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A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain

(receptor tyrosine kinases)

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ABSTRACT **The Shc adaptor protein, hereafter referred to as ShcA, possesses two distinct phosphotyrosine-recognition modules, a C-terminal Src homology 2 (SH2) domain and an N-terminal phosphotyrosine-binding (PTB) domain, and is itself phosphorylated on tyrosine in response to many extracellular signals. Phosphorylation of human ShcA at Tyr-317 within its central (CH1) region induces binding to the Grb2 SH2 domain and is thereby implicated in activation of the Ras pathway. Two *shc*-related genes (*shcB* and *shcC*) have been identified in the mouse. *shcB* is closely related to human *SCK*, while *shcC* has not yet been found in other organisms. The ShcC protein is predicted to have a C-terminal SH2 domain, a CH1 region with a putative Grb2-binding site, and an N-terminal PTB domain. The ShcC and ShcB SH2 domains bind phosphotyrosine-containing peptides and receptors with a specificity related to, but distinct from, that of the ShcA SH2 domain. The ShcC PTB domain specifically associates *in vitro* with the autophosphorylated receptors for nerve growth factor and epidermal growth factor. These results indicate that ShcC has functional SH2 and PTB domains. In contrast to *shcA*, which is widely expressed, *shcC* RNA and proteins are predominantly expressed in the adult brain. These results suggest that ShcC may mediate signaling from tyrosine kinases in the nervous system, such as receptors for neurotrophins.**

The mammalian *shcA* gene encodes three overlapping proteins of 46, 52, and 66 kDa, which differ only in the extent of their N-terminal sequences (1). These *shcA* gene products share a C-terminal Src homology 2 (SH2) domain, a central proline-rich region (CH1), and a more N-terminal phosphotyrosine-binding (PTB) domain (2, 3). The ShcA SH2 domain binds preferentially to phosphotyrosine sites with the sequence Tyr(*P*)-(hydrophobic/Glu)-Xaa-(Ile/Leu/Met), and recognizes specific autophosphorylation sites in the activated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (4–6). In contrast, the ShcA PTB domain has recently been shown to bind with high affinity to phosphotyrosine sites with the consensus sequence Leu/Ile-Xaa-Asn-Pro-Xaa-Tyr(*P*). Such sites are found in a number of growth factor receptors, notably the nerve growth factor receptor (Trk), and in polyoma middle T antigen (7–9). The ShcA PTB domain, therefore, recognizes phosphotyrosine in the context of amino-terminal residues, as distinct from SH2 domains, which recognize amino acids C-terminal to phosphotyrosine (10). The p66 ShcA isoform, which is generated by alternative splicing, has an additional proline-rich N-terminal sequence (CH2) (E. Migliaccio, S. Mele, A. E. Salcini, G. Pelicci, K.-A. V. Lai, G. Superti-Furga, P. P. DiFiore, T.P., L. Lanfrancone, and P. G. Pelicci, unpublished results).

These results indicate that ShcA proteins have two modules that bind phosphotyrosine sites with entirely different specificities. Potentially for this reason, ShcA is a prominent substrate for tyrosine phosphorylation in cells stimulated with a wide variety of growth factors and cytokines and in lymphoid cells stimulated with antigen (11–15). In addition, ShcA proteins are phosphorylated by oncogenic receptor and cytoplasmic tyrosine kinases (16, 17).

The principal site of human ShcA phosphorylation is at Tyr-317, located in the central CH1 region within the motif Tyr-Val-Asn-Val (18). A very similar element is found in mouse ShcA (Tyr-Val-Asn-Ile, residues 313–316). Phosphorylation of this residue creates a high-affinity binding site for the SH2 domain of a second adaptor protein, Grb2, which binds preferentially to phosphotyrosine sites with Asn at the +2 position (4). Grb2 is, in turn, associated through its Src homology 3 domains with Pro-rich motifs in the C-terminal tail of mSos1, a Ras guanine nucleotide exchange factor. ShcA phosphorylation, therefore, induces the formation of a ternary complex containing ShcA, Grb2, and mSos1, which may activate the Ras pathway (10). Consistent with this possibility, ShcA overexpression induces transformation of rodent fibroblasts in a fashion that is dependent on Tyr-317 (18). ShcA overexpression also elicits Ras-dependent neurite outgrowth in PC-12 neuronal cells (19). This latter observation suggests that the binding of autophosphorylated Trk to the ShcA PTB domain, and ensuing ShcA phosphorylation and association with Grb2, is one mechanism by which the Trk tyrosine kinase might activate the Ras pathway (20, 21).

The significance of ShcA in signal transduction has been underscored by the identification of a *Drosophila* Shc protein that interacts through its PTB domain with the activated *Drosophila* EGF receptor (DER) (22). Analysis of *Drosophila* Shc has also raised the possibility that Shc proteins have functions in addition to Ras activation. To pursue the role of Shc proteins in signaling by mammalian tyrosine kinases, we have investigated the possibility that mammalian cells contain additional *shcA*-related genes. Two previously undescribed mouse genes have been identified (*shcB* and *shcC*),‖ and the *shcC* gene product has been characterized. On the basis of homology, *shcB* most likely represents the mouse homolog of the recently identified human *shcA*-like gene *SCK* (3). ShcC is

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Abbreviations: SH2, Src homology 2; PTB, phosphotyrosine-binding; EGF, epidermal growth factor; EGFR, EGF receptor; NGF, nerve growth factor; GST, glutathione *S*-transferase; RT-PCR, reverse transcriptase-mediated PCR.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U46854).

very similar in its overall sequence, domain structure, and biochemical properties to ShcA. However, unlike *shcA*, which is widely expressed, *shcC* is specifically expressed in the brain. These results suggest that ShcC may mediate signaling downstream of tyrosine kinases in the nervous system.

MATERIALS AND METHODS

Cell Culture and Reagents. A431 and AF6295 cells (Axl-transformed NIH 3T3 cells, kindly provided by Edison Liu, University of North Carolina, Chapel Hill) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin in the presence of 5% CO_2/95% air. PC-12 cells (kindly provided by Patricia Maness-Tidwell, University of North Carolina, Chapel Hill) were grown in DMEM containing 15% horse serum, 5% fetal bovine serum, penicillin, and streptomycin in the presence of 10% CO_2. NIH 3T3 cells expressing TrkA were kindly provided by Mariano Barbacid (23). EGF was a kind gift of H. Shelton Earp and used at a concentration of 100 ng/ml. Mouse 7S nerve growth factor (NGF) was purchased from Upstate Biotechnology (Lake Placid, NY) and used at a concentration of 100–200 ng/ml. PY20 and EGF receptor (EGFR) antibodies were purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase (HRP)-linked anti-glutathione *S*-transferase (GST) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The λDash 129 genomic library was kindly provided by Janet Rossant (Mt. Sinai Hospital, Toronto). The mouse brain and NIH 3T3 cDNA libraries were purchased from Stratagene.

Cloning of *shcB*. Approximately 1.4×10^6 recombinants from a λDash 129 genomic library were screened in duplicate with a ^{32}P-labeled polymerase chain reaction (PCR) fragment corresponding to the human ShcA SH2 domain at reduced stringency (50% formamide/5× SSC/5× Denhardt's solution/0.5% SDS/200 μg of salmon sperm DNA per ml at 37°C). Filters (Hybond; Amersham) were washed twice at room temperature in 2× SSC/0.1% SDS followed by a single 20-min wash at 42°C. Filters were exposed for 3 days. An NIH 3T3 library (Stratagene) was screened as above with an exon fragment from the *shcB* genomic clone to isolate a partial *shcB* cDNA.

Cloning of *shcC*. A mouse brain library (Stratagene) ($\approx 1 \times 10^6$ recombinants) was screened with a ^{32}P-labeled PCR fragment corresponding to the *shcB* cDNA under high stringency, resulting in the isolation of a second *shcA*-related cDNA, *shcC*. Further 5′ sequence of *shcC* was obtained by 5′ rapid amplification of cDNA ends (RACE) using 5′ RACE-ready cDNA derived from mouse brain (Clontech). The validity of sequence obtained from RACE clones was confirmed by PCR amplification of these sequences from reverse-transcribed RNA derived from brain tissue. The PCR products were sequenced. Deoxyinosine was included in the PCR buffer to overcome problems with GC content of the 5′ end of *shcC* (24).

Northern Blot and Reverse Transcriptase-Mediated PCR (RT-PCR) Analysis of *shcC* Expression. Mouse multiple tissue Northern blots were purchased from Clontech and probed with ^{32}P-labeled PCR fragments under high stringency (50% formamide/10× Denhardt's solution/5× SSC/1% SDS/100 μg of salmon sperm DNA per ml at 42°C overnight). The filter was washed twice at 44°C with 2× SSC/0.1% SDS for 30 min each, followed by a single wash at 55°C in 0.2× SSC/0.1% SDS for 30–40 min. RT-PCR was performed as previously described (25). Briefly, 5 μg of total RNA from the indicated tissues was reverse transcribed in the presence of random hexamers by using the Superscript Moloney murine leukemia virus reverse transcriptase (BRL) in a final volume of 20 μl. A portion of this cDNA reaction was PCR amplified in the presence of *shcC* primers (5′-GTCATTGGCTCCATTCGGACA; 3′-ATCCTGGCATCCGGGGCTCT) and then fractionated on a 3% NuSieve GTG agarose gel (FMC Bioproducts).

Antibody Production, *in Vitro* Binding, and Western Blot Analysis. The ShcA SH2 domain has been previously described (1). GST fusion proteins of the SH2 domains of ShcB and ShcC were constructed by PCR amplification of the regions encoding mouse ShcB and ShcC SH2 domains and subcloning into the pGEX2T bacterial expression vector (Pharmacia). A fusion construct of the PTB domain of ShcC was constructed by PCR amplification of the corresponding region of ShcC from amino acids 28–213 (see Fig. 1). Fragments were subcloned into pGEX4T1. The fidelity of the inserts was confirmed by sequencing. Bacterial cultures containing the fusion constructs of SH2 domains were grown in 2× YT medium containing ampicillin at 100 μg/ml and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 3–5 hr at 37°C. Bacteria were pelleted, then lysed by sonication in phosphate-buffered saline/1% Triton X-100/1% Tween 20/1 mM dithiothreitol supplemented with leupeptin at 10 μg/ml and aprotinin at 10 μg/ml (PBS-A buffer). Lysates were cleared, then incubated with glutathione-Sepharose beads. Beads were pelleted and washed extensively to remove nonspecifically bound proteins. GST-PTB fusion proteins were isolated as above except that the beads were washed with phosphate-buffered saline/20% (vol/vol) glycerol/0.5% Tween-20/1 mM dithiothreitol supplemented with leupeptin at 10 μg/ml and aprotinin at 10 μg/ml. Antibodies were raised against fusion proteins of the SH2 domain of ShcC as described for ShcA (1). *In vitro* binding experiments and Western blotting were performed as described in ref. 1. For peptide competition of the PTB binding to TrkA, experiments were performed as in ref. 22, using peptide at a concentration of 10 μM.

Peptide Selection by SH2 Domains. The selectivities of the isolated SH2 domains of ShcB and ShcC were determined by using a degenerate peptide library screen as previously described (4, 26).

RESULTS

Identification of Two Mouse Genes Related to *shcA*. During the course of screening a mouse genomic library with a human *shcA* probe, we isolated a genomic clone derived from an *shc*-like gene (*shcB*). Sequence analysis of the genomic *shcB* clone identified an exon encoding part of an SH2 domain similar to that found in human ShcA. However, several lines of evidence suggested that the protein product of this genomic clone was distinct from ShcA. In particular, the predicted sequence of the mouse ShcB SH2 domain showed only 68% identity to the mouse ShcA SH2 domain. Sequence comparison of *shcB* suggests that it is the mouse homolog of the recently described human *shc*-like gene, *SCK* (T. Saxton, J.P.O., and T.P., unpublished results).

While we were pursuing the isolation of *shcB* cDNAs, a mouse brain cDNA library was screened with an *shcB* probe. Analysis of one cDNA clone isolated in this screen gave a predicted protein sequence distinct from both mouse ShcA and ShcB, suggesting the presence of a third member of the *shc* gene family (*shcC*). The *shcC* clone contained an open reading frame encoding 474 amino acids with a potential initiating methionine. This sequence apparently encodes the p55 isoform of ShcC described below (J.P.O., unpublished results).

Comparison of the predicted protein sequences of mouse ShcA and ShcC (Fig. 1) indicates that these proteins are highly related (59% identity). Ninety-six amino acids at the C terminus of ShcC have all the residues characteristic of an SH2 domain, including residues required for phosphotyrosine binding (27, 28), and are 69% identical with the corresponding region of ShcA. At its N terminus, ShcC has a region of 163 amino acids that is closely related to the ShcA PTB domain (78% identity). The most highly conserved sequences between

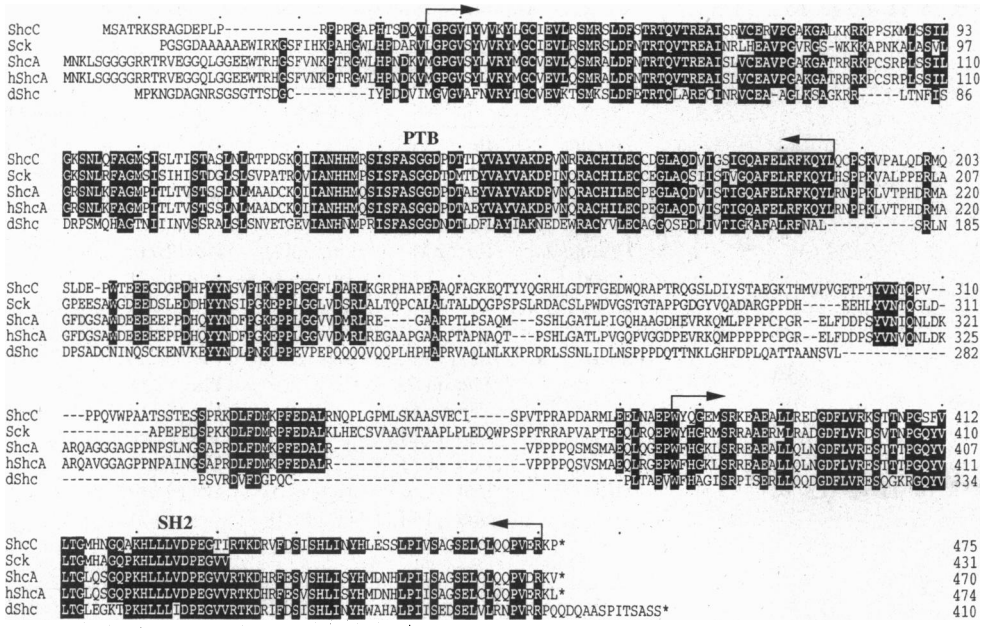

FIG. 1. Alignment of the Shc family of proteins. ShcA and ShcC represent the predicted peptide sequences of the respective mouse genes. dShc represents the predicted sequence of the *Drosophila shc* gene (22). The predicted peptide sequence of the partial human *SCK* cDNA is shown (3). hShcA is the predicted peptide sequence of the human *shcA* gene (1). Sequences were aligned by using the PILEUP program of the Genetics Computer Group software analysis package. The results of this comparison were imported into the MALIGNED multiple sequence alignment program. Amino acids which are identical in at least 4 of 5 sequences are shown in reverse type. The consensus SH2 and PTB domains are marked with arrows.

ShcA and ShcC are those corresponding to the ShcA SH2 and PTB domains, suggesting that ShcC might also have two distinct phosphotyrosine-recognition modules that bind activated tyrosine kinases. The central CH1 regions of ShcA and ShcC are less similar (40% identity) but contain at least three well-conserved motifs. A motif corresponding to the ShcA tyrosine phosphorylation site and Grb2-binding site (Tyr-Val-Asn-Ile, residues 313–316, in mouse ShcA) is present in ShcC and human Sck (Tyr-Val-Asn-Thr). In addition, a motif with the consensus Tyr-Tyr-Asn-Xaa-Xaa-Pro-Xaa-Lys-Xaa-Pro-Pro (where Xaa represents a variable amino acid) is present in the CH1 regions of ShcA, Sck, ShcC, and *Drosophila* Shc. The mammalian Shc proteins have an additional conserved motif (Lys/Arg-Asp-Leu-Phe-Asp-Met-Arg/Lys-Pro-Phe-Glu-Asp-Ala-Leu-Lys/Arg) in the CH1 region. On the basis of the precedent set by the binding of Grb2 to the Tyr-317 phosphorylation site of human Shc, it is probable that the conserved motifs in the CH1 regions of the Shc family of proteins contact downstream targets.

ShcC Is Preferentially Expressed in the Brain. Previous studies have determined that *shcA* is widely expressed. We therefore investigated the expression profile of *shcC*. Northern blot analysis of poly(A)$^+$ RNA from various mouse tissues indicated that *shcC* is specifically expressed in the brain as two transcripts, approximately 10 and 9.8 kb (Fig. 2*A*). Similarly, RT-PCR indicated that *shcC* is primarily expressed in the brain (Fig. 2*B*).

These results suggest that the expression of *shcC* is highly restricted. To investigate this point in more detail, antibodies were raised against a GST fusion protein containing the ShcC SH2 domain. Affinity-purified anti-ShcC antibodies specifically detected two major protein species of 55 kDa and 69 kDa in lysates from mouse cerebellum, cerebrum, forebrain, eye, and spinal cord, and these were absent from heart, intestine, kidney, liver, lung, pancreas, spleen, and stomach (Fig. 3*A*). These bands were not present when the antibody was incubated with fusion protein, but not with GST alone, and were not detected with preimmune serum. In addition, a larger immunoreactive polypeptide of 100 kDa was detected in a number of cell lines of neural origin (data not shown). The 55-kDa protein recognized by the anti-ShcC antibody is apparently encoded by the open reading frame within the cloned *shcC* cDNA. Since no 5′ in-frame stop codons have yet been identified, it is likely that additional 5′ sequences exist which may encode the 69- and 100-kDa immunoreactive proteins. Indeed, *shcA* encodes multiple protein isoforms (p46, p52, and p66) that differ solely in the extent of their N-terminal sequences and are all detected by using antibodies directed against the ShcA SH2 domain (Fig. 3*B*). The ShcC antibodies did not recognize specific proteins in several tissues which are known to express ShcA, indicating that these antibodies do not crossreact with ShcA polypeptides (Fig. 3). These results indicate that ShcC proteins are primarily expressed in the nervous system, consistent with the expression pattern of *shcC*

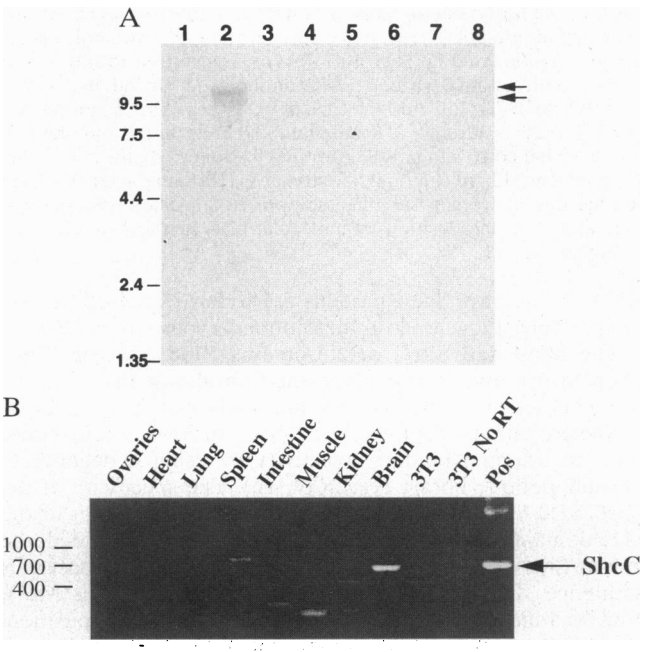

FIG. 2. *shcC* RNA is specifically expressed in brain tissues. (*A*) Northern analysis. Filters were hybridized, washed under stringent conditions, then exposed overnight at −70°C with an intensifying screen. Arrows mark the position of the two *shcC* transcripts. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; and 8, testis. Positions of length markers (in kb) are shown to the left. (*B*) RT-PCR analysis. PCR products were fractionated on an agarose gel and stained with ethidium bromide to visualize bands. Positions of molecular weight markers (in base pairs) are shown to the left. 3T3, NIH 3T3 cDNA; 3T3 No RT, as for 3T3 without reverse transcriptase; Pos, positive control of ShcC cDNA.

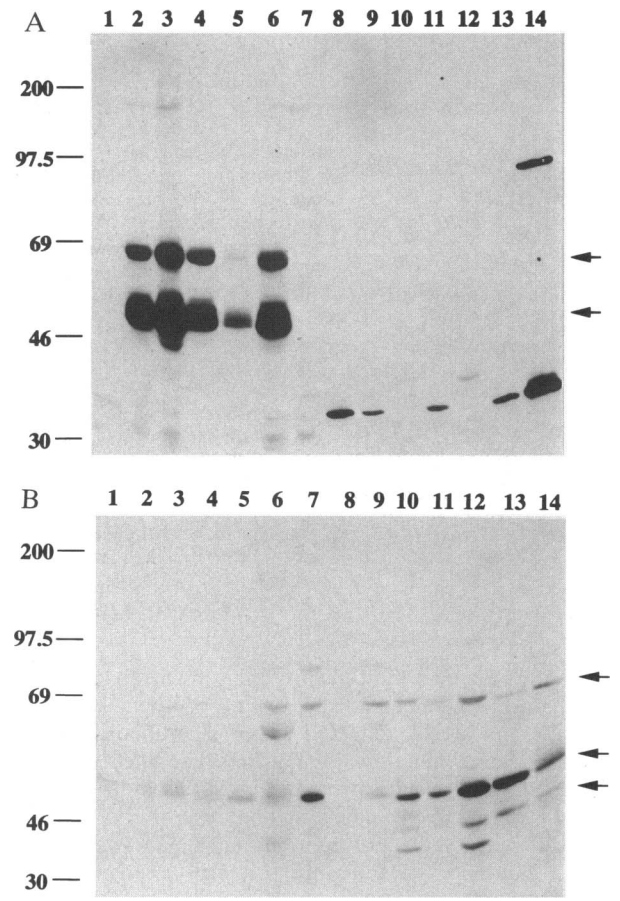

FIG. 3. ShcC proteins are specifically expressed in brain-derived tissues. (*A*) Fifty micrograms of total protein from each of the indicated mouse tissues, except for adrenal and eye, which only had 25 μg, was fractionated by SDS/10% PAGE, transferred to filters, and probed with affinity-purified ShcC antibody (1 μg/ml in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Tween-20/3% nonfat dry milk). Lanes: 1, adrenal; 2, cerebellum; 3, cerebrum; 4, forebrain; 5, eye; 6, spinal cord; 7, heart; 8, intestine; 9, kidney; 10, liver; 11, lung; 12, pancreas; 13, spleen; and 14, stomach. (*B*) Same as in A except staphylococcal protein-A-Sepharose-purified anti-ShcA antibody was used at 1–2 μg/ml. Positions of molecular mass markers (in kDa) are shown to the left.

RNA. In contrast, ShcA proteins are widely expressed but are present only at low levels in the central nervous system (Fig. 3).

The ShcB and ShcC SH2 Domains Bind Specific Phosphopeptides and Phosphotyrosine-Containing Proteins. To test whether the ShcC SH2 domain binds specific phosphotyrosine-containing peptides, GST-SH2 fusion proteins were used to examine binding selectivity by using a degenerate phosphopeptide library screen (4, 26). The specificity of the ShcC SH2 domain was compared with the specificities of the SH2 domains of ShcA and ShcB. As previously described, the human ShcA SH2 domain selected peptides with the consensus sequence Tyr(*P*)-(hydrophobic/Glu)-Xaa-(Ile/Leu/Met), with Xaa indicating little or no selectivity at the +2 position. The ShcB and ShcC SH2 domains gave similar, but distinct, profiles compared to ShcA (Table 1). In contrast to ShcA, ShcB and ShcC showed a preference for hydrophobic amino acids at the +2 position. As with ShcA, both ShcB and ShcC selected hydrophobic amino acids at the +1 and +3 positions. However, the ShcB SH2 domain bound preferentially to Phe and Tyr at +3, binding which was not seen with the other Shc family members. These data suggest that the ShcA, ShcB, and ShcC SH2 domains are similar in their binding specificity for phosphotyrosine-containing peptides, as anticipated from their close sequence relationship. However, there are discernable differences in their selectivity for residues at the +1 to +3 positions, suggesting that their binding to cellular phosphoproteins is not identical.

Table 1. Peptide selectivity of the SH2 domains of Shc family members

SH2 domain	Residue (enrichment value) pY+1	pY+2	pY+3	Selectivity
Human ShcA*	Ile (2.1) Glu (2.0) Tyr (1.7) Leu (1.6)	Xaa	**Ile** (3.4) **Leu** (3.2) **Met** (3.2)	7
Drosophila Shc	Ile (2.4) Leu (1.6)	Leu (1.7) Tyr (1.7) Ile (1.5)	Ile (2.9) Leu (2.4) Met (2.4) Val (1.8)	12
ShcB	Ile (1.9) Met (1.7) Thr (1.6) Asp (1.6) Leu (1.5)	Met (1.9) Ile (1.6)	Met (2.2) Phe (2.1) Ile (2.1) Tyr (1.9) Val (1.9)	8
ShcC	Leu (1.6) Met (1.6) Ile (1.5) Gln (1.5)	Met (1.5) Tyr (1.5)	**Ile** (3.1) Leu (2.7) Met (2.7) Val (1.7)	7

The numbers in parentheses indicate the enrichment value of the amino acid residue. Calculation of enrichment values and selectivity are described in ref. 26. Those residues with enrichment values greater than 3 are printed in boldface type.
*The numbers for human ShcA are taken from ref. 4.

The ability of the SH2 domains of the Shc family members to bind phosphotyrosine-containing proteins in cell lysates was therefore investigated directly. The ShcA, ShcB, and ShcC SH2 domains all bound specifically to the autophosphorylated EGFR in EGF-stimulated cells. However, the ShcB SH2 domain appeared to bind more efficiently than ShcC SH2, which in turn was more efficient than the ShcA SH2 domain (Fig. 4). In contrast, in lysates from NIH 3T3 cells which overexpress the Axl RTK (25), the ShcA SH2 domain bound most tightly to autophosphorylated Axl, followed by the ShcB SH2, which was more effective than ShcC SH2 (Fig. 4). These results are consistent with the data from the phosphopeptide library selection, indicating that the SH2 domains of the different Shc family members have similar binding specificities, but they also display differences which may affect their relative abilities to recognize specific phosphotyrosine-containing proteins *in vivo*.

ShcC Has a Functional PTB Domain. ShcC has sequences related to the ShcA PTB domain. To test whether this region might function as a phosphotyrosine-binding module, the presumptive PTB domain of ShcC was expressed as a GST fusion protein and assessed for its ability to bind to phosphotyrosine-containing proteins in lysates of EGF-stimulated cells as compared to the PTB domain of ShcA. The ShcC PTB domain bound to the autophosphorylated EGFR and additional phosphotyrosine-containing proteins from a lysate of EGF-stimulated cells, in a similar fashion to the ShcA PTB domain (Fig. 5*A*). Comparison of phosphoproteins bound by the ShcC PTB and SH2 domains showed that they recognized overlapping but distinct sets of proteins. These results suggest that the ShcC PTB domain is active in binding to specific phosphotyrosine-containing proteins.

Since ShcC is expressed primarily in neural-derived tissues, we tested whether ShcC could couple to neural-specific receptor tyrosine kinases. ShcA has been shown to specifically interact with the TrkA receptor (21, 29) which is expressed in neural crest-derived sensory neurons (30). This interaction occurs through the PTB domain of ShcA which binds to the motif Ile-Glu-Asn-Pro-Gln-Tyr(*P*) in the juxtamembrane re-

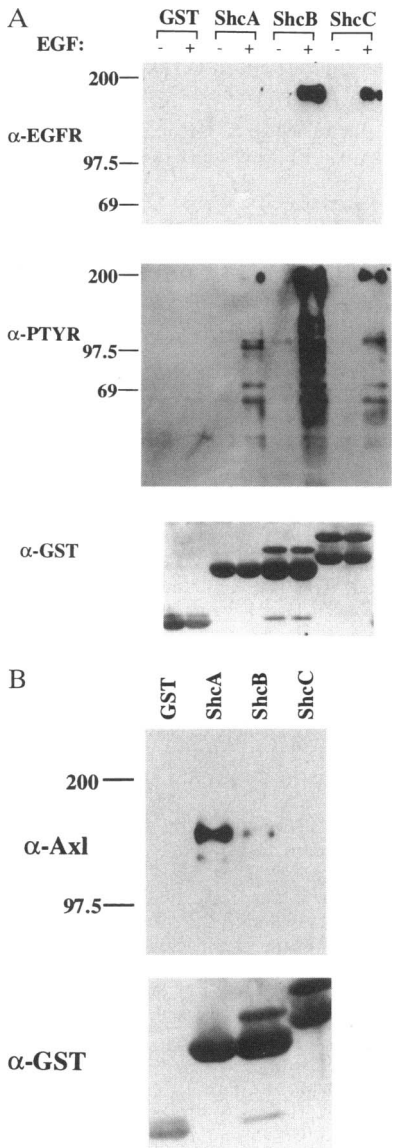

FIG. 4. The SH2 domains of ShcB and ShcC bind tyrosine-phosphorylated proteins. (*A*) A431 cells were grown in the presence or absence of EGF (100 ng/ml) for 1 min, then lysed in cold PLC-LB (1). Equal amounts of fusion proteins were mixed with equivalent amounts of lysate, spun down, washed, fractionated by SDS/10% PAGE, then probed with the indicated antibodies (α-). Anti-GST is shown to confirm that equal amounts of fusion proteins were used in the mixes. Positions of molecular mass markers (kDa) are shown to the left. (*B*) Binding experiments were performed as in *A* except with lysates of NIH 3T3 cells transformed by the Axl tyrosine kinase receptor.

gion of activated TrkA. The ShcC PTB domain also bound *in vitro* to autophosphorylated TrkA, present in lysates of NIH 3T3 cells that ectopically express TrkA (Fig. 5*B*). This interaction was blocked by inclusion of a phosphopeptide modeled on the Tyr-490 juxtamembrane autophosphorylation site of TrkA. These results demonstrate that ShcC can specifically interact with neural receptors and that the ShcC PTB domain recognizes phosphorylated sequences with an Asn-Pro-Xaa-Tyr(*P*) motif.

DISCUSSION

A *shc* Gene Family. Previous work has suggested that ShcA adaptor proteins play a central role in transducing signals from transmembrane and cytoplasmic tyrosine kinases (1, 17). Several biochemical attributes of ShcA apparently contribute to its ability to couple to multiple tyrosine kinases. Notably,

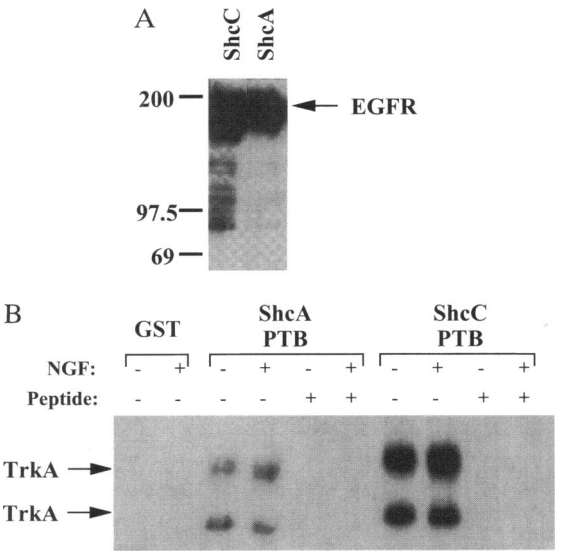

FIG. 5. The PTB domains of ShcC and ShcA bind to activated growth factor receptors. (*A*) *In vitro* binding experiments were performed as described for Fig. 4*A*. Equivalent amounts of lysate from EGF-stimulated A431 cells were incubated with 10 μg of either ShcA or ShcC GST-PTB fusion proteins. Bound proteins were fractionated by SDS/PAGE and then transferred to Immobilon and probed with anti-phosphotyrosine antibody. No binding was seen with GST alone (data not shown). (*B*) *In vitro* binding experiments were performed as in *A* with lysates from NIH 3T3 cells which overexpress TrkA (23). Lysates from cells treated with (+) or without (−) NGF (100 ng/ml) were incubated with the indicated GST-PTB fusion protein either in the presence (+) or in the absence (−) of competing phosphopeptide. The filter was probed with anti-phosphotyrosine antibody. Anti-TrkA antibody was also used to confirm that the tyrosine phosphorylated pp140 and pp110 proteins were the TrkA mature form and precursor (data not shown). TrkA is tyrosine phosphorylated in these cells even prior to NGF stimulation, possibly owing to the high receptor density.

mammalian ShcA and its *Drosophila* homolog are the only proteins identified thus far that contain both functional SH2 and PTB domains. The potential significance of the *shcA* gene is underscored by its conservation between invertebrates and mammals. Here, we have identified two additional *shc*-related genes in the mouse and have analyzed some of the properties of their protein products. The observation that the mammalian genome encodes a family of Shc-related proteins raises the possibility that Shc polypeptides are more diverse in function than previously anticipated. Since there are multiple mammalian *shc* genes, we have designated the mouse genes described here as *shcB* and *shcC* and the original *shc* gene as *shcA*. The product of a partial human cDNA, Sck, is very similar to the mouse ShcB protein (T. Saxton, J.P.O., and T.P., unpublished results), and it therefore seems probable that *shcB* is the mouse homolog of *SCK* (3).

Modular Construction of Shc Family Members. ShcA proteins have two distinct phosphotyrosine-recognition modules, the SH2 and PTB domains, that flank a central region that contains the principal ShcA tyrosine phosphorylation site. On the basis of the observation that Grb2 binds the Tyr-317 phosphorylation site of human ShcA, it is probable that the central CH1 region of ShcA provides binding sites for downstream targets. This possibility suggests a model in which the PTB and SH2 domains couple ShcA to upstream tyrosine kinases, while the CH1 domain is an effector region that provides an output to cytoplasmic signaling proteins.

ShcA and ShcC share a common structural organization. ShcC has an N-terminal PTB domain that is 78% identical to the ShcA PTB domain. We have identified an Arg residue in ShcA PTB (Arg-175 of p52shc) that is critical for binding of phosphorylated ligands and might play a role in phosphoty-

rosine recognition (9). This Arg is conserved in ShcC (Fig. 1). These observations suggested that the PTB domains of ShcB and ShcC might bind specific phosphotyrosine sites as observed for ShcA PTB. Indeed, the ShcC PTB domain associated *in vitro* with the autophosphorylated NGF and EGF receptors and with a number of additional phosphotyrosine-containing proteins in EGF-stimulated cells (Fig. 5).

Both ShcB and ShcC have a C-terminal SH2 domain which shows binding specificity *in vitro* similar to that of the ShcA SH2 domain. However, there are differences in the binding properties of the SH2 domains of the three Shc family members which suggest they may have distinct, albeit related, binding activities *in vivo*. The predicted peptide specificities of the SH2 domains do not shed light on why ShcB SH2 has the greatest affinity for the activated EGFR compared with ShcC and ShcA. The known ShcA SH2 binding site on the EGFR, Tyr-1173 [Tyr(*P*)-Leu-Arg-Val], is not obviously a better ShcB SH2 target site. However, it is possible that the amino acids other than the +1 through +3 positions affect the specificity. The solution structure of the ShcA SH2 domain complexed with a tyrosine-phosphorylated peptide from the T-cell receptor has recently been described (31). Fifteen amino acids of the SH2 domain form contacts with the phosphopeptide, of which 13 are identical in ShcC, consistent with the finding that these SH2 domains have similar peptide selectivities. Taken together, these results indicate that ShcC has functional SH2 and PTB domains through which it can interact with tyrosine kinases and phosphotyrosine-containing proteins.

The central effector region of the Shc family members is less well conserved, sharing only 32–40% identity. However, several short motifs are conserved in the CH1 domains of all three proteins which might provide contact sites for distinct effectors. Both ShcC and Sck have potential Grb2-binding sites (Tyr-Val-Asn-Thr) which are similar to the known Grb2 binding site of human ShcA (Tyr-Val-Asn-Val) (Fig. 1), although it is possible that this sequence in ShcC binds an SH2 domain other than, or in addition to, Grb2. Another motif in the CH1 region, Tyr-Tyr-Asn-Xaa-Xaa-Pro-Xaa-Lys-Xaa-Pro-Pro, is conserved in the three mammalian Shc family members and is also present in *Drosophila* Shc, which lacks a Grb2-binding site. It is not known whether the Tyr residues in this motif are phosphorylated. However, this element is a strong candidate for an effector binding site. The more variable residues in the CH1 domains of Shc family members may play a structural role or may contact distinct effectors.

Signaling Functions of ShcC. A striking difference between ShcC and ShcA is in their pattern of expression. *shcC* RNA and protein are specifically expressed in the mouse brain. Although *shcB* RNA is also predominantly found in the brain, it is more widely expressed than ShcC (J.P.O., unpublished results). The expression patterns of ShcB and ShcC are complementary to that of ShcA, which is broadly expressed but low in brain. These data raise the possibility that the newly discovered Shc family members, especially ShcC, play a specific role in signaling from tyrosine kinases in the nervous system. Both the TrkB and TrkC receptor tyrosine kinases, which are activated by brain-derived neurotrophic factor and neurotrophin 3 or 4/5, possess a juxtamembrane motif corresponding to the Tyr-490 PTB-binding site in TrkA, which is recognized by the ShcC PTB domain. Hence, ShcC may participate in signaling from these neurotrophin receptors, as well as other tyrosine kinases such as Pyk2 which are primarily expressed in the brain (32).

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