A *Drosophila shc* Gene Product Is Implicated in Signaling by the DER Receptor Tyrosine Kinase

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Antibodies to the human Shc adaptor protein were used to isolate a cDNA encoding a *Drosophila* Shc protein (dShc) by screening an expression library. The *dshc* gene, which maps to position 67B-C on the third chromosome, encodes a 45-kDa protein that is widely expressed throughout the *Drosophila* life cycle. In flies, the dShc protein physically associates with the activated *Drosophila* epidermal growth factor receptor homolog (DER) and is inducibly phosphorylated on tyrosine by DER. The 45-kDa dShc protein is closely related both in overall organization and in amino acid sequence (46% identity) to the 52-kDa mammalian Shc isoform. In addition to a C-terminal Src homology 2 (SH2) domain, dShc contains an N-terminal phosphotyrosine-binding (PTB) domain, which associates in vitro with the autophosphorylated DER receptor tyrosine kinase and with phosphopeptides containing an Asn-Pro-X-pTyr motif, where pTyr stands for phosphotyrosine. A potential binding site for the dShc PTB domain is located at Tyr-1228 of DER. These results indicate that the *shc* gene has been conserved in evolution, as have the binding properties of the Shc PTB and SH2 domains. Despite the close relationship between the *Drosophila* and mammalian Shc proteins, dShc lacks the high-affinity Grb2-binding site found in mammalian Shc, suggesting that Shc proteins may have functions in addition to regulation of the Ras pathway.

The human *shc* gene encodes three overlapping proteins of 46, 52, and 66 kDa that are implicated in signaling by a wide range of protein-tyrosine kinases (29). The p46 and p52 Shc isoforms are apparently generated by differential use of translation initiation sites (29), while the 66-kDa isoform is formed by differential splicing and has an additional proline-rich N-terminal extension (24). Shc proteins contain a C-terminal Src homology 2 (SH2) domain, which binds preferentially to phosphotyrosine (pTyr)-containing peptides with the consensus sequence pTyr-Ile-X-Ile (46), and possess a central glycine/proline-rich region. The p46 and p52 Shc polypeptides possess at their amino terminus a novel pTyr-binding (PTB) domain of approximately 160 amino acids in length, which associates with a variety of pTyr-containing proteins from cells transformed by v-*src* and binds directly to the activated epidermal growth factor (EGF) receptor and nerve growth factor (NGF) receptor (Trk) (3, 18, 49). The Shc PTB domain is functionally similar to an SH2 domain, in the sense that it binds its ligands in a pTyr-dependent fashion. However, binding of the PTB domain appears dependent on residues N terminal to the pTyr, in contrast to the SH2 domain, which recognizes amino acids C terminal to pTyr. In particular, the Shc PTB domain binds motifs containing the consensus sequence Asn-Pro-X-pTyr (NPXpY) (49), as found at Shc-binding sites in Trk, the EGF receptor, ErbB3, and polyomavirus middle T antigen (2, 7, 12, 25, 30, 47). Hence, mammalian Shc proteins have two pTyr-binding modules of quite different specificities.

Possibly for this reason, Shc proteins are prominent substrates for phosphorylation by a considerable number of tyrosine kinases in vivo. Shc polypeptides are phosphorylated on tyrosine in cells transformed by oncogenic tyrosine kinases such as v-Src, v-Fps, and Bcr-Abl (23, 32), following cell stimulation by a wide range of growth factors and cytokines (6, 8, 9, 19, 29, 31, 34, 42, 50) and upon activation of antigen receptors in lymphoid cells (35, 41). Shc proteins also become strongly phosphorylated on tyrosine in mice treated with EGF (39), indicating that this may be of physiological relevance.

The principal site of p52^{shc} phosphorylation has been mapped to Tyr-317 (located between the PTB and SH2 domains), within the sequence Tyr-Val-Asn-Val (40). Phosphorylation of this site induces binding of the Grb2 SH2 domain, which associates selectively with motifs with the consensus pTyr-X-Asn-X (38, 45). Since Grb2 is implicated in regulation of the Ras pathway, through its ability to interact with Sos guanine nucleotide exchange factors (5, 14, 15, 21, 37), this provides a means by which Shc may participate in control of the Ras pathway. Consistent with this possibility, overexpression of Shc in rodent fibroblasts leads to malignant transformation (29, 40). Shc transforming activity is abrogated by changing Tyr-317 to phenylalanine, suggesting that Grb2 binding may be important for Shc-induced transformation. Shc overexpression in the neuronal cell line PC12 induces neurite outgrowth in a Ras-dependent manner, indicating that Shc may normally play a role in coupling Trk to Grb2 and thereby to the Ras pathway (38).

Many of the gene products and biochemical pathways that mediate signaling downstream of receptor tyrosine kinases are conserved in *Drosophila melanogaster*. In flies, receptor tyrosine kinases such as sevenless (sev), the EGF receptor homolog (DER), and torso have been shown genetically to signal via the Ras pathway (10, 13, 44). Furthermore, a *Drosophila* homolog of Grb2 (termed Drk) and *Drosophila* Sos are re-

quired for efficient signaling from these tyrosine kinases to Ras (4, 28, 33, 43, 44). Here we report the molecular cloning and expression of a *Drosophila* homolog of mammalian *shc* and the characterization of its product. We present biochemical evidence that dShc may play a role in signaling downstream of the DER tyrosine kinase.

MATERIALS AND METHODS

Isolation and analysis of cDNA clones. An affinity-purified antibody directed towards the SH2 domain of human Shc was used to screen a *Drosophila* cDNA expression library (Novagen) made from mRNA isolated from embryos at 20 to 22 h of development. Phage were allowed to infect cells of the BL21(pLysS) strain of *Escherichia coli*, which were plated on 2× YT plates at a density of 3×10^4 PFU/20-cm-diameter dish. Plates were incubated at 37°C for approximately 8 h and then overlaid with nitrocellulose filters (Schleicher & Schuell), presoaked in 1 mM IPTG (isopropyl-D-thiogalactoside), and allowed to incubate at 37°C for another 12 h. Filters were blocked for 1 h at room temperature in 5% nonfat dry milk in TBS-T (20 mM Tris [pH 7.5], 150 mM sodium chloride, and 0.05% Triton X-100) and probed with 1 μg of affinity-purified anti-Shc antibodies per ml (29) for 2 h. Filters were processed by using an epichemiluminescence kit (Amersham). Approximately 2×10^6 phage were screened, and a single clone, A2-1, was isolated. Additional overlapping *dshc* cDNA clones were obtained by screening a similar library with a 280-bp *Sac*I fragment from clone A2-1 as a probe by using standard DNA hybridization protocols (17). One such clone, 9B, contains the entire open reading frame. DNA sequence analysis was performed on plasmid subclones by using the Sequenase system (United States Biochemical Corp.). Mouse *shc* cDNA clones were isolated from an NIH 3T3 cDNA library (18a).

In situ hybridization. Digoxigenin-labeled DNA probes were prepared by random priming according to the directions in the Boehringer Mannheim application manual, with the exception that a 20-fold higher hexanucleotide concentration was used. Embryos were collected and fixed for 15 min in 4% paraformaldehyde–0.2% Tween 20–0.2% sodium deoxycholate. In situ hybridization and detection of bound probe were carried out as described elsewhere (48). A similar probe was used for in situ hybridization to polytene chromosomes (1).

In vitro transcription-translation. For in vitro transcription of the *dshc* open reading frame, the *Eco*RI-*Apa*I 1.2-kb fragment of clone A2-1 from the Ex-Lox bacteriophage vector was subcloned into the pBluescript vector (pBSKS-dShc p42). A full-length cDNA (pBSKS-dShc p45) was constructed by replacing the 0.9-kb *Eco*RI-*Pst*I insert of this pBSKS-dShc p42 vector with an *Eco*RI-*Pst*I 1.1-kb fragment of clone 9B. The resulting recombinant plasmids were used for in vitro transcription and subsequent translation in a rabbit reticulocyte system by using commercial kits according to the manufacturer's recommendations (Promega Corporation, Madison, Wis.).

Fusion proteins and generation of antibodies. The dShc N-terminal region (residues 1 to 203), the C-terminal SH2 domain (residues 299 to 403), and the C-terminal half of the protein (residues 185 to 403) were cloned into the pGEX-KT vector (Pharmacia) to make glutathione *S*-transferase (GST) fusion proteins dShcN, dShcB, and dShcC, respectively. PCR was used to create restriction endonuclease sites to facilitate cloning of *dshc* coding regions into the expression vector. Specifically, *Bam*HI (5′) and *Eco*RI (3′) sites were added in order to clone *dshc* sequences into pGEX-KT. The resulting pGEX-dShcN, pGEX-dShcB, and pGEX-dShcC plasmids were transformed into *E. coli* DH5α. Recombinant protein was induced for 3 h with 1 mM IPTG, starting when the bacterial culture reached an optical density at 600 nm of 0.5 to 0.8. Bacterial pellets were sonicated three times for 20 s each time in PLC-lysis buffer {50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.5], 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N*′,*N*′-tetraacetic acid [EGTA], 1.5 mM magnesium chloride, 100 mM sodium fluoride, 1 mM dithiothreitol, 10 mM sodium PP_i, and 10 μg each of aprotinin and leupeptin per ml} and clarified by centrifugation. All GST fusion proteins were purified with glutathione-agarose (Pharmacia) and eluted with 10 mM glutathione as described previously (28). Anti-dShc antibodies were prepared by immunizing New Zealand White rabbits individually with purified GST-dShcB or dShcC fusion proteins. Affinity-purified anti-pTyr and anti-Drk antibodies were used as described previously (28). Polyclonal anti-DER antiserum was generously provided by B.-Z. Shilo.

Western blotting (immunoblotting) and immunoprecipitation. For immunoprecipitation, *Drosophila* embryos, pupae, larvae, or adult flies were homogenized in PLC-lysis buffer containing 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation and incubated with a 1:200 dilution of anti-dShcB or anti-dShcC and protein A-Sepharose for 90 min at 4°C. Immunoprecipitates were washed three times in 1 ml of HNTG (20 mM HEPES [pH 7.5], 150 mM sodium chloride, 10% glycerol, 0.1% Triton X-100, and 1 mM sodium orthovanadate) and then boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer. All samples were fractionated by electrophoresis on SDS–10% polyacrylamide gels and transferred to nitrocellulose in a semidry blotting apparatus at 0.8 mA/cm² for 1 h. Filters were blocked for 1 h at room temperature in 5% nonfat dry milk in TBS-T and probed with a 1:200 dilution of either anti-dShcC or anti-DER. For anti-pTyr immunoblots,

filters were blocked in 5% bovine serum albumin–1% ovalbumin in TBS-T and probed with 1 μg of affinity-purified anti-pTyr antibodies per ml. Immunoblots were developed by using either an ECL kit (Amersham) or ^{125}I-protein A (Amersham).

In vitro binding studies. GST fusion proteins were induced and purified by binding to glutathione-agarose beads. Adult flies or NIH 3T3 cells overexpressing the human EGF receptor (HER 14) were lysed in PLC-lysis buffer, clarified, and incubated with immobilized GST or GST fusion proteins (1 to 2 μg) for 90 min at 4°C. The protein complexes were washed three times with HNTG and boiled for 5 min in SDS sample buffer. Complexes were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Filters were blocked and probed with antibodies as described above.

Drosophila strains. Flies carrying transgenes encoding activated torso-sev or torso-DER fusion proteins (with fusion of the extracellular domain of torso[4021] to the intracellular kinase domains of sev or DER) under the control of the *hsp70* promoter in the pW8 transformation vector (11, 36) were stimulated at 37°C for 45 min and then allowed to recover at room temperature for appropriate periods.

The Df(3L)AC1/TM3 stock containing a deficiency of the region 67A2 to 67D11-13 was obtained from the Bloomington Stock Center. To obtain flies with this deficiency for polytene squashes, Df(3L)AC1/TM3 was crossed with TM3/TM6,B. The resulting Df(3L)AC1/TM6,B was then crossed with w,m,f to generate Df(3L)AC1/w,m,f.

Northern (RNA) blot analysis. Northern blots derived from *D. melanogaster* at various growth stages were a gift from L. Rabinow. These were prepared as described previously (51). A [32P]UTP-labeled antisense RNA probe was synthesized with T3 RNA polymerase by using pBSKS-dShc p45 as a template (linearized with *Eco*RI). Loading controls were performed by rehybridizing the blots with a probe for *rp49* (27).

Peptide competition assays. Synthetic phosphopeptides (DER peptide PVS-VDNPEpYLLNAQK and Trk peptide HIIENPQpYFSD) were prepared by using Fmoc chemistry with HBTU activation. Products were purified by reverse-phase high-pressure liquid chromatography and authenticated by amino acid analysis and mass spectrometry. For competition experiments, immobilized GST or GST-dShcN fusion proteins were incubated with lysates of EGF-stimulated HER 14 cells or with heat-shocked *Drosophila* lysates containing activated DER in the absence or presence of peptide (10 μM) in a total volume of 1 ml for 90 min at 4°C. The protein complexes were washed three times with HNTG, boiled for 5 min in SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were blocked and probed with antibodies as described above.

RESULTS

Isolation of a *Drosophila* cDNA encoding a protein related to mammalian SHC. To investigate whether the *shc* gene has been conserved in evolution, we set out to isolate *shc*-related cDNAs from *D. melanogaster*. To this end, a Western blot was performed with a *Drosophila* embryonic protein lysate by using affinity-purified polyclonal antibodies raised against the SH2 domain of human Shc (29). A single protein of approximately 45 kDa specifically cross-reacted with this antibody, suggesting that *D. melanogaster* expresses at least one polypeptide antigenically related to human Shc (data not shown).

To identify *Drosophila* cDNAs that might encode this Shc-related protein, the same anti-Shc antibody was used to screen a *Drosophila* cDNA expression library made from embryos at 20 to 22 h of development. A single phage clone was isolated, which upon sequence analysis was found to contain an open reading frame encoding 397 amino acids. This cDNA was then used as a probe to rescreen a similar cDNA expression library made from embryos at 22 to 24 h of development. A longer 1.5-kb cDNA was isolated, with an open reading frame predicted to encode a protein of 409 amino acids (Fig. 1A). This cDNA has two potential ATG translational initiation sites that are preceded by several in-frame stop codons.

Alignment of the predicted *Drosophila* protein sequence with human and murine Shc proteins showed a high degree of amino acid identity and a similar overall organization for the invertebrate and mammalian polypeptides. The *Drosophila* sequence has several short deletions compared with its mammalian counterparts (Fig. 1B and C). The human *shc* gene has two
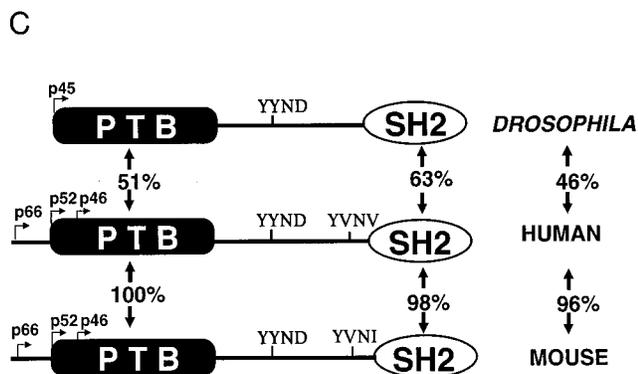
A

FIG. 1. Sequence of dShc. (A) The nucleotide sequence of the *dshc* cDNA and the predicted amino acid sequence of the dShc protein are shown. The termination codon is indicated by an asterisk. (B) Comparison of the *Drosophila*, human, and mouse Shc proteins. Identical residues are highlighted in reverse type. The conserved PTB domain and SH2 domain are indicated by long horizontal arrows. Arrowheads indicate tyrosine motifs discussed in the text. (C) Domain structure of Shc family proteins and percent amino acid identity between individual regions.

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   1  TCGGTACCGGATCGAATTCAAGCGCTTGAATTCAAGCGGTCCCACCTTGAGTAAT
  56  ATCCGAATTACTAATAACAAAGGCTTGCAGCCTACAGCTGCGGAAAAAGGACCAG
 111  CAAGAAGCCAAATCGCTGCCAAGCCCCACAGCCACCGCACAAATGCCTAAGAATG
                                                      M  P  K  N  G    5
 166  GCGATGCCGGCAACCGGAGCGGTTCCGGGACAACCAGCGACGGCTGCATCTATCC
        D  A  G  N  R  S  G  S  G  T  T  S  D  G  C  I  Y  P   24
 221  GGACGACGTCATAATGGGCGTGGGTGTTGCATTCAACGTGCGGTACACGGGATGC
        D  D  V  I  M  G  V  G  V  A  F  N  V  R  Y  T  G  C   42
 276  GTTGAGGTCAAGACCTCAATGAAATCCCTGGACTTCGAGACCCGTACGCAGTTGG
        V  E  V  K  T  S  M  K  S  L  D  F  E  T  R  T  Q  L  A   60
 331  CGCGAGAGTGCATCAATCGAGTTTGCGAGGCTGCCGGTTTAAAGTCTGCTGGCAA
        R  E  C  I  N  R  V  C  E  A  A  G  L  K  S  A  G  K   79
 386  GCGGGAGATTAACAAACTTTATATCGGATCGACCCAGCATGCAGCACGCCGGCACC
        R  R  L  T  N  F  I  S  D  R  P  S  M  Q  H  A  G  T   97
 441  AACATCATCATTAATGTATCGAGCCGAGCTCTGTCGCTGAGCAACGTGGAAACGG
        N  I  I  I  N  V  S  S  R  A  L  S  L  S  N  V  E  T  G   115
 496  GCGAGGTTATAGCCAATCACAATATGCCGCGCATATCGTTCGCGTCCGGCGGTGA
        E  V  I  A  N  H  N  M  P  R  I  S  F  A  S  G  G  D   134
 551  CAATGACACCCTCGACTTCCTCGCCTACATAGCCAAGAATGAGGATGAATGGGCG
        N  D  T  L  D  F  L  A  Y  I  A  K  N  E  D  E  W  A   152
 606  GCATGTTATGTCCTGGAATGCGCTGGCGGCCAAAGCGAGGATCTAATAGTGACCA
        A  C  Y  V  L  E  C  A  G  G  Q  S  E  D  L  I  V  T  I   170
 661  TTGGCAAGGCGTTTGCCTTGCGCTTCAACGCTCTCAGTAGGCTCAATGATCCCTC
        G  K  A  F  A  L  R  F  N  A  L  S  R  L  N  D  P  S   189
 716  GGCGGATTGCAACATCAATCAAAGCTGCAAGGAGAATGTGAAGGAGTACTACAAC
        A  D  C  N  I  N  Q  S  C  K  E  N  V  K  E  Y  Y  N   207
 771  GATTTGCCAAACAAGCTGCCACCAGAAGTGCCCGGAACCACAACAACAGCAGGTGC
        D  L  P  N  K  L  P  P  E  V  P  E  P  Q  Q  Q  Q  V  Q   225
 826  AGCAGCCTCTCCATCCGCATGCGCCTCGTGTGGCACAACTGAATCTGAAGAAGCC
        Q  P  L  H  P  H  A  P  R  V  A  Q  L  N  L  K  K  P   244
 881  TCGCCGATCGCCTGAGCAGCAACCTCATCGACCTCAACTCCCCGCCTCCCGATCAG
        R  D  R  L  S  S  N  L  I  D  L  N  S  P  P  P  D  Q   262
 936  ACGACCAATAAGTTAGGCCACTTTGATCCCCTCCAAGCGACCACAGCTGCCAATT
        T  T  N  K  L  G  H  F  D  P  L  Q  A  T  T  A  A  N  S   280
 991  CTGTTTTGCCCTCTGTTCGCGATGTGTTCGACGGACCGCAATGCCCGCTCACAGC
        V  L  P  S  V  R  D  V  F  D  G  P  Q  C  P  L  T  A   299
1046  GGAGGTGTGGTTCCATGCGGGCATATCCAGACCAATATCGGAGCGTTTGCTGCAG
        E  V  W  F  H  A  G  I  S  R  P  I  S  E  R  L  L  Q   317
1101  CAGGACGGCGACTTTCTGGTGCGCGAGTCACAGGGAAAGCGGGGCCAGTACGTAC
        Q  D  G  D  F  L  V  R  E  S  Q  G  K  R  G  Q  Y  V  L   335
1156  TAACCGGATTAGAAGGCAAGACGCCCAAGCATCTGCTTCTCATCGATCCCGAGGG
        T  G  L  E  G  K  T  P  K  H  L  L  L  I  D  P  E  G   354
1211  CGTGGTCCGAACCAAAGATCGCATCTTCGACAGCATTAGCCATCTGATCAACTAC
        V  V  R  T  K  D  R  I  F  D  S  I  S  H  L  I  N  Y   372
1266  CACTGGGCACACGCGCTGCCCATCATTTCGGAGGATTCCGAGCTGGTCTTGCGTA
        H  W  A  H  A  L  P  I  I  S  E  D  S  E  L  V  L  R  N   390
1321  ATCCGGTGCGAAGACCCCAGCAGGATCAGGCTGCGTCGCCCATTACATCAGCATC
        P  V  R  R  P  Q  Q  D  Q  A  A  S  P  I  T  S  A  S   409
1376  TTCCTGAACCGGAGGGAACAATCATACGTATAGCTACCACCCATCATATACATAA
        S  *                                                    427
1431  CAAATAATTATATATACTGGCATCAGCGTACACGATTAGATTTAAATATTGTGCA
1486  CTCGTAGAATATCTTTTATATATCGTCTGTATAAAAACGAACCCATATATTTATG
1541  GATTGAATATAAACATATTTACAAATATGTGCATGTCTACAGTATTTTCCATTCA
1596  AATGCGAATAAAGATGATAAAGT
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B

C

expression, total RNA was isolated from *Drosophila* embryos, larvae, pupae, and adults at various time points and probed with ^{32}P-labeled *dshc* antisense RNA in a Northern blot. A specific *dshc* transcript of 1.6 kb was identified in all the samples. The highest level of this *dshc* RNA transcript was observed in early (0- to 4-h) embryos (Fig. 2). The lowest level of expression was in late larval and early pupal stages, although even at these stages *dshc* RNA was readily detectable.

Identification of a dShc protein during *Drosophila* development. To investigate the expression of *dshc* gene products in vivo, polyclonal antibodies to bacterial dShc fusion proteins were generated. dShc residues 299 to 403, containing the SH2 domain, were expressed in *E. coli* as a GST fusion protein (GST-dShcB). A similar fusion protein was constructed by using dShc residues 185 to 403 (GST-dShcC). These purified fusion proteins were used to immunize rabbits. Both polyclonal

in-frame ATG codons at positions 153 (ATG-153) and 234 (ATG-234) which apparently represent the initiation sites for translation of the 52- and 46-kDa Shc isoforms (29). These two ATG codons are conserved in the *dshc* gene, although only the most 5′ ATG initiation site seems to be effectively utilized in vivo (see below). The N-terminal 178 amino acids of *Drosophila* Shc (dShc) shows a strong sequence relationship (51% identity) with the corresponding region of mammalian Shc, which contains the Shc PTB domain. A number of basic residues that might participate in the pTyr-binding properties of the PTB domain are conserved between the fly and mammalian proteins. The central region of dShc (residues 179 to 300), corresponding to the Gly/Pro-rich region of mammalian Shc (termed CH1), is less well conserved (~30% identity). In particular, the major tyrosine phosphorylation site of mammalian Shc, Tyr-317, is not found in dShc. However, Tyr-204 of human Shc is present in the human, mouse, and *Drosophila* Shc proteins and is located within a conserved motif, YYNDX-PXKXPP. At its C terminus, the dShc protein has a sequence of 96 amino acids (residues 301 to 396) with all the hallmarks of an SH2 domain. This region of dShc shows the highest degree of sequence relationship with the mammalian Shc proteins (63% identity). This presumably accounts for the ability of antibodies to the human Shc SH2 domain to recognize the dShc polypeptide. Overall there is 46% amino acid identity (63% similarity) between dShc and mammalian p52 Shc.

Cytogenetic localization of *dshc*. The cytological map position of the *dshc* gene was determined by in situ hybridization to polytene chromosomes. The entire *dshc* cDNA was labeled with digoxigenin. Hybridization was detected on the left arm of the third chromosome at position 67B-C (data not shown). To confirm the localization of *dshc*, polytene chromosomes bearing a deficiency for the region from 67A2 to 67D11-13, termed AC-1 (20), were hybridized with the same probe. Large deletions in the heterozygous state cause bulges in the polytene chromosomes at the site of the deletion. The *dshc* hybridization signal was specifically localized to the loop that results from the AC-1 deficiency (data not shown). This confirms that the *dshc* gene is located in the region of 67B-C.

Distribution of *dshc* transcripts in whole embryos and during *Drosophila* development. To investigate the distribution of *dshc* transcripts in *Drosophila* embryos, RNA in situ hybridization was performed with a digoxigenin-labeled *dshc* cDNA probe and *dshc* was detected by using alkaline phosphatase. *dshc* was widely and generally expressed throughout the embryo (Fig. 2). An *engrailed* cDNA used as a control to probe *Drosophila* embryos gave the anticipated segmented expression pattern.

As an alternative approach towards examining *dshc* gene

FIG. 2. Expression of *dshc* transcript. (A) Expression pattern of *dshc* in embryos. A digoxigenin *dshc* probe was used for in situ hybridization of a whole embryo (left). As a control, embryos were also hybridized with an *engrailed* probe, since *engrailed* is expressed in a well-characterized pattern during embryogenesis (right). (B) Transcript distribution of *dshc* throughout *Drosophila* development. A ^{32}P-labeled RNA probe was hybridized to a blot of RNAs from different stages of development. A transcript of 1.6 kb is visible in all cases.

FIG. 3. Identification and expression of dShc proteins. Protein lysates isolated from *Drosophila* embryos, larvae, pupae, and adults (Dros. lysates) were immunoprecipitated with anti-dShcC antibodies (αdShc). As a control, 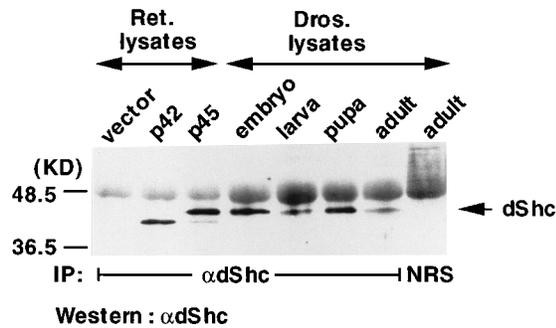the adult *Drosophila* lysate was also immunoprecipitated with nonimmune rabbit serum (NRS). For comparison, vector (pBluescript), dShc-p42, or dShc-p45 plasmids were transcribed and translated in a rabbit reticulocyte lysate (Ret. lysates) and the in vitro translation products were immunoprecipitated with anti-dShcC antibodies. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted with anti-dShcC antibodies. The position of p45[dshc] is indicated (dShc).

antisera specifically immunoprecipitated from *Drosophila* extracts a single protein of approximately 45 kDa, which was recognized by anti-dShc antibodies in Western blots of the immunoprecipitates (Fig. 3). The 45-kDa protein precipitated by anti-dShc antibodies could be identified in lysates of embryos, pupae, larvae, and adult flies, consistent with the broad distribution of the 1.6-kb *dshc* RNA during the *Drosophila* life cycle.

The *dshc* cDNA has two potential translational initiation codons, which would yield overlapping proteins of 42 and 45 kDa (Fig. 1). In order to identify the actual site of translation initiation for the single dShc protein identified in vivo and to determine whether the polypeptide identified with anti-dShc antibodies corresponds to the predicted *dshc* gene product, the cloned *dshc* cDNA was subjected to coupled in vitro transcription-translation. For this purpose, two constructs, one of which contains only the potential p42 ATG start site (dShc-p42) while the other contains both the p42 and p45 potential start sites (dShc-p45), were generated in the pBluescript vector. The proteins expressed from these cDNAs in vitro were subjected to immunoprecipitation and blotting with anti-dShc antibodies and were analyzed in parallel with dShc proteins precipitated from *Drosophila* extracts (Fig. 3). In vitro translation of dShc-p42 gave a polypeptide of the expected size (42 kDa), while translation of dShc-p45 gave two polypeptides of approximately 42 and 45 kDa. The larger 45-kDa in vitro translation product comigrated exactly with the 45-kDa protein immunoprecipitated with anti-dShc antibodies from *Drosophila* lysates, suggesting that this polypeptide is indeed encoded by the *dshc* gene. These results suggest that the principal in vivo *dshc* product (p45[dshc]) uses the first ATG translation initiation site. Although we have not detected a 42-kDa protein in vivo, it might possibly be expressed at levels below the sensitivity of the techniques employed here.

The 45-kDa dShc protein associates with the activated DER receptor tyrosine kinase. Since mammalian Shc proteins bind to the autophosphorylated EGF receptor (2, 29, 38), we tested the possibility that the dShc polypeptide might associate with the closely related *Drosophila* receptor DER (22) and potentially contribute to DER signaling. To investigate the interactions of p45[dshc] with DER, coimmunoprecipitation experiments were performed with lysates of *D. melanogaster* inducibly expressing an activated variant of the DER tyrosine

kinase. A constitutively activated form of DER has been previously created by fusing the extracellular domain of a mutant torso receptor (torso[4021]) to the intracellular region of DER (36). Transgenic flies, in which this chimeric gene product is under the control of the *hsp70* heat shock promoter, were heat shocked for 2.5 h to induce expression of the activated torso[4021]-DER fusion protein. Clarified protein lysates of heat-shocked flies were then immunoprecipitated with anti-dShc antibodies, and the immune complexes were immunoblotted with antibodies to pTyr or to dShc (Fig. 4). Although the levels of p45[dshc] were equivalent before and after heat shock, a prominent pTyr-containing protein of approximately 150 kDa was specifically associated with dShc in lysates of heat-shocked flies. The mobility of this protein corresponds to that of the activated torso[4021]-DER receptor tyrosine kinase, which can also be seen in anti-Drk immunoprecipitates (Fig. 4). Immunoblotting of anti-dShc immunoprecipitates with anti-DER antibodies confirmed that this 150-kDa protein is indeed the chimeric receptor (data not shown). These results indicate that dShc forms a specific complex with autophosphorylated DER. In addition, a low level of pTyr was specifically detected in dShc isolated from heat-shocked flies expressing the activated DER tyrosine kinase. Hence, dShc is likely to be a substrate for DER in vivo. Despite the fact that dShc is detectably tyrosine phosphorylated in flies expressing an activated DER variant, we have been unable to detect association of dShc with the adaptor protein Drk (Fig. 4).

The association between dShc and DER was investigated in more detail by monitoring the kinetics of this interaction upon heat shock of flies carrying the *torso[4021]-DER* transgene. As can be seen in Fig. 5, the association of dShc with activated DER, and other pTyr-containing proteins, was maximal after approximately 2.5 h of heat shock. Similarly, tyrosine phosphorylation of dShc itself was concomitant with the appearance of dShc-DER complexes (data not shown). These results are consistent with the suggestion that the induction of the activated torso[4021]-DER tyrosine kinase is directly responsible for dShc association with pTyr-containing proteins, including autophosphorylated torso[4021]-DER itself, and for dShc tyrosine phosphorylation.

The ability of dShc to associate with the sev receptor ty-
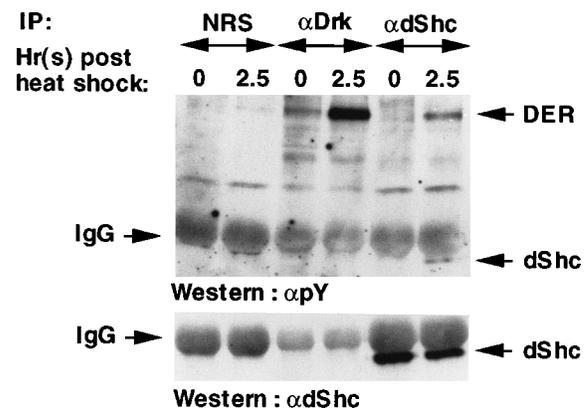
FIG. 4. dShc associates with the activated DER receptor tyrosine kinase. Transgenic flies carrying the *torso[4021]-DER* minigene were lysed prior to heat shock or were heat shocked for 2.5 h, and lysates were immunoprecipitated (IP) with anti-dShcC (αdShc) or anti-Drk (αDrk) antibodies or with nonimmune rabbit serum (NRS). Complexes were resolved by SDS-PAGE and immunoblotted with antibodies to pTyr (αpY, upper panel) or dShcC (lower panel). The positions of autophosphorylated torso-DER receptors (DER), p45 dShc (dShc), and the immunoglobulin heavy chain (IgG) are indicated.

IP: αdShc
Western : αpY

Western : αdShc

In vitro binding: Gst-dShcN
Western : αpY
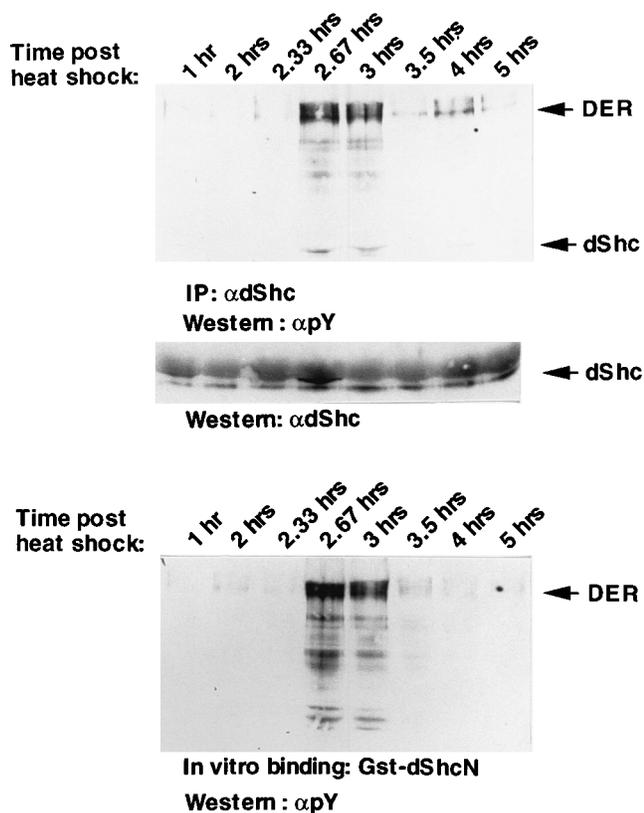
FIG. 5. In vivo and in vitro association of dShc with activated DER following heat shock. Flies carrying the *torso-DER* receptor transgene were heat shocked for the indicated time periods, and protein lysates were incubated with anti-dShcC (αdShc) antibodies (upper panel) or the GST-dShcN fusion protein containing the PTB domain (lower panel). Complexes were resolved by SDS-PAGE and immunoblotted with anti-pTyr (αpY) or anti-dShcC (αdShc) antibodies. The mobilities of the activated receptors (DER) and p45 dShc are indicated. IP, immunoprecipitation.
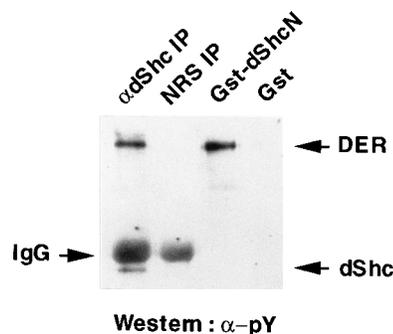

Western : α-pY

FIG. 6. The dShc PTB domain binds DER in vitro. The PTB domain of dShc was expressed as a GST fusion protein (GST-dShcN) containing dShc residues 1 to 203. Lysates containing activated torso-DER were incubated with immobilized GST or GST-dShcN, and associated torso-DER was identified by immunoblotting with anti-pTyr (α-pY) antibodies. Arrows indicate the positions of the chimeric receptor (DER) and tyrosine-phosphorylated dShc. IgG, immunoglobulin G; αdShc, anti-dShc antibody; NRS, nonimmune rabbit serum; IP, immunoprecipitation.

dShcN polypeptide, examined as a function of time following heat shock of torso[4021]-DER transgenic flies, were very similar to the pTyr proteins that coprecipitated with dShc in anti-dShc immunoprecipitates (Fig. 5). The N-terminal dShc PTB domain may therefore play a significant role in the interactions of dShc with activated receptors and other pTyr-containing proteins in vivo. We have also detected binding of the dShc SH2 domain to pTyr-containing proteins in lysates of flies expressing activated DER and an activated *Drosophila* fibroblast growth factor receptor homolog (data not shown).

The dShc PTB domain binds the human EGF receptor and phosphopeptides with NPXpY motifs. Since both Shc and the EGF receptor tyrosine kinase have apparently been conserved in evolution, we tested whether the *Drosophila* dShc PTB domain could recognize the autophosphorylated human EGF receptor in vitro. Indeed, immobilized GST-dShc fusion protein, containing the *Drosophila* PTB domain, bound specifically to the human EGF receptor in lysates of mammalian cells stimulated with EGF (Fig. 7).

The structural and functional similarity between the dShc PTB domain and the PTB domain of mammalian Shc suggested that the *Drosophila* domain might also recognize phosphopeptides with NPXpY motifs. The DER receptor tyrosine

rosine kinases (16) was tested by using transgenic flies that inducibly express sev as a torso[4021] fusion protein under the control of the *hsp70* promoter. With an approach similar to that described above for torso[4021]-DER, no association of the dShc protein was seen with activated sev (data not shown). Hence, it appears that dShc selectively associates with autophosphorylated DER.

The dShc PTB domain binds autophosphorylated DER in vitro. The p45[*dshc*] protein has two potential pTyr-binding domains, the C-terminal SH2 domain and the N-terminal PTB domain, that might account for its ability to recognize activated receptor tyrosine kinases such as DER. To investigate the binding properties of the dShc N-terminal region, corresponding to the potential PTB domain, dShc residues 1 to 203 were expressed in *E. coli* as a GST fusion protein (GST-dShcN). The dShcN fusion protein was immobilized and incubated with lysates of heat-shocked *D. melanogaster* containing the torso[4021]-DER transgene. The resulting complexes were immunoblotted with antibodies to pTyr (Fig. 6). The GST-dShcN polypeptide bound the autophosphorylated torso[4021]-DER tyrosine kinase and several other pTyr-containing proteins present in lysates of *D. melanogaster* expressing activated DER. These results suggest that the N-terminal dShc sequence is a functional PTB domain, in the sense that it possesses an intrinsic ability to bind tyrosine-phosphorylated proteins. The pTyr-containing proteins that associated in vitro with the GST-

Western : αpY

FIG. 7. The dShc PTB domain binds the activated human EGF receptor and phosphopeptides with NPXpY motifs. HER 14 cells, expressing the human EGF receptor, were stimulated (stim.) with EGF (+) or left unstimulated (−), as indicated. Cells were lysed and incubated with GST or the GST-dShcN fusion protein in the absence or presence of phosphopeptides (10 μM) corresponding to the Tyr-1228 site (PVSVDNPEpYLLNAQK) of DER (DER peptide) or the Tyr-490 autophosphorylation site (HIIENPQpYFSD) of Trk (Trk peptide), as indicated. Autophosphorylated EGF receptor (EGFR) was identified by immunoblotting with anti-pTyr (αpY antibodies).

kinase possesses a Tyr at residue 1228, in the C-terminal tail, which lies within an NPXY motif. Autophosphorylation of Tyr-1228 could potentially provide a binding site for the dShc PTB domain. To test this notion, a phosphopeptide corresponding to the presumptive Tyr-1228 DER autophosphorylation site (PVSVDNPEpYLLNAQK) was synthesized. This phosphopeptide bound efficiently to the dShc PTB domain, as measured by competition for binding to the activated EGF receptor in vitro, consistent with the possibility that DER Tyr-1228 represents a PTB-binding site (Fig. 7). Mammalian Shc has been shown to bind through its PTB domain to an autophosphorylation site at Tyr-490 of Trk, which contains an NPXpY motif (49). A phosphopeptide modeled on this Trk PTB-binding site (HIIENPQpYFSD) also bound strongly to the dShc PTB domain in a competition assay (Fig. 7). No binding was observed with nonphosphorylated peptides (data not shown). These results suggest that the dShc PTB domain recognizes phosphopeptides containing NPXpY motifs, requires tyrosine phosphorylation for binding, and has a binding specificity related to that of the Shc PTB domain.

DISCUSSION

Isolation of *dshc*, a *Drosophila* homolog of mammalian *shc*. We have isolated a *Drosophila* homolog of mammalian *shc* by screening a *Drosophila* embryonic cDNA expression library, using an antibody directed towards the SH2 domain of human Shc. This cDNA encodes a 45-kDa protein closely related in sequence to the mammalian p52[shc] isoform. The identification of a *Drosophila shc*-related gene, and its product, indicates that mammalian *shc* belongs to a gene family that is present in invertebrates and has therefore been conserved in evolution. The strong conservation of *shc* sequences from *D. melanogaster* to humans argues that this gene is functionally important. Although we have detected only a single *shc* gene in *D. melanogaster*, mammals have at least one additional *shc*-like gene (18, 26). It therefore remains possible that there are additional *Drosophila shc*-related sequences.

dShc is implicated in signaling by the DER receptor tyrosine kinase in *D. melanogaster*. In mammalian cells, Shc proteins physically associate with a variety of receptor tyrosine kinases and become phosphorylated on tyrosine in response to a wide range of extracellular signals, including growth and differentiation factors, cytokines, and antigens. These results have prompted us to examine whether dShc might also become coupled to activated receptor tyrosine kinases in *D. melanogaster* and therefore represent a potential receptor target. Indeed, dShc was identified in complexes with an activated form of DER but was not associated with sev. The very broad expression of dShc throughout *Drosophila* development, together with its ability to associate with a biologically important receptor such as DER, suggests that dShc plays a role in linking receptor tyrosine kinases to downstream signaling pathways. This notion is supported by the finding that dShc is phosphorylated on tyrosine in flies expressing activated DER.

Structure, function, and binding properties of dShc. While the mammalian *shc* gene encodes three distinct protein isoforms, in flies only a single 45-kDa dShc protein, which most closely resembles mammalian p52[shc] in sequence and structure, has been identified. p52[shc] is generally the most highly expressed Shc protein in mammalian cells (29). We have obtained no evidence to suggest that the *dshc* gene might encode an isoform analogous to p66[shc]. While *dshc* potentially encodes a 42-kDa isoform similar to mammalian p46[shc], such a polypeptide has not yet been detected in *Drosophila* lysates.

Hence, the expression of *dshc* appears less complex than that of its mammalian counterpart.

A comparison of Shc protein sequences from *D. melanogaster* and mammals has suggested several possible functions of the *Drosophila* dShc polypeptide. As anticipated from its recognition by antibodies directed towards the SH2 domain of human Shc, dShc has an SH2 domain very similar in sequence to that of the mammalian Shc proteins. As we have previously reported (49), the N-terminal ~180 residues of dShc are closely related to the PTB domain of mammalian Shc. This observation suggests that the PTB domain, like the SH2 domain, has been conserved in evolution and is present in invertebrates, consistent with the possibility that it plays a significant role in cell signaling. The mammalian Shc PTB domain recognizes pTyr sites containing the consensus motif NPXpY but apparently requires at least 5 residues N terminal to pTyr for efficient binding (49). The presence of a PTB-like domain in the dShc protein indicates that this polypeptide may also possess the ability to bind such pTyr motifs. This notion is substantiated by the observation that the dShc PTB domain binds in vitro to autophosphorylated DER and other pTyr-containing proteins from *Drosophila* lysates. It is of interest that a single PTB-binding consensus is found in the C-terminal DER tail (VDNPEY[1228]LLNAQ) and that a very similar motif is found in members of the EGF receptor family in other species, including the human EGF receptor (VGNPEY[1114]LNTVQ), human ErbB2 (VENPEY[1196]LTPQ), and the avian EGF receptor (VDNPEY[1149]LNTNQ). Consistent with the conservation of this potential PTB-binding motif, we have found that the dShc PTB domain also stably associates with the activated human EGF receptor in vitro. This interaction was inhibited by competition with a phosphopeptide containing the C-terminal DER NPXpY motif. These data suggest that autophosphorylation of DER Tyr-1228 creates a binding site for the dShc PTB domain and likely provides a primary site for the association of dShc with DER in flies. The ability of the dShc PTB domain to recognize other phosphopeptides with an NPXpY motif, such as a phosphopeptide modeled on the Trk Tyr-490 autophosphorylation site, indicates that the binding properties of the dShc PTB domain are similar to those of its mammalian counterpart. Functional PTB domains, like SH2 domains, are therefore present in invertebrates as well as in vertebrates. The high degree of sequence identity observed between the PTB and SH2 domains of dShc and mammalian Shc likely reflects their conserved pTyr-binding activities.

The PTB and SH2 domains flank a central region that bears more limited sequence identity to the corresponding region of mammalian Shc. Of particular interest, the principal tyrosine phosphorylation site in Shc, whose occupancy leads to Grb2 binding and is proposed to contribute to Ras activation, is not present in dShc. Consistent with this observation, we have not detected any association of dShc with Drk, the *Drosophila* homolog of Grb2, despite the fact that dShc becomes phosphorylated on tyrosine by DER. The most highly conserved motif in the central region of *Drosophila* and mammalian Shc proteins is the element Tyr-Tyr-Asn-Asp-X-Pro-X-Lys-X-Pro-Pro. This sequence contains the only Tyr residues in the central region of dShc and the only Tyr residues in the central region other than Tyr-317 in mammalian Shc. The presence of this motif in both *Drosophila* and mammalian Shc argues that it is functionally significant. Since substitution of Tyr-317 of human Shc with Phe greatly reduces but does not abolish Shc phosphorylation (40), it is possible that one or both of these conserved tyrosines can be phosphorylated, especially since they are preceded by acidic amino acids. Phosphorylation of these residues might induce the binding of downstream SH2- or

PTB-containing proteins, or might have some other binding activity. In principle, phosphorylation of this motif might also create a Grb2/Drk-binding site, but this has not yet been observed to occur in either the mammalian or the *Drosophila* system (reference 40 and this study). It is of interest that DER itself has a Tyr-Tyr-Asn-Asp motif within its C-terminal tail.

A general scheme to explain the structure of Shc and Shc-related proteins is as follows. These proteins possess both SH2 and PTB domains, which allow them to associate with distinct tyrosine phosphorylation sites and, in principle, might allow binding to multiple pTyr-containing proteins. These pTyr-binding regions likely serve to receive inputs from activated tyrosine kinases. In contrast, the central Shc region allows for a signal output, for mammalian Shc by coupling to Grb2 and possibly other downstream binding partners. The downstream targets of dShc have yet to be identified but apparently do not include Drk. These observations suggest that Shc and Shc-related proteins may have multiple functions in signaling, in addition to their proposed involvement in regulating the Ras pathway. Indeed, there are as yet no data to indicate that dShc is involved in signaling through Ras.

The *dshc* gene has been mapped to a region of the third chromosome that has been well characterized by deletion analysis, by ethyl methanesulfonate- and diepoxybutane-induced mutations, and by P-element insertions. Mutagenesis screens designed to identify components of the sev, DER, and torso signaling pathways have not yet identified genes that map in close proximity to *dshc*. However, in these screens maternal-effect mutations in *dshc* would go undetected. It is also possible that the high-level expression of *dshc* throughout development may prevent a detectable mutant phenotype in the heterozygous state, which is the basis for the screens undertaken thus far. It will be of great interest to identify mutant alleles of *dshc* in order to understand the role of *dshc* in *Drosophila* development and in signaling downstream of receptor tyrosine kinases.

ACKNOWLEDGMENTS

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