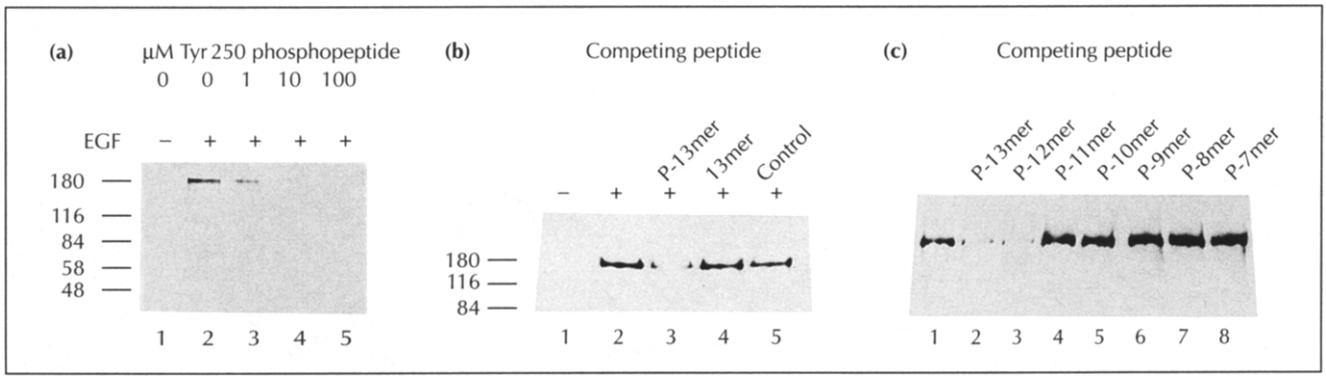

Fig. 5. A phosphopeptide containing an NPXpY motif competes efficiently for binding of GST–ShcB to the activated EGF receptor. **(a)** GST–ShcB bound to glutathione–Sepharose beads was incubated with Triton X100 lysates from control (lane 1) and EGF-stimulated (lanes 2–5) HER14 cells in the absence (lanes 1,2) or the presence (lanes 3–5) of 1, 10, and 100 μM of a phosphopeptide based on the sequence around the Shc-binding site (Y250) in polyoma middle T antigen (LLSNPTpYSVMRSK). **(b)** GST–ShcB bound to glutathione–Sepharose beads was incubated with Triton X100 lysates from control (lane 1) and EGF-stimulated (lanes 2–5) HER14 cells in the absence (lanes 1,2) or the presence (lanes 3–5) of 10 μM competing peptides — the phosphorylated polyoma middle T 13mer (lane 3); the unphosphorylated polyoma middle T 13mer (lane 4); or the irrelevant phosphotyrosine-containing control peptide GMKIpYIDPFTpYEDPNEAVK (lane 5). **(c)** GST–ShcB bound to glutathione–Sepharose beads was incubated with Triton X100 lysates from EGF-stimulated HER14 cells in the absence (lane 1) or the presence (lanes 2–8) of 10 μM of a series of sequentially truncated phosphotyrosine-containing peptides based on the sequence around Y250 in polyoma middle T (LLSNPTpYSVMRSK) — the 13mer starting at L_{-6} (lane 2); the 12mer starting at L_{-5} (lane 3); the 11mer starting at S_{-4} (lane 4); the 10mer starting at N_{-3} (lane 5); the 9mer starting at P_{-2} (lane 6); the 8mer starting at T_{-1} (lane 7); and the 7mer starting at the phosphotyrosine, pY (lane 8).

electrophoresis and transferred electrophoretically to a PVDF membrane. This membrane was incubated for 60 minutes with lysates of *Escherichia coli* cells expressing GST–ShcB. After incubation with this bacterial lysate, the membrane was washed and then probed for the presence of GST–ShcB with an anti-GST antiserum (Fig. 4b). The results indicate that GST–ShcB is able to bind directly to EGF receptors from EGF-stimulated HER14 cells. Only weak binding to the EGF receptors from unstimulated cells was observed. This may be due to a low level of receptor activation in resting cells resulting from overexpression of the receptor, as receptors from quiescent cells contained some phosphotyrosine (data not shown). These data clearly show that the Shc amino-terminal region binds to the activated EGF receptor, and that binding results from direct recognition of the autophosphorylated receptor.

A phosphopeptide containing an NPXpY motif competes with the activated EGF receptor for binding to the amino-terminal Shc region

As Shc proteins bind *in vivo* to tyrosine residues within the sequence NPXpY, of which two are present in the EGF receptor, it is possible that the Shc amino-terminal region recognizes such sequences. To investigate this possibility, lysates from EGF-stimulated cells were incubated with GST–ShcB in the presence of increasing amounts of a phosphopeptide containing an NPXpY motif. This peptide corresponds to the Shc-binding site of the polyoma middle T antigen, centered on tyrosine residue Y250 (LLSNPTpYSVMRSK). This peptide competed efficiently for binding of the fusion protein to the EGF receptor (Fig. 5a). An approximately 50 % reduction in binding was observed in the presence of 1 μM peptide. Neither unphosphorylated polyoma middle T peptide, nor an irrelevant phosphotyrosine-containing peptide (GMKIpYIDPFTpYEDPNEAVK), competed with GST–ShcB for binding to the EGF receptor (Fig. 5b). This indicates that the binding of GST-ShcB to the EGF receptor depends on the presence of a phosphorylated tyrosine residue within a specific amino-acid sequence.

To investigate further the contribution of peptide residues amino-terminal to the phosphorylated tyrosine residue to binding by the amino-terminal Shc region, a series of polyoma middle T peptides, sequentially truncated at the amino terminus one residue at a time, were tested for their ability to compete with the autophosphorylated EGF receptor for binding to GST–ShcB. The original phosphorylated 13mer, which includes six residues amino-terminal to the phosphotyrosine, and the 12mer with five amino-terminal residues, but none of the shorter peptides, competed efficiently with the EGF receptor for binding to GST–ShcB (Fig. 5c). These results, summarized in Table 1, suggest that the binding of a peptide to the amino-terminal region of Shc depends on residues amino-terminal to the phosphotyrosine. The aliphatic leucine five residues amino-terminal to the phosphotyrosine (the –5 position) appears to be important for binding.

Table 1. Binding of phosphopeptides to the amino-terminal region of Shc.

Phosphopeptide	Binding to Shc amino terminus
G-M-K-I-pY-I-D-P-F-T-**pY**-E-D-P-N-E-A-V-K	–
L-**L**-S-**N-P**-T-**pY**-S-V-M-R-S-K	+
L-S-**N-P**-T-**pY**-S-V-M-R-S-K	+
S-**N-P**-T-**pY**-S-V-M-R-S-K	–
N-P-T-**pY**-S-V-M-R-S-K	–
P-T-**pY**-S-V-M-R-S-K	–
T-**pY**-S-V-M-R-S-K	–
pY-S-V-M-R-S-K	–

Amino acids are given in the single-letter code; phosphotyrosine is abbreviated as pY. Residues that are conserved in Shc-binding sites, or demonstrated to be required for binding to the Shc amino-terminal domain are shown in bold.

The amino-terminal Shc domain binds specifically to tyrosine 490 in the NGF receptor

To test whether the receptor-binding activity of the amino-terminal Shc domain has a more general role, we assessed its ability to bind to the nerve growth factor receptor, Trk, which is known to associate with Shc *in vivo*. Several autophosphorylation sites have been identified in Trk, of which tyrosine residue Y490 functions as the principal Shc-binding site, and Y785 is required for binding to phospholipase C γ (PLCγ) [40,41]. Wild-type Trk, as well as mutants in which tyrosine residues Y490 and Y785 were substituted by phenylalanines (F490 and F785, respectively) and kinase-inactive Trk mutants were expressed using the baculovirus expression system in Sf9 cells. Approximately equal amounts of receptors were immunoprecipitated from lysates of 1.5×10^6 cells,

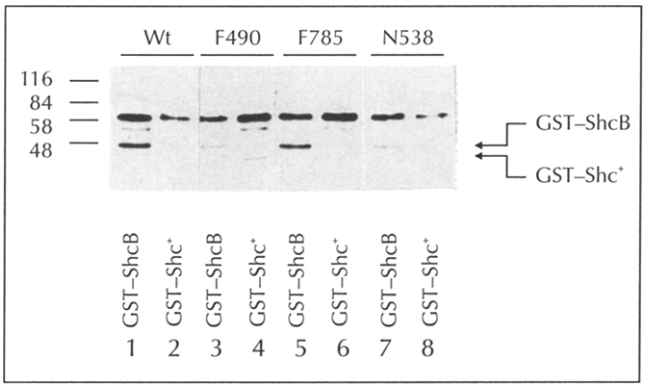

Fig. 6. Binding of the amino-terminal Shc domain to tyrosine residue Y490 in Trk. Trk immunoprecipitates from 1.5×10^6 Sf9 cells expressing wild-type (Wt, lanes 1, 2), F490 (lanes 3, 4), F785 (lanes 5, 6) and kinase-inactive (N538, lanes 7,8) Trk were autophosphorylated *in vitro* and subsequently incubated with bacterial lysates containing equal amounts of GST–ShcB (lanes 1, 3, 5, 7) or GST–Shc$^+$ (lanes 2, 4, 6, 8). Bound proteins were analyzed by immunoblotting with an anti-GST antiserum.

autophosphorylated *in vitro* in the presence of ATP and subsequently incubated with bacterial lysates containing equal amounts of GST–ShcB or GST–Shc$^+$. GST-fusion proteins that bound to activated Trk were analyzed by immunoblotting with an anti-GST antiserum. GST–ShcB bound efficiently to autophosphorylated wild-type Trk and to the F785 mutant, but poorly to the F490 mutant and kinase-inactive Trk (Fig. 6).GST–Shc$^+$ polypeptide did not bind appreciably to any of the autophosphorylated Trk receptors tested (Fig. 6).

These results indicate that the isolated Shc amino-terminal domain binds specifically to the tyrosine 490 Trk autophosphorylation site, reflecting the specificity of Shc binding *in vivo*. As the Shc SH2 domain apparently plays a lesser role in binding to activated Trk, it is likely that the amino-terminal domain is responsible for binding of Shc to Trk *in vivo*, an interaction which appears to be important in coupling nerve growth factor stimulation to neurite outgrowth in PC12 cells [42,43].

Discussion

Analysis of the amino-acid sequences of human, mouse and *Drosophila* Shc homologues indicates that, in addition to the carboxy-terminal SH2 domain, a second highly conserved domain of approximately 160 amino acids is located at the amino terminus (V.K-M.L., J.P.O. and T.P., unpublished observations). The presence of this element in both mammalian and *Drosophila* Shc suggests that it is functionally important and might therefore represent a module involved in protein–protein interactions. Consistent with this possibility, the amino-terminal region of Shc expressed as a GST-fusion protein bound a number of unidentified phosphotyrosine-containing proteins in v-*src*-transformed cells. This domain also bound specifically to the EGF receptor following stimulation with EGF, whereas a shorter fusion protein that lacked part of the conserved Shc sequence failed to associate with the activated EGF receptor.

The activated EGF receptor also associated with the Shc amino-terminal region in lysates boiled in the presence of SDS, which disrupts previously formed protein complexes. The fact that denatured EGF receptors bind less well to this novel domain than native receptors suggests that optimal binding may require some degree of structure. In a GST-fusion protein, the Shc amino-terminal region also binds to EGF receptors present on PVDF membranes. These data show that the Shc amino-terminal region directly recognizes the autophosphorylated EGF receptor. The identities of phosphotyrosine-containing polypeptides from v-*src*-transformed and EGF-stimulated cells that bind to Shc are currently under investigation.

Previous work has suggested that Shc binds *in vivo* to sites with a conserved motif, NPXpY, found in a variety of receptors and cytoplasmic proteins. These include Trk, the EGF receptor, ErbB3 and the polyoma middle T antigen [36–40]. The Shc SH2 domain binds preferentially *in vitro* to phosphopeptides with the sequence pY(I/E/Y)X(I/L/M), suggesting that, like other SH2 domains, it may primarily recognize residues carboxy-terminal to the phosphorylated tyrosine residue [13]. Furthermore, the Shc SH2 domain does not bind with high affinity to phosphopeptides containing the sequence NPXpY (S.E.S., unpublished observations). In contrast, a phosphopeptide with the NPXpY motif, based on the sequence flanking tyrosine residue Y250 of the polyoma middle T antigen Shc-binding site, competed efficiently with the EGF receptor for binding to the amino-terminal Shc domain [37,38]. These results raised the possibility that the amino-terminal Shc domain might participate in the recognition of NPXpY sites. Such a Shc-binding motif is found at tyrosine residue Y490 of the Trk NGF receptor [40,42]. The Shc SH2 domain does not bind efficiently to autophosphorylated Trk *in vitro*; in contrast the amino-terminal Shc domain bound to wild-type Trk, but not to a Trk mutant specifically lacking the Y490 autophosphorylation site.

The activated PDGFβ receptor does not contain any NPXpY sequences in its cytoplasmic domain [44,45] and does not bind to the Shc amino-terminus *in vitro*, although it does associate with the Shc SH2 domain [46]. These results indicate that the Shc amino-terminal region directly recognizes phosphotyrosines, but unlike SH2 domains it may require specific residues amino-terminal to the phosphotyrosine for high-affinity binding. Consistent with this possibility, deletion of the leucine residue at the −5 position from the polyoma middle T phosphopeptide completely abolished its ability to compete for binding of the EGF receptor to the Shc amino-terminal region. We have obtained identical results when the series of polyoma middle T phosphopeptides were assayed directly for binding to the Shc amino-terminal

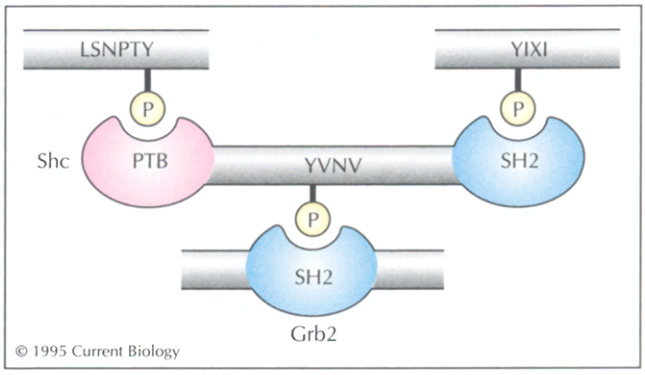

Fig. 7. Shc mediates multiple phosphotyrosine-dependent protein–protein interactions. Shc contains two domains that bind phosphotyrosine — the PTB domain and an SH2 domain. The PTB domain apparently recognizes phosphotyrosine in the context of amino-terminal residues (with consensus sequence NPXpY), while the SH2 domain recognizes phosphotyrosine and carboxy-terminal residues. The PTB and SH2 domains of Shc may potentially bind distinct sites on the same protein — the activated EGF receptor for example — or might associate with two different polypeptides. In addition, phosphorylation of Shc at tyrosine Y317 creates a binding site for the Grb2 SH2 domain.

domain by surface plasmon resonance, using a Biacore (unpublished observations). This suggests that the Shc amino-terminal domain recognizes at least five residues amino-terminal to the phosphorylated tyrosine. Interestingly, a non-polar residue is present at the −5 position of most Shc binding sites identified thus far [38].

These results raise the possibility that Shc has two receptor-binding domains — a conventional carboxy-terminal SH2 domain required for association with the PDGF receptor and a novel, amino-terminal phosphotyrosine-binding (PTB) domain that binds receptors with NPXpY motifs, such as Trk (Fig. 7). The activated EGF receptor binds stably to both the amino- and carboxy-terminal Shc domains *in vitro*, which may account for the very potent phosphorylation of Shc observed in cells or animals stimulated with EGF [16,20]. In principle, the two phosphotyrosine-binding modules of Shc might also recognize sites on different proteins, which would then be brought into a common complex.

The results presented here suggest that the amino-terminal Shc domain behaves functionally as an SH2 domain, in that it directly recognizes specific phosphotyrosine-containing sites. At first glance, there is no obvious sequence relationship between the amino-terminal Shc region and known SH2 domains. However, a sequence can be identified immediately amino-terminal to the p46 initiation site of Shc, WLHPND, which is reminiscent of the amino termini of conventional SH2 domains (for example, the Syp amino-terminal SH2 domain begins with WFHPNI) [47]. This is followed by the sequence YLVR, which resembles the motif F/YLVR found in many SH2 domains and contains the only invariant SH2 residue, arginine βB5 [14]. In SH2 domains, this arginine lies at the base of the positively-charged phosphotyrosine-binding pocket and forms hydrogen bonds with the phosphate oxygens. Indeed, the Shc amino-terminal region has a substantial number of basic residues that are conserved in the *Drosophila* homologue. Therefore, it is possible that the Shc amino-terminal domain has a phosphotyrosine-binding pocket that resembles that of SH2 domains, but differs in the rest of its ligand-binding surface. This may account for the apparent ability of Shc to bind tyrosine phosphorylation sites with conserved features involving residues amino-terminal to the phosphotyrosine. The mechanism by which the Shc amino-terminal region recognizes such sites awaits further investigation.

The precise extent of the Shc amino-terminal domain required for binding to the activated EGF receptor and other tyrosine phosphorylated proteins is not yet defined. However, 1a fusion protein containing the first 108 residues of $p52^{Shc}$ did not bind the EGF receptor, whereas a protein containing the first 225 residues bound tightly to the receptor. Our data are in agreement with two recent studies defining a novel Shc phosphotyrosine-binding domain between residues 46 and 208 of $p52^{Shc}$ [48,49]. The region of high sequence identity between mammalian and *Drosophila* Shc proteins spans residues 40–205 of $p52^{Shc}$, which correspond roughly to the receptor-binding domain (see Fig. 1). We have recently identified a sequence closely related to those found at the amino-terminal regions of mammalian and *Drosophila* Shc in a distinct mouse protein (T. Saxton, J. O'Bryan and T.P., unpublished observations), suggesting that this novel receptor-binding domain might be of general importance in signal transduction.

Conclusions

By comparing the amino-acid sequences of mammalian and *Drosophila* homologs, we have identified a conserved domain at the amino terminus of Shc. This novel domain binds phosphotyrosine-containing proteins in v-*src*-transformed and EGF-stimulated fibroblasts. One of the proteins bound in lysates from EGF-stimulated fibroblasts is the EGF receptor itself. Binding appears to be direct and specific for phosphorylated tyrosine residues within the sequence NPXpY, which is conserved in many Shc binding sites. An aliphatic amino acid five residues amino-terminal of the phosphotyrosine was also shown to be important for high affinity binding. Binding experiments with the NGF receptor, which contains only one well-defined Shc-binding site, suggests that the Shc amino-terminal domain may be important for its interaction with activated protein-tyrosine kinase receptors *in vivo*. The Shc amino-terminal region has only limited sequence identity to known SH2 domains, suggesting that it represents a new phosphotyrosine-binding module. The binding of Shc to activated receptors through its amino terminus, which recognises phosphotyrosine in the context of amino-terminal residues, could leave the carboxy-terminal SH2 domain free for other interactions. Thus, Shc may function as an adaptor protein that brings together two tyrosine-phosphorylated proteins.

Materials and methods

Cells lines and antibodies

NIH3T3 cells overexpressing human EGF receptor (HER14 cells) [50] were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and 400 μg ml^{-1} G418; v-*src* transformed Rat-2 cells [51] were grown in DMEM containing 10 % FBS. A polyclonal anti-Shc antiserum was raised against a GST–Shc SH2 fusion protein. A polyclonal anti-phosphotyrosine antiserum was raised and affinity purified as described [52]. An anti-EGF receptor monoclonal antibody used for immunoprecipitations was purchased from UBI (Lake Placid, New York, USA). Polyclonal anti-EGF receptor antisera were used for immunoblotting.

GST-fusion proteins

pGEX GST–Shc fusion constructs were generated by PCR subcloning of human $p52^{Shc}$ cDNA fragments encoding residues 1–108 (GST–ShcA), 1–225 (GST–ShcB) or 1–307 (GST–ShcC) in pGEX4T–2. The PCR products were sequenced to ensure fidelity. GST-Shc^{+} contains $p52^{Shc}$ residues 280–473 [26]. Expression of fusion proteins was

induced by incubation in the presence of 100 μM IPTG for several hours at 30 °C. Bacteria were lysed by sonication in phosphate buffered saline (PBS) containing 1 mM DTT, 1 mM Benzamidine, 10 μg ml^{-1} Aprotinin and 10 μg ml^{-1} Leupeptin. Triton-X 100 was added to a final concentration of 1 %, nuclei were removed by centrifugation for 10 min at 10 000 x *g* at 4 °C, and fusion proteins were purified by binding to glutathione–Sepharose. Glutathione–Sepharose beads were collected by centrifugation and washed five times with PBS containing 1 % Triton X100, 1 mM DTT and 1 mM Benzamidine. Fusion proteins were stored at 4 °C bound to beads in PBS, containing 1 % Triton X100, 1 mM DTT, 1 mM Benzamidine, 0.02 % sodium azide and 10 % glycerol.

Immunoprecipitation and immunoblotting

Cells were grown to subconfluence on 10 cm tissue culture dishes. If cells were to be stimulated with EGF, they were starved for 14–16 h in DMEM containing 0.5 % FBS and 20 mM HEPES pH 7.2. Control cells or cells stimulated with 100 ng ml^{-1} EGF for 5 min at 37 °C were rinsed twice with cold PBS and lysed in 1 ml 50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % Triton X100, 1.5 mM $MgCl_2$ 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 500 μM sodium vanadate, 1 mM PMSF, 10 μg ml^{-1} Aprotinin and 10 μg ml^{-1} Leupeptin (PLC-lysis buffer) per 10 cm tissue culture dish. Lysates were cleared by centrifugation at 10 000 rpm in a microcentrifuge at 4 °C, incubated with 5 μl polyclonal antiserum or 1 μg monoclonal antibody for 1 h on ice and subsequently with 100 μl 10 % Protein A-Sepharose or anti-mouse IgG-Sepharose for 1 h at 4 °C on an agitator. Sepharose beads were collected by centrifugation and washed four times with PLC-lysis buffer. Immunoprecipitates were boiled for 3 min in 62.5 mM Tris/Cl pH 6.8, 10 % glycerol, 5 % β-mercaptoethanol, 5 mM DTT, 2.3 % SDS, and 0.025 % bromophenol blue (SDS-sample buffer) and resolved by SDS-PAGE.

To disrupt previously formed protein–protein complexes, cells from a 10 cm tissue culture dish were collected and boiled for 3 min in 200 μl 10 mM sodium phosphate, 0.5 % SDS, 1 mM EDTA and 1 mM DTT. After boiling, the lysates were diluted in 800 μl 50 mM sodium phosphate, 150 mM NaCl, 1 % Nonidet P40, 1 % sodium deoxycholate, 2 mM EDTA, 50 mM NaF and 100 μM sodium orthovanadate.

For *in vitro* binding studies, fibroblast cell lysates were incubated for 1 h at 4 °C on a rocker with 25 μl glutathione-Sepharose beads containing 5–10 μg of fusion protein. Beads were washed four times with PLC-lysis buffer and bound proteins were analyzed by SDS-PAGE and immunoblotting.

For immunoblotting, proteins were transferred to PVDF membranes using a Biorad semi-dry blotting apparatus at 50 mA per gel for 60 min at room temperature. Membranes were blocked for 1 h at room temperature in 10 mM Tris/Cl pH 7.4, 150 mM NaCl, 0.2 % Tween 20 (TBST) containing 5 % dried milk and incubated with a 1:200 dilution of polyclonal antisera in TBST + 5 % milk for 1 h at room temperature. Blots were washed 2 x 10 min with TBST and 2 x 5 min with 10 mM Tris/Cl pH 7.4 and 150 mM NaCl (TBS). Membranes were then incubated for 1 h with HRP–Protein A (Biorad, Hercules, California 94547, USA) diluted 1:10 000 in TBST and washed as before. Reactive proteins were visualized by ECL (Amersham Canada, Oakville, Ontario, L6L 5T7). For immunoblotting with anti-phosphotyrosine antiserum, membranes were blocked with TBST containing 5 % bovine serum albumin (BSA) followed by an incubation with the antiserum diluted in TBST + 5 % BSA. Washes and detection were done exactly as described for other antisera.

For binding of fusion proteins to EGF receptors immobilized onto PVDF membranes, membranes were blocked by incubation for 1 h at 4 °C in TBST containing 5 % BSA and subsequently incubated for 1 h at 4 °C with 3 ml bacterial lysate containing ~2 μg of GST–ShcB diluted 1:3 in TBST + 5 % BSA. Bound proteins were detected by immunoblotting with an anti-GST antiserum.

Baculovirus expression of Trk proteins and in vitro *binding to bacterially expressed GST–Shc fusion proteins*

Wild-type and mutant Trk proteins were expressed using a baculovirus expression system in Sf9 cells, immunoprecipitated and phosphorylated *in vitro* in the presence of 100 nM ATP as described before [42]. Following *in vitro* autophosphorylation, immunoprecipitated receptors obtained from 1.5 x 10^6 cells were incubated with 1 ml bacterial lysate, containing approximately 5 μg GST-Shc fusion protein, for 1 h at 4 °C; immunocomplexes were washed four times with PLC-lysis buffer and analyzed by immunoblotting with a polyclonal anti-GST serum.

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