The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein–protein interactions

Peter van der Geer*, Sandra Wiley*, Gerald D. Gish* and Tony Pawson*†

Background: Signal transduction initiated by a wide variety of extracellular signals involves the activation of protein-tyrosine kinases. Phosphorylated tyrosine residues in activated receptors or docking proteins then function as binding sites for the Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains of cytoplasmic signalling proteins. Shc is an adaptor protein that contains both PTB and SH2 domains and becomes phosphorylated on tyrosine in response to many different extracellular stimuli. These results have suggested that Shc is a prominent effector of protein-tyrosine kinase signalling. Thus far, only a single Shc phosphorylation site, the tyrosine at position 317 (Y317) has been identified. Phosphorylation of Y317 has been implicated in Grb2 binding and activation of the Ras pathway.

Results: Here, we report the identification of two major and novel Shc tyrosine phosphorylation sites, Y239 and Y240. These residues are present in the central proline-rich (CH1) region and are conserved in all isoforms of Shc. Y239/240 are co-ordinately phosphorylated by the Src protein-tyrosine kinase *in vitro*, and in response to epidermal growth factor stimulation or in v-*src*-transformed cells *in vivo*. Mutagenesis studies indicate that Y239/240 make an important contribution to the association of Shc with Grb2. Phosphopeptide-binding studies suggest that these two tyrosine residues may be involved in interactions with a number of cellular proteins.

Conclusions: Shc is the most prominent general substrate for protein-tyrosine kinases *in vivo*. The identification of two novel Shc phosphorylation sites indicates that Shc has the potential to interact with multiple downstream effectors. Shc Y239/240 are highly conserved in evolution, suggesting that the phosphorylation of these residues is of fundamental importance. We propose that distinct Shc phosphorylation isomers form different signalling complexes and thereby activate separate downstream signalling cascades.

Addresses: *Programme in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. †Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

Correspondence: Tony Pawson
E-mail: pawson@mshri.on.ca

Received: 29 July 1996
Revised: 22 August 1996
Accepted: 13 September 1996

Current Biology 1996, Vol 6 No 11:1435–1444

Introduction

Many different extracellular signals activate protein-tyrosine kinases [1–3], which in turn initiate cascades of protein–protein interactions that lead to changes in gene expression, cytoskeletal architecture and cell metabolism. These protein–protein interactions are mediated by Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, which bind to specific tyrosine phosphorylation sites [4–6].

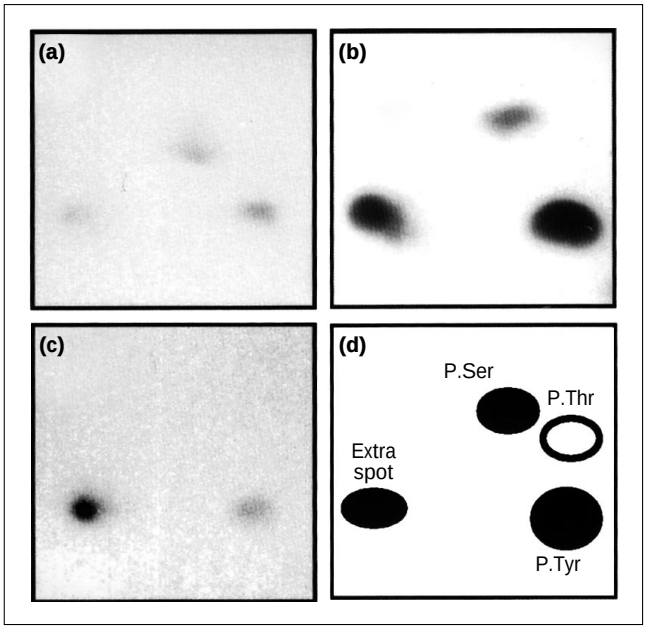
The adaptor protein Shc contains an amino-terminal PTB domain, a central proline-rich region (CH1), and a carboxy-terminal SH2 domain [7]. The Shc PTB domain was recognized recently as a protein module of approximately 160 amino-acid residues that binds to phosphotyrosine (pY) in the context of specific amino-terminal residues, recognizing the sequence ϕXNPXpY (single-letter amino-acid code; ϕ indicates a large non-polar residue and X any amino acid) [8–11]. Shc PTB-binding sites have been identified in a number of growth factor receptors [4,5], and two potential Shc PTB-binding sites are present in the SHIP inositol phosphatase [12–14]. The CH1 region of Shc apparently provides a scaffold for further protein interactions: the tyrosine at position 317 (Y317) is contained within a binding site for the SH2 domain of Grb2, and some stretches that are rich in proline residues may constitute binding sites for Src-homology 3 (SH3) domains [7,15,16]. The SH2 domain at the carboxyl terminus of Shc binds, *in vitro*, to phosphorylated tyrosine residues in the context of a large non-polar amino acid at the +3 position [17], but physiological Shc SH2 domain-binding sites have not been identified with certainty.

The *Shc* gene encodes three widely expressed proteins, $p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$, which differ only in the extent of their amino-terminal sequences [7]. Two mammalian

Shc-related polypeptides, ShcB (also known as Sck) and ShcC, have been identified recently, and have been shown to be expressed predominantly in the brain [9,18]. The sequence conservation between different mammalian Shc family members is highest within their PTB and SH2 domains [9,18]; the central CH1 region contains only three short stretches of conserved amino acids, of which two centre around tyrosine residues. One such conserved motif (YYNS/DϕPXKXPP) contains Y239 and Y240 of Shc, the second motif (YVNXQ) includes the Shc Y317 phosphorylation site [7,15], and the third conserved motif, which associates with adaptins, is predicted to form a turn [19]. A Shc protein has also been identified recently in *Drosophila* [20]. It is of interest to note that the CH1 residues homologous to Shc Y239 and Y240 are present in *Drosophila* Shc, whereas the Shc Y317-containing motif is absent.

Shc polypeptides become phosphorylated on tyrosine and serine residues in response to a wide variety of stimuli [4,21]. Tyrosine-phosphorylated Shc can associate with Grb2 [15,16,22], and through its association with the Grb2/Sos complex may be linked to activation of the Ras/MAP kinase pathway [23–27]. Our understanding of Shc phosphorylation is limited to the characterization of the Y317 phosphorylation site [15]. Y317 is present within a consensus binding site for the Grb2 SH2 domain (pYXNX) [17], and it is generally accepted that Grb2 associates with Shc through Y317. Here, we have analyzed tyrosine phosphorylation of Shc in detail and have identified Y239 and Y240 as two novel and major tyrosine phosphorylation sites. Our data indicate that these two phosphotyrosine sites contribute significantly to the interaction of Shc with Grb2 following growth factor stimulation. Furthermore, Y239 and Y240 may be involved in binding several tyrosine-phosphorylated proteins.

Figure 1



Acid hydrolysis of Shc isolated from *in vivo* ^{32}P-labeled cells reveals a product that does not comigrate with phosphoserine (P.Ser), phosphothreonine (P.Thr), or phosphotyrosine (P.Tyr). Shc polypeptides isolated from ^{32}P-orthophosphate-labeled EGF-stimulated HER14 or v-*src*-transformed fibroblasts were transferred to PVDF membranes and hydrolyzed in hydrochloric acid. The hydrolysis products were separated in the presence of P.Ser, P.Thr, and P.Tyr markers by electrophoresis at pH 1.9 and pH 3.5 in two dimensions on TLC plates. (a) Shc from EGF-stimulated cells. (b) Shc from v-*src*-transformed cells. (c) Phosphoamino-acid analysis of the material present in the extra spot shown in (b). (d) Schematic presentation of the mobilities of the unlabeled phosphoamino-acid markers and the extra spot following separation in two dimensions. P.Thr is shown in white as no labeled P.Thr was detected in this experiment.

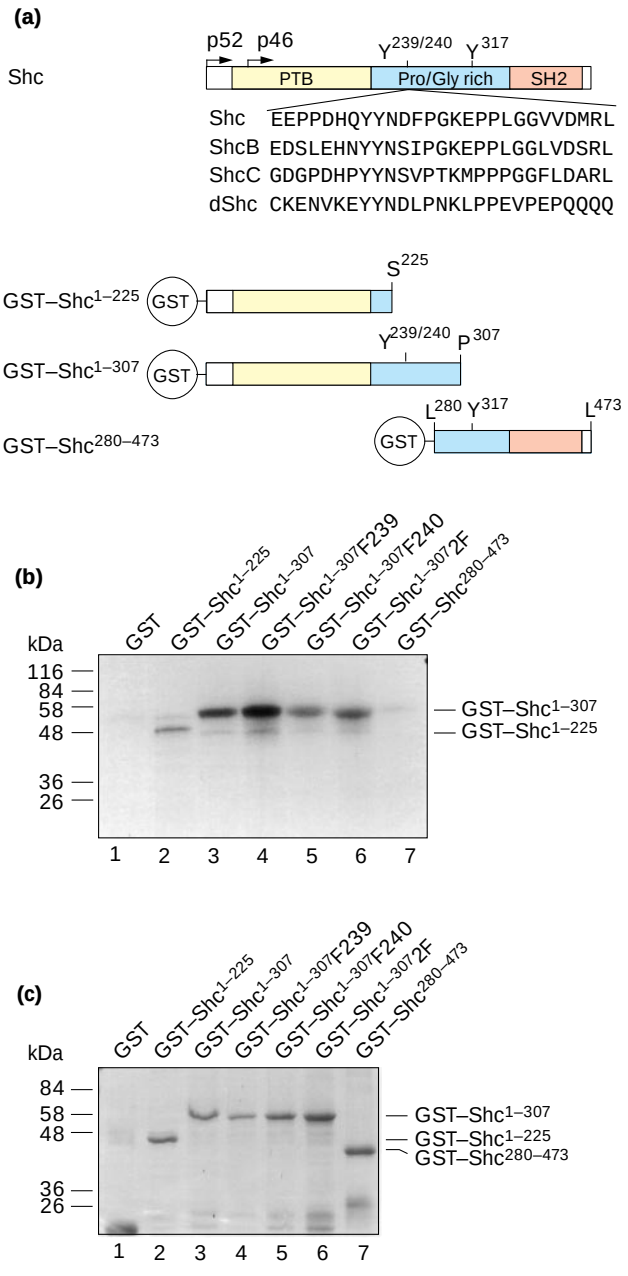
Results

Shc is co-ordinately phosphorylated at Y239 and Y240 by v-Src *in vitro*

Shc becomes phosphorylated on tyrosine and serine residues in response to numerous stimuli, suggesting that its phosphorylation is of general importance for signal transduction. Phosphoamino-acid analysis performed in the initial study to characterize Shc phosphorylation in response to epidermal growth factor (EGF) showed a spot with a mobility different from phosphoserine, phosphothreonine or phosphotyrosine [7]. To confirm this initial observation, NIH3T3 cells overexpressing the EGF receptor were labeled with ^{32}P-orthophosphate and stimulated with EGF; a similar ^{32}P-labeling was undertaken with v-*src*-transformed Rat-2 cells. Shc was isolated from these cells and subjected to partial acid hydrolysis. Separation of the hydrolysis products by electrophoresis in two dimensions on thin-layer cellulose (TLC) plates showed the presence of an additional radiolabeled spot that did not correspond to any of the phosphorylated hydroxy-amino acids (Fig. 1). The migration of this novel product suggested that it may represent a dimer of two phosphorylated amino acids. This hydrolysis product was recovered from the TLC plate and subjected to further acid hydrolysis and a subsequent round of phosphoamino-acid analysis. Acid hydrolysis generated phosphotyrosine in addition to the original compound (Fig. 1c), but not phosphoserine or phosphothreonine, consistent with the possibility that this hydrolysis product is composed of two phosphotyrosine residues.

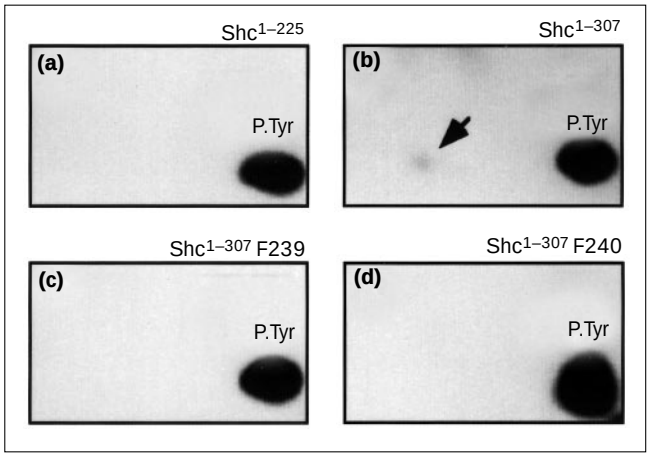
Shc contains only one site with two neighbouring tyrosine residues, at positions 239 and 240. Co-incident phosphorylation of these adjacent tyrosines within the same molecule could generate a di-phosphopeptide. To test this possibility, two glutathione S-transferase (GST)-fusion proteins containing Shc residues 1–225 (GST–Shc$^{1–225}$) or 1–307 (GST–Shc$^{1–307}$) were phosphorylated *in vitro* using purified v-Src kinase. As a potential substrate for protein-tyrosine kinases, GST–Shc$^{1–307}$ differs from GST–Shc$^{1–225}$

Figure 2



The amino-terminal half of Shc contains sites for phosphorylation by the Src protein-tyrosine kinase *in vitro*. (a) Schematic representation of the different fusion proteins used in this study; the sequence around Y239 and Y240 in different members of the Shc family is also shown. (b) Different GST fusion proteins were incubated with purified v-Src protein-tyrosine kinase in the presence of γ-[^{32}P]ATP and divalent cations. Phosphorylated proteins were resolved by SDS–PAGE, the gel was stained and dried, and phosphorylated proteins were visualized by autoradiography. Lane 1: GST; lane 2: GST–Shc$^{1–225}$; lane 3: GST–Shc$^{1–307}$; lane 4: GST–Shc$^{1–307}$F239; lane 5: GST–Shc$^{1–307}$F240; lane 6: GST–Shc$^{1–307}$2F; lane 7: GST–Shc$^{280–473}$. (c) Coomassie blue staining of a gel containing identical amounts of the proteins analyzed in (b).

Figure 3



Phosphorylation of Y239 and Y240 by Src *in vitro* gives rise to the extra spot seen in phosphoamino-acid analysis of tyrosine phosphorylated Shc isolated from v-*src*-transformed cells. *In vitro* phosphorylated GST–Shc fusion proteins isolated from the gel shown in Figure 2b were hydrolyzed in hydrochloric acid; hydrolysis products were separated by electrophoresis at pH 1.9 and 3.5 in two dimensions on TLC plates and visualized by autoradiography in the presence of an intensifier screen. (a) GST–Shc$^{1–225}$. (b) GST–Shc$^{1–307}$. (c) GST–Shc$^{1–307}$F239. (d) GST–Shc$^{1–307}$F240.

by the presence of only two additional tyrosine residues, Y239 and Y240 (Fig. 2a). A fusion protein that contains Shc Y317, but not Y239/240 (GST–Shc$^{280–473}$), was used as a control. GST–Shc$^{1–307}$ was a better substrate for the Src protein-tyrosine kinase than GST–Shc$^{1–225}$, suggesting that Y239 and Y240 may be good sites for phosphorylation by v-Src (Fig. 2b,c). To test this possibility directly, Y239 and Y240 were mutated to phenylalanine, individually and in combination, in the GST–Shc$^{1–307}$ fusion protein. Substitution of both Y239 and Y240 with phenylalanine significantly reduced the extent of phosphorylation of the GST–Shc$^{1–307}$ fusion protein by v-Src (Fig. 2b,c).

The GST–Shc$^{1–225}$ and the GST–Shc$^{1–307}$ fusion proteins were phosphorylated with v-Src, resolved by SDS–PAGE and isolated from the dried gel after autoradiography, and were hydrolyzed for phosphoamino-acid analysis or digested with trypsin for phosphopeptide mapping. Phosphoamino-acid analysis showed that phosphorylated GST–Shc$^{1–307}$ had the novel extra spot whereas phosphorylated GST–Shc$^{1–225}$ did not (Fig. 3). The intensity of the extra spot was low following phosphorylation by Src *in vitro* — this may be caused by low stoichiometry of phosphorylation *in vitro*, or may indicate that the singly phosphorylated site is a poor substrate for Src. Substitution of either Y239 or Y240 with phenylalanine resulted in disappearance of the extra spot in the phosphoamino-acid analysis (Fig. 3), further suggesting that the novel spot represents a dipeptide containing phosphorylated tyrosine residues 239 and 240.

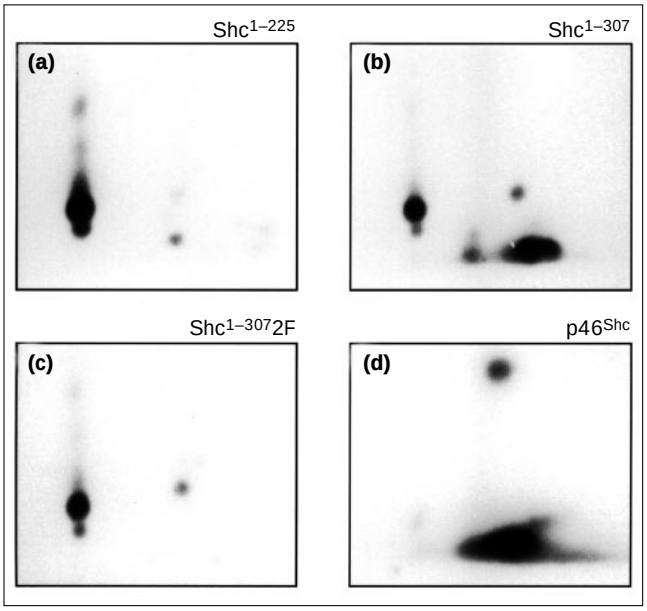
Phosphopeptide mapping of the two GST fusion proteins phosphorylated *in vitro* by v-Src showed that GST–Shc$^{1–307}$ contained a phosphopeptide that was missing in phosphorylated GST–Shc$^{1–225}$ (Fig. 4a,b). This phosphopeptide migrated in the same position as a major phosphopeptide present in tryptic digests of *in vivo* ^{32}P-labeled p46^{Shc} isolated from v-*src* transformed cells (Fig. 4d). Substitution of both Y239 and Y240 with phenylalanine resulted in disappearance of the phosphopeptide unique to GST–Shc$^{1–307}$ (Fig. 4c). These data add support to the idea that Y239 and 240 are phosphorylated by v-Src *in vitro*, and suggest that these sites are also phosphorylated *in vivo*.

Y239 and Y240 are highly phosphorylated by v-Src and the activated EGF receptor *in vivo*

To study Shc phosphorylation *in vivo*, the mutations affecting Y239 and Y240 were introduced into a cDNA encoding the full-length 52 kDa isoform of Shc with an haemagglutinin (HA) epitope at the amino terminus. Wild-type, F239, F240 and F239/240 (2F) mutant proteins were then expressed by transient transfection in COS1 cells. To compare the phosphorylation of the wild-type and mutant Shc proteins, the COS1 cells were co-transfected with a v-*src* expression vector. Analysis of anti-HA immunoprecipitates by immunoblotting with an anti-Shc polyclonal antiserum, showed that wild-type and mutant Shc proteins were expressed at very similar levels in transfected cells (Fig. 5a). Immunoblotting of parallel HA-immunoprecipitates with a polyclonal anti-phosphotyrosine antiserum showed that wild-type protein was highly tyrosine-phosphorylated in these v-Src-expressing COS1 cells (Fig. 5b). However, the 2F mutant Shc contained considerably lower levels of phosphotyrosine, and the single mutants F239 and F240 showed intermediate levels of tyrosine phosphorylation. These data indicate that both Y239 and Y240 are phosphorylated in cells expressing the v-Src cytoplasmic protein-tyrosine kinase.

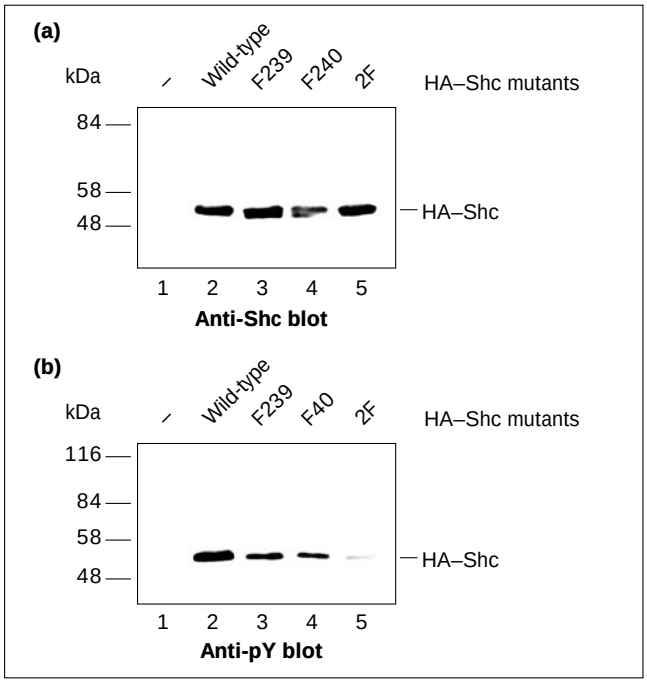
To study phosphorylation of these Shc proteins in response to activation of a receptor protein-tyrosine kinase, COS1 cells were transiently transfected with the Shc expression constructs, starved for 24 hours in serum-free medium and stimulated with EGF for 5 minutes at 37 °C. Wild-type and mutant Shc proteins were expressed

Figure 4



GST–Shc$^{1–307}$ phosphorylated *in vitro* by Src and p46^{Shc} isolated from Src-transformed cells have a tryptic phosphopeptide in common. GST–Shc fusion proteins phosphorylated *in vitro* by purified Src (see Fig. 2b), and p46^{Shc} isolated by immunoprecipitation from ^{32}P-labeled v-*src*-transformed fibroblasts, were isolated from the gel and digested with trypsin. The digestion products were separated in two dimensions by electrophoresis at pH 1.9 and chromatography in phosphochromatography buffer on TLC plates. Phosphopeptides were visualized by autoradiography in the presence of an intensifier screen. (a) GST–Shc$^{1–225}$ phosphorylated *in vitro* by Src. (b) GST–Shc $^{1–307}$ phosphorylated *in vitro* by Src. (c) GST–Shc$^{1–307}$2F phosphorylated *in vitro* by Src. (d) p46^{Shc} isolated from ^{32}P-labeled v-*src*-transformed cells.

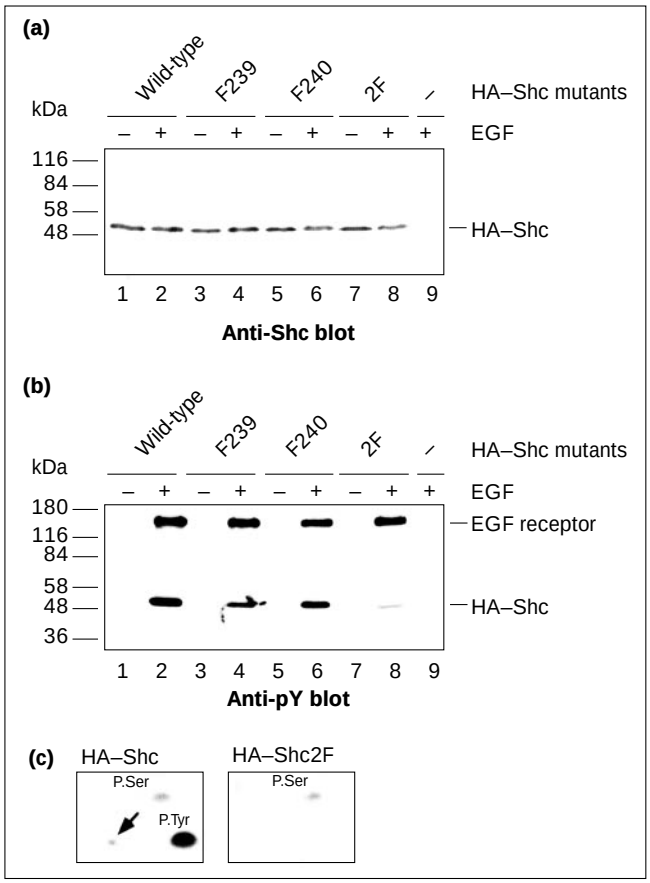
Figure 5



Mutation of Y239 and Y240 to phenylalanine results in decreased tyrosine phosphorylation of Shc in v-Src-expressing COS1 cells. HA-tagged wild-type and mutant Shc proteins were co-expressed with v-Src by transient transfection in COS1 cells. Shc proteins were isolated by immunoprecipitation with an anti-HA monoclonal antibody and analyzed by immunoblotting with (a) an anti-Shc polyclonal serum and (b) an anti-pY polyclonal serum. The gels show anti-HA immunoprecipitates from COS1 cells transiently expressing v-Src (lane 1), wild-type HA–Shc and v-Src (lane 2), HA–ShcF239 and v-Src (lane 3), HA–ShcF240 and v-Src (lane 4), or HA–Shc2F and v-Src (lane 5).

at very similar levels (Fig. 6a). Anti-phosphotyrosine blotting of anti-HA immunoprecipitates showed strong tyrosine phosphorylation of the wild-type Shc protein in response to EGF. In contrast, the 2F mutant was more weakly tyrosine phosphorylated and the single F239 and F240 mutants were phosphorylated to intermediate levels, as also observed in the v-Src-expressing cells. Wild-type and mutant proteins showed equivalent association with the activated EGF receptor (Fig. 6b). Phosphoamino-acid analysis of HA-tagged Shc proteins (labeled with ^{32}P *in vivo*) isolated from EGF-stimulated COS1 cells showed that mutant proteins with both Y239 and Y240 substituted with phenylalanine lacked the extra spot characteristic of wild-type Shc. These data indicate that Y239 and Y240 are major *in vivo* sites of phosphorylation in response to growth factor stimulation. Furthermore, the prominent representation of the di-phosphopeptide in acid hydrolysates of Shc phosphorylated *in vivo* suggests that both tyrosines are commonly phosphorylated in the same molecule.

Figure 6



Y239 and Y240 are major sites of Shc phosphorylation in EGF-stimulated COS1 cells. Transiently expressed wild-type and mutant HA-tagged Shc proteins were isolated by immunoprecipitation with an anti-HA monoclonal antibody from control and EGF-stimulated COS1 cells and analyzed by immunoblotting with **(a)** an anti-Shc polyclonal serum and **(b)** an anti-pY polyclonal serum. The gels show anti-HA immunoprecipitates from serum-starved control (lanes 1,3,5,7) and EGF-stimulated (lanes 2,4,6,8,9) COS1 cells transiently expressing wild-type HA–Shc (lanes 1,2), HA–ShcF239 (lanes 3,4), HA–ShcF240 (lanes 5,6), or HA–Shc2F (lanes 7,8), or from COS1 cells transfected with an empty expression vector (lane 9). **(c)** Transiently expressed wild-type and 2F mutant HA–Shc proteins were isolated from ^{32}P-labeled EGF-stimulated COS1 cells and hydrolyzed in hydrochloric acid. The hydrolysis products were separated in the presence of unlabeled markers by electrophoresis in two dimensions at pH 1.9 and pH 3.5 on TLC plates. Phosphoamino acids were visualized by autoradiography in the presence of an intensifier screen. The spot containing the Y239/240 di-phosphopeptide is indicated with an arrow.

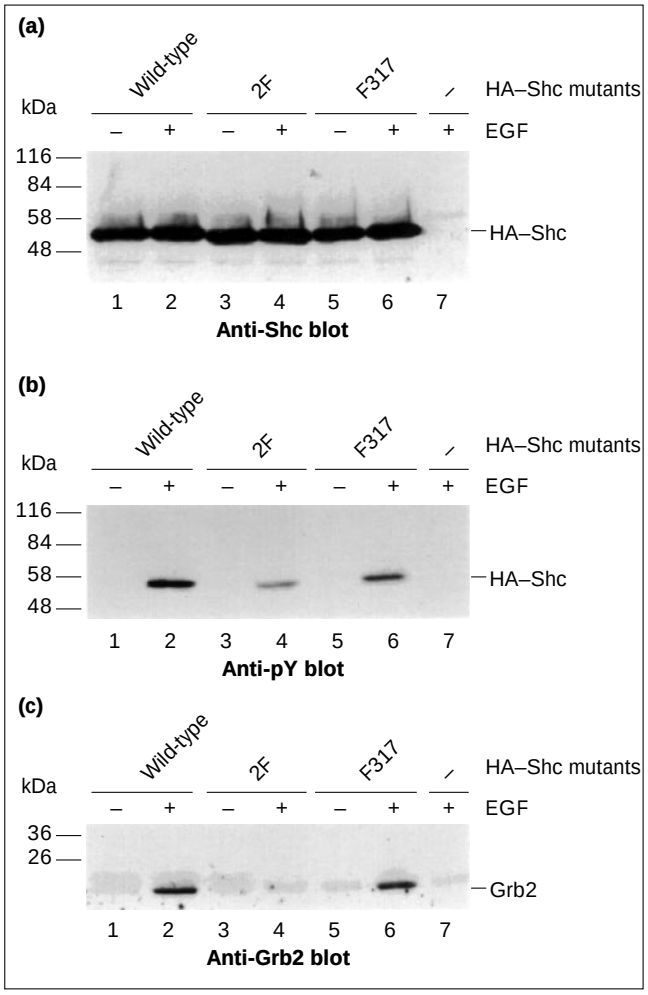
The relative extent to which Shc was phosphorylated at either Y239/240 or Y317 was directly compared by transiently expressing HA-tagged wild-type or mutant proteins in COS1 cells. Substitution of Y317 with phenylalanine resulted in a decrease in EGF-induced Shc tyrosine phosphorylation. However, substitution of Y239/240 caused a more marked reduction in the EGF-induced tyrosine phosphorylation of Shc than did substitution of Y317 (Fig. 7b). These data indicate that Y239/240, as well as Y317, are important Shc phosphorylation sites.

Phosphorylation of Shc Y239/240 induces binding of cellular proteins

A likely function of the Y239/240 phosphorylation sites is to provide binding sites for modules that recognize phosphotyrosine, such as SH2 or PTB domains. Examination of the amino-acid sequence around these sites showed that Y239 is present within a consensus binding sequence for the Grb2 SH2 domain, pYXNX [17], and might therefore interact with Grb2. To test this possibility, 12-amino acid peptides based on the sequence around Y239 and Y240 (residues 236–247) were tested for their ability to bind to Grb2. The unphosphorylated peptide (YY), peptides phosphorylated on either Y239 or Y240 (pY-Y and Y-pY, respectively), and the doubly phosphorylated peptide (pY-pY) were biotinylated, immobilized on Streptavidin-agarose and incubated with cell lysates from v-Src-expressing fibroblasts. Bound proteins were analyzed by anti-Grb2 immunoblotting. Peptides phosphorylated on Y239 and peptides phosphorylated on both Y239 and Y240 bound to Grb2 present in these cell lysates (Fig. 8a), whereas unphosphorylated peptides or peptides phosphorylated on Y240 did not.

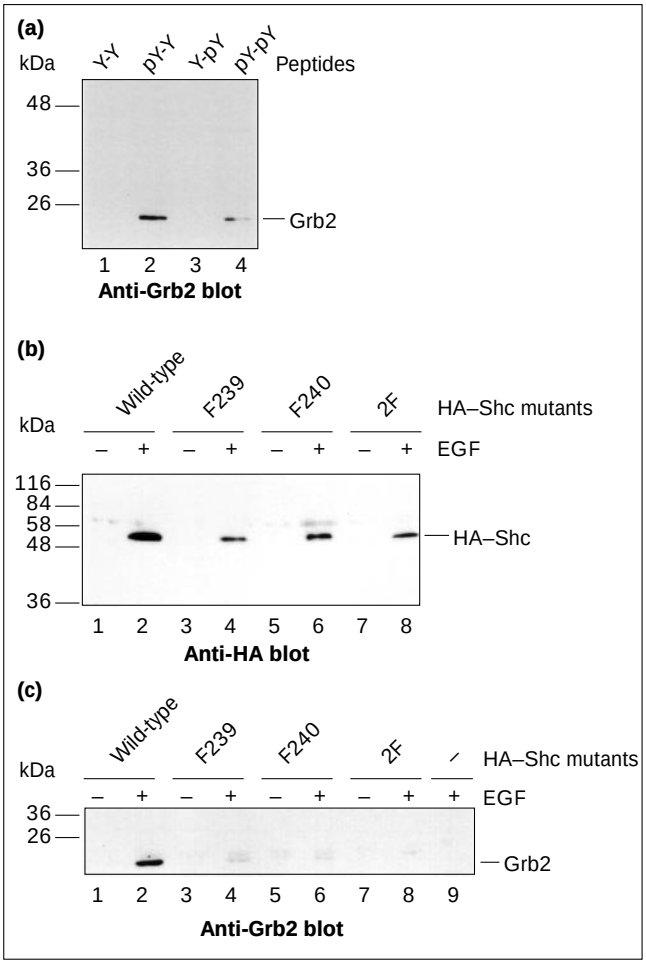
To extend this analysis to full-length Shc, lysates from control and EGF-stimulated COS1 cells expressing wild-type and mutant HA-tagged Shc proteins were incubated with immobilized GST–Grb2 SH2 domain fusion proteins. In this experiment, wild-type HA-tagged Shc from EGF-stimulated cells bound strongly to the Grb2 SH2 domain *in vitro*, whereas the single or double mutant proteins with substitutions at Y239/240 bound significantly less well (Fig. 8b). To test for association of Grb2 with HA-tagged Shc *in vivo*, HA immunoprecipitates from transiently transfected

Figure 7



Both Y239/240 and Y317 are major Shc phosphorylation sites. Transiently expressed wild-type and mutant HA-tagged Shc polypeptides were immunoprecipitated with an anti-HA antibody and immunoblotted with anti-Shc (a), anti-pY (b) and anti-Grb2 (c) sera. The gels show anti-HA immunoprecipitates from serum-starved (lanes 1,3,5) and EGF-stimulated (lanes 2,4,6,7) COS1 cells expressing wild-type HA–Shc (lanes 1,2), HA–Shc2F (lanes 3,4), or HA–ShcF317 (lanes 5,6), or from cells containing the empty expression vector (lane 7).

Figure 8



Phosphorylation of Shc at Y239 and Y240 forms a binding site for the Grb2 SH2 domain. (a) Biotinylated phosphopeptides based on the sequence around Y239 and Y240 were immobilized on streptavidin–Sepharose and incubated with lysates from v-*src*-transformed fibroblasts. Bound proteins were analyzed by immunoblotting with an anti-Grb2 polyclonal serum; lysates were incubated with unphosphorylated peptide (Y-Y, lane 1), a peptide phosphorylated on Y239 (pY-Y, lane 2), a peptide phosphorylated on Y240 (Y-pY, lane 3) and a peptide phosphorylated on both tyrosine residues (pY-pY, lane 4). (b) Lysates from serum-starved control (lanes 1,3,5,7) or EGF-stimulated (lanes 2,4,6,8) COS1 cells transiently expressing HA-tagged wild-type or mutant Shc proteins were incubated with GST–Grb2 SH2 fusion proteins immobilized on glutathione–Sepharose. Bound proteins were analyzed by anti-HA immunoblotting. Lysates were from cells expressing wild-type Shc (lanes 1,2), ShcF239 (lanes 3,4), ShcF240 (lanes 5,6), or Shc2F (lanes 7,8). (c) HA immunoprecipitates from serum-starved control (lanes 1,3,5,7) and EGF-stimulated (lanes 2,4,6,8,9) COS1 cells transiently expressing HA-tagged wild-type or mutant Shc proteins were analyzed by immunoblotting with an anti-Grb2 polyclonal serum. Lysates were from cells expressing wild-type Shc (lanes 1,2), ShcF239 (lanes 3,4), ShcF240 (lanes 5,6), or Shc2F (lanes 7,8), or from cells transfected with an empty expression vector (lane 9).

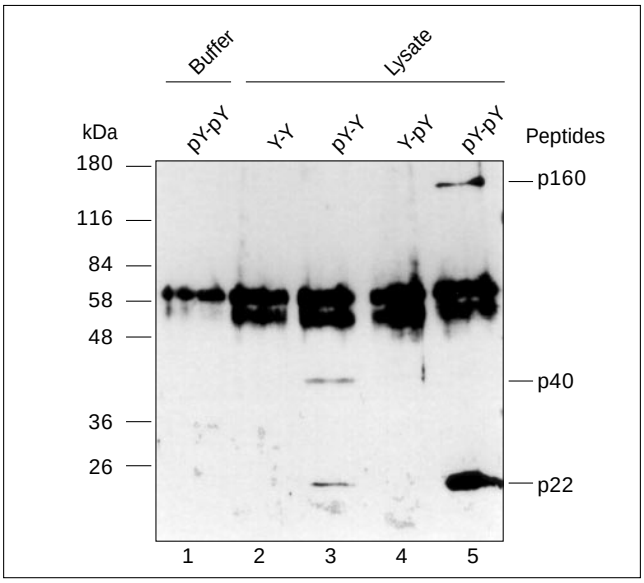
control and EGF-stimulated COS1 cells were analyzed by anti-Grb2 immunoblotting. Grb2 was easily detected in HA immunoprecipitates from EGF-stimulated COS1 cells expressing the wild-type HA-tagged Shc protein. The F239 and F240 single, and the 2F double mutant proteins coprecipitated significantly less Grb2, consistent with a model in which the twin tyrosine residues constitute a major Grb2 binding site in Shc (Fig. 8c).

The relative involvement of Y239/240 and Y317 in Grb2-binding were then compared directly. Whereas mutation of Y317 moderately reduced binding of HA-tagged Shc to Grb2 following EGF stimulation, the substitution of Y239/240 with phenylalanines resulted in a large decrease in Grb2 binding (Fig. 7c). These data suggest that, while the Y239/240 and Y317 sites both interact with Grb2,

Y239/240 is the major Grb2 binding site in the cells analyzed here. Consistent with this possibility, quantitative analysis has shown that the di-phosphorylated Y239/240 peptide binds the Grb2 SH2 domain with a five-fold greater affinity than a phosphopeptide containing the Y317 site (our unpublished results).

To test whether these phosphorylation sites could be involved in interactions with proteins other than Grb2, we used the phosphotyrosine-containing peptides based on the sequence around Y239 and Y240 as affinity reagents. Immobilized peptides were incubated with cell lysates of v-Src expressing cells and bound proteins were eluted and analyzed by anti-phosphotyrosine blotting. Unphosphorylated peptides or peptides phosphorylated on Y240 did not specifically bind any phosphotyrosine-containing proteins. Peptides phosphorylated on Y239 or phosphorylated on both Y239 and Y240 bound to a number of tyrosine phosphorylated proteins. We reproducibly observed binding of proteins with molecular weights of ~22, ~40 and ~160 kDa. Interestingly, the peptide phosphorylated singly on Y239 appeared to bind to p40 with greater affinity than the doubly phosphorylated peptide; in contrast, the doubly phosphorylated peptide appeared to bind with greater affinity to p22 and p160 (Fig. 9). These data suggest that Shc may change binding partners depending on the phosphorylation status of Y239 and Y240.

Figure 9



Phosphorylated peptides based on the sequence around Y239 and Y240 bind specific phosphotyrosine-containing proteins in lysates from v-*src*-transformed cells. Biotinylated phosphopeptides based on the sequence around Y239 and Y240 immobilized on streptavidin–Sepharose were incubated with lysates from v-*src*-transformed fibroblasts. Bound proteins were analyzed by immunoblotting with an anti-pY monoclonal antibody. Lysis buffer was incubated with the doubly phosphorylated peptide as a negative control (pY-pY, lane 1); cell lysates were incubated with the unphosphorylated peptide (Y-Y, lane 2), a peptide phosphorylated on Y239 (pY-Y, lane 3), a peptide phosphorylated on Y240 (Y-pY, lane 4) and a peptide phosphorylated on both tyrosine residues (pY-pY, lane 5).

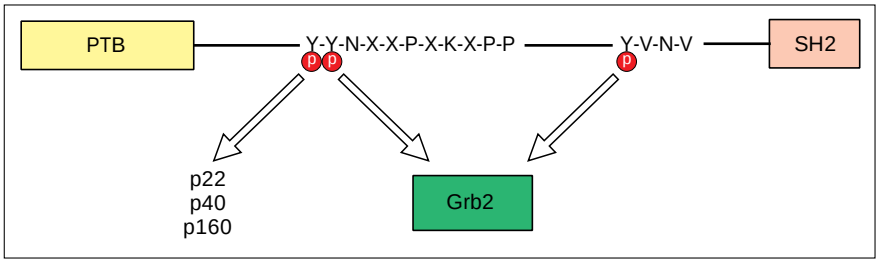
Discussion

A wealth of data suggest that the phosphorylation of Shc is an important step in signal transduction initiated by a wide variety of extracellular stimuli. Y317, located in the carboxy-terminal half of the central CH1 region of Shc, has been identified previously as an important site of phosphorylation [7,15]. Here, we have analyzed Shc phosphorylation and have identified Y239 and Y240 as two additional phosphorylation sites. A combination of phosphopeptide mapping, phosphoamino-acid analysis, and site-directed mutagenesis have shown that Y239 and Y240 are principal sites of Shc phosphorylation by v-Src *in vitro*, and by v-Src and the activated EGF receptor *in vivo*. Identification of a di-phosphopeptide containing phosphorylated Y239/240 indicates that both sites are efficiently phosphorylated within individual Shc molecules, for example in EGF-stimulated cells. The remaining tyrosine phosphorylation observed when Y239 and Y240 are both changed to phenylalanine can be accounted for by phosphorylation at Y317. Consistent with our results, previous work indicated that a full-length mutant Shc protein with phenylalanine at residue 317 could still be phosphorylated on tyrosine *in vivo* [15].

We have compared the sequence around Y239/240 of Shc with the corresponding sequences in the ShcB and ShcC polypeptides [9,18]. Both tyrosine residues and the surrounding amino-acid sequences are conserved between the three mammalian Shc family members (Fig. 1). The sequence relationship amino-terminal to the twin tyrosine residues is limited to the presence of acidic amino-acid residues that may be involved in recognition by protein-tyrosine kinases. Sequence conservation is quite extensive within the 18 residues carboxy-terminal to the adjacent tyrosines, suggesting that the function of Y239 and Y240 is conserved between the Shc family members. It is intriguing that the sequence conservation extends considerably beyond the 3–5 residues needed to provide a binding surface for an SH2 domain [17,28], suggesting that multiple functions may be performed by this island of conserved amino-acid residues. Two adjacent tyrosine residues are also present in *Drosophila* Shc, and are found within a YYNDXPXKXPP motif which is conserved between the human and the *Drosophila* polypeptides. This is in contrast to Y317, which is not found in *Drosophila* Shc [20] — suggesting that the function of Y239 and 240 has been established early in evolution and may be conserved between *Drosophila* and man, whereas the function of Y317 may have evolved more recently.

Y239 is present within the context of a Grb2 SH2 domain consensus binding site, pYXNX [17]. Peptide-binding

Figure 10



Shc contains three tyrosine phosphorylation sites in the proline-rich CH1 region – Y239 and Y240 identified here, and Y317 identified previously [15]. Both the tandem phosphorylation sites and Y317 contribute to Grb2 binding. Y239/240 appear to be involved in the interaction with a number of additional phosphotyrosine-containing proteins in v-*src*-transformed cells.

studies show that both the Y239 mono-phosphorylated and the di-phosphorylated peptides can bind to Grb2 present in cell lysates. The unphosphorylated peptide or the peptide phosphorylated on Y240 did not bind to Grb2. *In vivo*, wild-type HA-tagged Shc bound strongly to Grb2, but mutant Shc proteins in which Y239 and Y240 had been changed, either individually or together, to phenylalanine demonstrated a strongly reduced ability to associate with Grb2, suggesting that Y239 constitutes a major Grb2 binding site. It not clear why the F240 mutant binds Grb2 with greatly reduced affinity compared with the wild-type protein *in vivo*. It is possible that the phenylalanine at the 240 position interferes with Grb2 recognition. Alternatively, Y239 may be phosphorylated less efficiently when it is followed by phenylalanine, perhaps because the protein-tyrosine kinase that phosphorylates Y239 preferentially recognizes this site in the context of a phosphotyrosine at position 240. A third possibility is that the double-phosphorylated site has a higher affinity for the Grb2 SH2 domain. With this in mind, it is of interest to note that adjacent tyrosine residues followed by an asparagine are present in the *Drosophila* and *Caenorhabditis elegans* homologs of the EGF receptor, DER and Let23 [29,30]. Both receptor protein-tyrosine kinases have been shown genetically to interact with Grb2 [31–34]. A YYN motif is also present in the recently described Daughter-of-Sevenless (Dos) protein, which contains a pleckstrin homology domain [35]; both Dos and Drk, the *Drosophila* homolog of Grb2, are required for efficient signalling downstream of Sevenless.

The presence of two tyrosine residues within one SH2 binding site raises the possibility of signal integration. It is possible that complete phosphorylation of these two sites depends on the activation of two different protein-tyrosine kinases; activation of the first kinase would result in phosphorylation of one of the two tyrosine residues, creating a phosphorylation site for the second kinase. Thus, complete phosphorylation of this site may result from activation of two signalling pathways. In this context, it is of interest that, *in vitro*, peptides phosphorylated on Y239 appear to bind a tyrosine-phosphorylated protein that is not bound by the doubly phosphorylated peptide. Conversely, the doubly phosphorylated peptide appears to bind to tyrosine phosphorylated proteins that are not bound by peptides phosphorylated on Y239 alone, whereas both peptides bind Grb2. This suggests that different signalling pathways might be activated downstream of Shc depending on the phosphorylation of Shc at the Y239 and Y240 sites (Fig. 10). We are currently investigating the proteins that interact with phosphorylated Y239 and Y240. Their identification may provide further insights in the role of Shc during signal transduction.

Conclusions

Shc polypeptides are general substrates for protein-tyrosine kinases that become activated in response to a variety of extracellular stimuli. Analysis of Shc tyrosine-phosphorylation has been limited to the characterization of Y317. Here, we have identified two novel tyrosine phosphorylation sites, Y239 and Y240. These twin tyrosine residues are phosphorylated *in vitro* by purified Src kinase, and *in vivo* in v-*src*-transformed and EGF-stimulated cells. These novel sites are highly phosphorylated *in vivo*, and both tyrosine residues are frequently phosphorylated in the same polypeptide. The Y239/240 Shc sites are highly conserved in evolution, suggesting that they are of physiological importance.

Y239 is present within a consensus-binding site for the Grb2 SH2 domain, and phosphorylation of Shc at both tyrosine residues is important for its association with Grb2 *in vivo*. This raises the possibility that the Grb2 SH2 domain may recognize sites that contain two phosphorylated tyrosine residues. Phosphopeptides based on the sequence around Y239/240 bind Grb2 and several tyrosine-phosphorylated proteins in lysates of v-*src*-transformed cell. These data suggest that these phosphorylation sites may function as binding sites for one or more signalling proteins. Their identification may lead to further understanding of Shc's role in normal and malignant signal transduction.

Materials and methods

Cells lines and antibodies

HER14 human EGF receptor overexpressing NIH3T3 cells [36] were grown in Dulbecco-Vogt modified Eagles medium (DMEM) containing 10 % fetal bovine serum (FBS) and 400 μg ml^{-1} G418; S7A v-*src*-transformed Rat-2 cells [37] were grown in DMEM containing 10 % FBS; v-*src*-transformed embryonic fibroblasts were grown in DMEM

containing 5 % calf serum (CS); and COS1 cells [38] were grown in DMEM containing 10 % CS. Polyclonal anti-Shc [7] and anti-pY [39] sera were raised as described. Polyclonal anti-Grb2 serum C-23, monoclonal anti-pY 4G10, and monoclonal anti-HA 12CA5 were purchased from Santa Cruz Biotechnology (Santa Cruz, California), UBI (Lake Placid, New York), and Babco (Richmond, California), respectively.

GST fusion proteins

GST–Shc$^{1–225}$ and GST–Shc$^{1–307}$ are identical to GST–ShcB and GST–ShcC [8]. GST–Shc$^{280–473}$ is identical to GST–Shc^{+} and contains p52Shc residues 280–473 [15]. The GST–Grb2 SH2 fusion protein has been described before [16]. Mutants were generated using a PCR-based strategy; mutant cDNA fragments were sequenced to ensure fidelity and cloned back into the pGEX constructs. Fusion proteins were induced and purified as described before [8].

Expression of wild-type and mutant HA-tagged Shc polypeptides in mammalian cells

Wild-type and mutant human Shc cDNAs were cloned into the mammalian expression vector pcDNA3, a Kozak consensus sequence for initiation of protein synthesis and a sequence encoding the HA epitope starting with a methionine, were linked in frame by a sequence encoding 13 amino acids to the Shc cDNA starting at the first residue of the 52 kDa isoform. COS1 cells at 50 % confluency were transfected with 5 μg pcDNA3 HA-Shc with or without 1 μg v-*src* cloned into pECE; transfections were carried out using Lipofectin and Optimem (Gibco-BRL, Burlington, Ontario L7P 1A1) according to manufacturers directions. Cells were lysed 72 h after transfection.

Immunoprecipitation, peptide-affinity purification 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 lysed in PLC-lysis buffer and immunoprecipitations were carried out exactly as described before [8]. To test for binding of phosphotyrosine-containing proteins to phosphopeptides, PLC lysates were incubated with biotinylated peptides that had been immobilized on streptavidin–Sepharose beads. Sepharose beads were collected by centrifugation and washed 4 times with PLC-lysis buffer. Immunoprecipitates were boiled for 3 min in 62.5 M 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. Peptide bound proteins were eluted with 100 mM triethylamine, boiled in SDS-sample buffer, and resolved by SDS–PAGE. Transfer to PVDF membranes and immunoblotting were carried out as described [8].

Phosphoamino-acid analysis and tryptic phosphopeptide mapping

Cells were grown to confluence on 10 cm plates, starved in serum-free medium if necessary, and labeled for 4 h in 5 ml phosphate-free DMEM containing 0.5 % dialyzed FBS and 1 mCi ml^{-1} ^{32}P-orthophosphate. v-Src-transformed cells were labeled in medium containing 5 % dialyzed FBS. Cells were rinsed twice with ice cold phosphate-buffered saline (PBS) and lysed in 1 ml PLC lysis buffer per 10 cm tissue culture dish. Shc proteins were isolated by immunoprecipitation with anti-Shc serum or anti-HA antibodies and resolved by SDS–PAGE. GST-fusion proteins were immobilized on glutathione–agarose phosphorylated *in vitro* by incubation with purified v-Src kinase (Oncogene Science, Cambridge, Massuchusetts) and γ-[^{32}P]ATP in 25 mM Hepes (pH 7.2), 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 1 mM DTT for 30 min at 30 °C. Following phosphorylation, the GST-fusion proteins bound to glutathione–agarose were washed twice with PLC-LB and samples were resolved by SDS–PAGE. For phosphopeptide mapping, proteins were recovered from the dried gel after autoradiography, concentrated by TCA precipitation and digested with trypsin as described [40]. Tryptic digests were resolved by electrophoresis at 1000 V for 30 min at pH 1.9 and chromatography in phosphochromobuffer. Phosphopeptides were visualized by autoradiography. For phosphoamino-acid analysis, proteins were hydrolyzed in 6 M HCl for 60 min at 110 °C after transfer to PVDF membrane or after isolation from the dried gel as described [40]. For phosphoamino-acid analysis of transiently expressed HA-tagged Shc, proteins were transferred to PVDF membranes; to reduce background, most phosphopeptides were eluted from the membrane during digestion with trypsin before acid hydrolysis. The phosphopeptide that gives rise to the di-phosphopeptide (pY-pY) following acid hydrolysis is not eluted from the membrane during digestion with trypsin.

Phosphopeptides were synthesized as described [41]. A biotin residue was added to the amino-terminal end of each peptide during the synthesis through addition of Fmoc-ε-aminocaproic acid (Bachem Bioscience, Torrance, California), followed by piperidine deprotection and the direct incorporation of biotin, predissolved in a minimal volume of DMSO, using HBTU activation. Cleavage of the peptide from the resin and deprotection was achieved through a 90 min incubation at room temperature in trifluoroacetic acid (10 ml) and a scavenger mixture composed of water, thioanisole, 1,2–ethanedithiol (5.0:0.2:0.1 % by volume) and solid phenol (75 mg). The product was precipitated with cold t-butyl ethyl ether, collected by centrifugation and purified using reverse phase HPLC. The authenticity of the phosphopeptides were confirmed by amino-acid analysis and mass spectroscopy.

Acknowledgements

The first two authors made equal contributions to this study. This research was supported by grants from the Human Frontiers Science Programme, Asahi chemical company, the Medical Research Council of Canada, and The National Cancer Institute of Canada (NCIC). T.P. is a Terry Fox Cancer Research Scientist of the NCIC and P.v.d.G. acknowledges a postdoctoral fellowship of the NCIC.

References

1. Fantl WJ, Johnson DE, Williams LT: Signalling by receptor tyrosine kinases. *Ann Rev Biochem* 1993, 62:453–481.
2. van der Geer P, Hunter T, Lindberg RA: Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* 1994, 10:251–337.
3. Schlessinger J, Ullrich A: Growth factor signaling by receptor tyrosine kinases. *Neuron* 1992, 9:383–391.
4. van der Geer P, Pawson T: The PTB domain a new protein module implicated in signal transduction. *Trends Biochem Sci* 1995, 20:277–280.
5. Eck MJ: A new flavor in phosphotyrosine recognition. *Structure* 1995, 3:421–424.
6. Mayer BJ, Baltimore D: Signalling through SH2 and SH3 domains. *Trends Biochem Sci* 1993, 3:8–13.
7. Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, *et al.*: A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 1992, 70:93–104.
8. van der Geer P, Wiley S, Lai V K-M, Olivier JP, Gish G, Stephens R, *et al.*: A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated growth factor receptors and phosphopeptides. *Curr Biol* 1995, 5:404–412.
9. Kavanaugh WM, Williams LT: An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* 1994, 266:1862–1865.
10. Blaikie P, Immanuel D, Wu J, Li N, Yainik V, Margolis, B: A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J Biol Chem* 1994, 269:32031–32034.
11. Zhou M-M, Ravichandran KS, Olejniczak ET, Petros AM, Meadows RP, Sattler M, *et al.*: Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature* 1995, 378:584–592.
12. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold R, Rohrschneider LR: p150^{ship}, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* 1996, 10:1084–1095.

13. Kavanaugh WM, Pot DA, Chin SM, Deuter-Reinhard M, Jefferson AB, Norris FA, *et al.*: Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and grb2. *Curr Biol* 1996, 6:438–445.
14. Damen JE, Liu L, Rosten P, Humphries RK, Jefferson AB, Majerus PW, Krystal G: The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3, 4, 5-triphosphate 5-phosphatase. *Proc Natl Acad Sci USA* 1996, 93:1689–1693.
15. Salcini AE, McGlade J, Pelicci G, Nicoletti I, Pawson, T, Pelicci PG: Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* 1994, 9:2827–2836.
16. Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Thomas S, *et al.*: Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in the activation of the Ras pathway by tyrosine kinases. *Nature* 1992, 360:689–692.
17. Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, *et al.*: Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* 1994, 14:2777–2785.
18. O'Bryan JP, Songyang Z, Cantley L, Der CJ, Pawson T: A mammalian adaptor protein with conserved SH2 and PTB domains is related to Shc and specifically expressed in brain. *Proc Natl Acad Sci USA* 1996, 93:2729–2734.
19. Okabayashi Y, Sugimoto Y, Totty NF, Hsuan J, Kido Y, Sakaguchi K, *et al.*: Interaction of Shc with adaptor protein Adaptins. *J Biol Chem* 1996, 271:5265–5269.
20. Lai K-MV, Olivier JP, Gish GD, Henkemeyer M, McGlade J, Pawson T: A ***Drosophila shc*** gene product is implicated in signalling by the DER receptor tyrosine kinase. *Mol Cell Biol* 1995, 15:4810–4818.
21. Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci, PG: Not all Shc's roads lead to Ras. *Trends Biochem Sci* 1996, 21:257–261.
22. McGlade J, Cheng A, Pelicci G, Pelicci PG, Pawson T: Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc Natl Acad Sci USA* 1992, 89:8869–8873.
23. Buday L, Downward J: Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of the receptor, Grb2 adaptor protein, and Sos nucleotide exchange factor. *Cell* 1993, 73:611–620.
24. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 1993, 363:45–51.
25. Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J, Bar-Sagi D: Grb2 mediates EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature* 1993, 363:88–92.
26. Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, *et al.*: Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* 1993, 363:85–88.
27. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D: The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* 1993, 363: 83–85.
28. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, *et al.*: SH2 domains recognize specific phosphopeptide sequences. *Cell* 1993, 72:767–778.
29. Livneh E, Glazer L, Segal D, Schlessinger J, Shilo BZ: The ***Drosophila*** EGF receptor gene homolog: conservation of both hormone binding and kinase domains. *Cell* 1985, 40:599–607.
30. Aroian RV, Koga M, Mendel JE, Ohshima Y, Sternberg PW: The ***let-23*** gene necessary for ***Caenorhabditis elegans*** vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* 1990, 348:693–699.
31. Doyle HJ, Bishop JM: Torso, a receptor tyrosine kinase required for embryonic pattern formation, shares substrates with the Sevenless and EGF-R pathways in ***Drosophila***. *Genes Dev* 1993, 7:633–646.
32. Clark SG, Stern MJ, Horvitz HR: ***C. elegans*** cell-signalling gene ***sem-5*** encodes a protein with SH2 and SH3 domains. *Nature* 1992, 356:340–344.
33. Sternberg PW, Horvitz HR: Signal transduction during ***C. elegans*** vulval induction. *Trends Genet.* 1991, 7:366–371.
34. Simon MA, Bowtell DD, Dodson GS, Laverty TR, Rubin GM: Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* 1991, 67:701–716.
35. Raabe T, Rieso-Escovar J, Liu X, Bausenwein BS, Deak P, Maröy P, Hafen E: DOS, a novel pleckstrin homology domain-containing protein required for signal transduction between Sevenless and Ras1 in ***Drosophila.*** *Cell* 1996, 85:911–920.
36. Honegger AM, Dull TJ, Felder S, Van Obberghen E, Bellot F, Szapary D, *et al.*: Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell* 1987, 51:199–209.
37. Brooks-Wilson AR, Ball E, Pawson T: The myristylation signal of $p60^{v\text{-}src}$ functionally complements the N-terminal fps-specific region of $P130^{gag\text{-}fps}$. *Mol Cell Biol* 1989, 9:2214–2219.
38. Gluzman Y: SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 1981, 23:175–182.
39. Kamps MP, Sefton BM: Identification of multiple novel polypeptide substrates of the v-src, v-yes, v-fps, v-ros and v-erb-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. *Oncogene* 1988, 2:305–315.
40. van der Geer P, Lou K, Sefton BM, Hunter T: Phosphopeptide mapping and phosphoamino acid analysis on cellulose thin-layer plates. In *Protein Phosphorylation; a Practical Approach*. Edited by Hardie DG. Oxford: IRL Press; 1993:31–59
41. van der Geer P, Wiley S, Gish GD, Lai VK-L, Stephens R, White MF, *et al.*: Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. *Proc Natl Acad Sci USA* 1996, 93: 963–968.