

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-

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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 × 10⁴ 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- β -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 μ g of affinity-purified anti-Shc antibodies per ml (29) for 2 h. Filters were processed by using an epichemiluminescence kit (Amersham). Approximately 2 × 10⁵ 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 *SacI* 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 a 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 *EcoRI*-*ApaI* 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 *EcoRI*-*PstI* insert of this pBSKS-dShc p42 vector with an *EcoRI*-*PstI* 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, *BamHI* (5') and *EcoRI* (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* DH5 α . 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 μ g 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 μ g of affinity-purified anti-pTyr antibodies per ml. Immunoblots were developed by using either an ECL kit (Amersham) or ¹²⁵I-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 μ g) 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 torso⁴⁰²¹ 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 *EcoRI*). Loading controls were performed by rehybridizing the blots with a probe for *rp49* (27).

Peptide competition assays. Synthetic phosphopeptides (DER peptide PVSVDNPEpYLLNAOK 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 μ M) 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.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank data bank under accession number U26445.

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

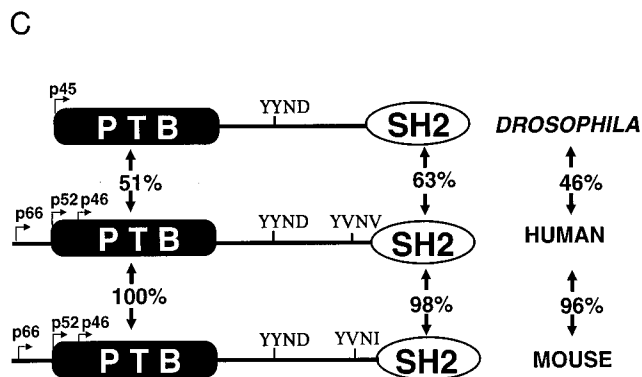
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1 TCGGTACCGGATCGAATTCGAAGCGCTTGAATTCGAAGCGGTCACCGCTTGCAGTAA
56 ATCCGAATTAATAAAGCGCTTGCAGCTACAGCTGCGGAAAAGGACCCAG
111 CAAGAAGCCAAATCGCTGCCAAGCCCCACAGCCACCGCACAAATGCCTAAGAATG
      M F K N G
166 GCGATGCGCGCAACCGGAGCGGTTCCGGGACAACCGGACCGGCTGCATCTATCC
      D A G N R S G S G T T S D G C I Y P
221 GGACGACGTATAATGGCGGTGGTGTGCATTCAACGTGCGGTACACGGGATGC
      D D V I M G V G V A F N V R Y T G C
276 GTTGAGGTCAAGACCTCAATGAAATCCCTGGACTTCGAGACCCGTACGCAAGTGG
      V E V K T S M K S L D F E T R T Q L A
331 CCGGAGAGTGCATCAATCGAGTTTGGAGGCTGCCGGTTTAAAGTCTGCTGGCAA
      R E C I N R V C E A A G L K S A G K
386 GCGGAGATTAAACAACCTTATATCGGATCGACCCAGCATGCAACGCGCCGACCC
      R R L T N F I S D R P S M Q H A G T
441 AACATCATCATTAAATGATCGAGCCGAGCTCTGCTGCTGAGCAACGTTGGAAACGG
      N I I I N V S S R A L S L S N V E T G
496 CCGAGGTTATAGCCAATACAAATATGCCCGCATATCGTTGCGCTCCCGGGGTGA
      E V I A N H N M P R I S F A S G G D
551 CAATGACACCCCTCGACTCTCTCGCTACATAGCCAAGATAGGATGATGGGGG
      N D T L D F L A Y T A K N E D E W A
606 GCATGTTATGTCCTGGAATGCGCTGGCGGCAAGGAGGATCTAATAGTGACCA
      A C Y V L E C A G G Q S E D L I V T I
661 TTGGCAAGGCGTTTGCCTTGGCTTCAAAGCTCTCAGTAGGCTCAATGATCCCTC
      G K A F A L R F N A L S R L N D P S
716 GCGGATTGCAACATCAATCAAAGCTGCAAGGAGATGTGAAGGACTACTACAAC
      A D C N I N Q S C K E N V K E Y Y N
771 GATTGCAAAACAAGCTGCCACCAGAAGTCCCGGAACCAACAACAGCAGGTGC
      D L P N K L P P E V P E P Q Q Q V V Q
826 ACCAGCCTCTCCATCCGATCGCCTCGTGTGGSCAACTGAATCTGAAGAAGCC
      Q P L H P H A P R V A Q L N L K K P
881 TCGGATCGCTGAGCAGCAACCTCACTGACCTCAACTCCCGCCTCCCGATCAG
      R D R L S S N L I D L N S P P P D Q
936 ACGACCAATAAGTATAGCCACTTTGATCCCTCCAAGCGACACAGCTGCCAATT
      T T N K L G H F D P L Q A T T A A N S
991 CTGTTTCCGCTCTGTTCCGATGTGTCCAGCAGCCCAATGCCGCTCACAGC
      L P S V R D V F D G P Q C P L T A
1046 GGAGTGTGTTCCATCGCGCATATCCAGACCAATATCGGACCGTTTGTCTGCA
      E V W F H A G I S R P I S E R L L Q
1101 CAGGACGCGACTTTCTGGTGGCGAGTCCAGGGAAGCGGGCCATACGTAC
      Q D G D F L V R E S O G K R G Q Y V L
1156 TAACCGGATAGAAAGCAAGCGCCAAAGCATCTGCTTCTCATCGATCCGAGGG
      T G L E G K T P K H L L L I D P E G
1211 CCGTCCGCAACAAAGATCGCATCTTCGACAGCATTAGCCATCTGATCAACTAC
      V V R T K D R I F D S I S H L I N Y
1266 CACTGGGCACACCGCTGCCCATCATTTCCGAGGATTCGAGCTGGTCTTCCGTA
      H W A H A L P I I S E D S E L V L R N
1321 ATCCGGTCCGAAGCCCGCAGGATCAGGCTCGCTCCGCCATATCATCAGCATC
      P V R R P Q Q D Q A A S P I T S A S
1376 TTCTGAAACGGAGGAAACAATCATACGTATAGCTACCCCATCATATACATAA
      S
1431 CAAATAATTATATACTGGCATCAGCGTACAGGATAGATTTAAATATTTGTGCA
1486 CTCTAGATAATCTTATATATAGCTGTATAAAAAGCAACCCATATATTTATG
1541 GATTGAATATAACATATACAAATATGTCATGTCTACAGTATTTTCATTCA
1596 AATGCGAATAAAGATGATAAAGT
    
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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.

B

	p52	p46	
human	M NKLS CGG CGRRTRVEGGQLGGEWTRH GSFV NKPTR GWLHP NKVMCPGVSYLVRY GCVEV LQSMRAL DFN TRTOV TR EALISLVCEAVPGAKGAT RRR KPCSRP		105
mouse	M NKLS CGG CGRRTRVEGGQLGGEWTRH GSFV NKPTR GWLHP NKVMCPGVSYLVRY GCVEV LQSMRAL DFN TRTOV TR EALISLVCEAVPGAKGAT RRR KPCSRP		105
<i>Drosophila</i>	M PKN CG AGNS----- G SG-- T SDG C LY P DDV I MG V AF N VRY I GC V EV K TS M KS L DF E TR T Q L ARE C I N RV C E A AG L K S AG R RL T ----		82
	PTB →		
human	LSS L GRSNL K F AG MP I L T VS T SS L N MA ADCK Q I T AN H M S I S F AS G GD P DA E V V A V AK D PVN Q R A CH I LE C PE G LA Q D V I S T I CA F EL R F K O Y L R N P P		210
mouse	LSS L GRSNL K F AG MP I L T VS T SS L N MA ADCK Q I T AN H M S I S F AS G GD P DA E V V A V AK D PVN Q R A CH I LE C PE G LA Q D V I S T I CA F EL R F K O Y L R N P P		210
<i>Drosophila</i>	- N F S D P S M Q H AG T I I N V S R A L S L S N V E T G VE T I AN H N M P R I S F A S G GD N D L D F L A Y I AK N E D W R AC V L E C A G G S E D L I V T I C K A F A L R F N A L S R ---		183
	▼		
human	K I W I F H DR M AG F D S A W D E E E E P PD H O Y Y N D F PK E P L GG V DM R L R E G A A P G A R E T A F N A O T P S H I G A T L P V G Q P V G D P E V R K Q M P P P P C P G R E L --- F D		313
mouse	K I W I F H DR M AG F D S A W D E E E E P PD H O Y Y N D F PK E P L GG V DM R L R E G --- G A A R P T L P S A Q M S S H L G A T L P I G Q H A A G D H E V R K Q M L P P P P C P G R E L --- F D		309
<i>Drosophila</i>	- L N D E S A D C N I N Q - S C K - E N V K E --- Y N D L E N K I P --- E V --- P E P Q Q Q V Q Q L H E R A P R V A Q L N L K K R D R L S S N L I D L N S --- P P P D Q T T N K L G H F D		270
	▼		
human	D F SY V N V Q N L D K A R Q A V G G A G P P N P A I N G S A P R D L F M K P F E D A L R V P P P Q S V S M A B Q L R G E P W F H G K L S R R E A E A L L Q L N G D F L V R E S I T T P G Q V L T G L Q S G		418
mouse	D F SY V N I Q N L D K A R Q A V G G A G P P N P S L N G S A P R D L F M K P F E D A L R V P P P Q S M S M A B Q L G E P W F H G K L S R R E A E A L L Q L N G D F L V R E S I T T P G Q V L T G L Q S G		414
<i>Drosophila</i>	- E --- L Q A T A N - S V L --- E S V --- R M F D ----- G P Q C P --- L T - A E - V --- W F H A G I S R P I S E R L L Q D D G D F L V R E S Q G K R Q Y V L T G L E G K		341
	SH2 →		
human	Q P K H L L L D P E G V V R T K D H R F E S V S H L I S V H M D N H L P I I S A G S E L C L Q Q P V E R K L		473
mouse	Q P K H L L L D P E G V V R T K D H R F E S V S H L I S V H M D N H L P I I S A G S E L C L Q Q P V D R K V		469
<i>Drosophila</i>	T P K H L L L D P E G V V R T K D R I F D S I S H L I N H W A H A L P I I S E D S E L V L R N V R R P Q D Q A A S P I T S A S S		409



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

expression, total RNA was isolated from *Drosophila* embryos, larvae, pupae, and adults at various time points and probed with ³²P-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

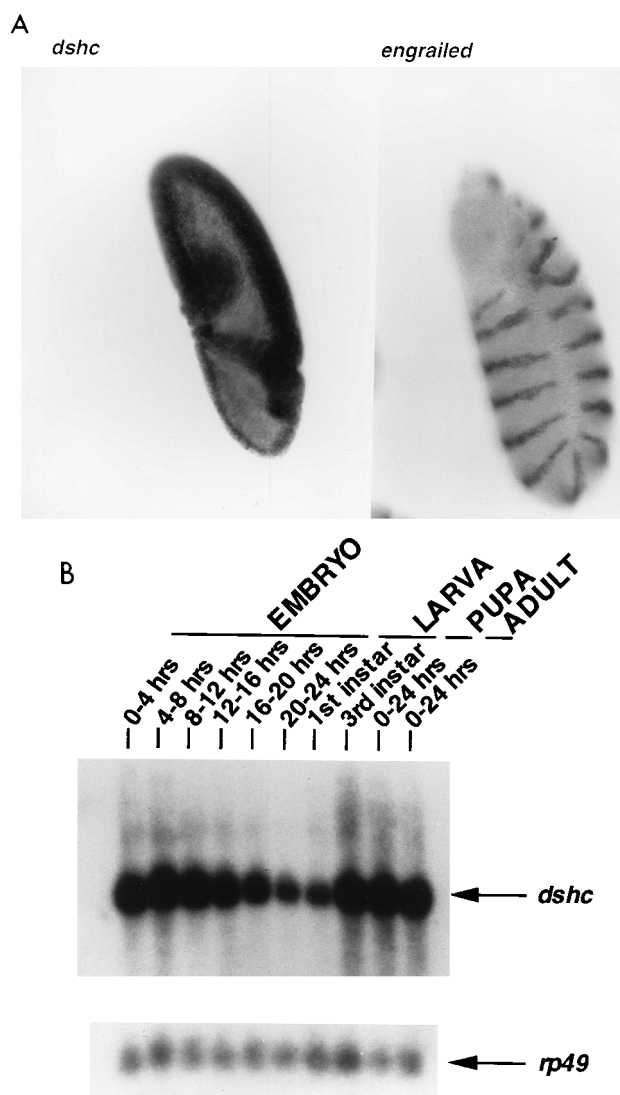


FIG. 2. Expression of *dshc* transcript. (A) Expression pattern of *dshc* in embryos. A digoxigenin *dshc* probe was used for in situ hybridization of a whole embryo (left). As a control, embryos were also hybridized with an *engrailed* probe, since *engrailed* is expressed in a well-characterized pattern during embryogenesis (right). (B) Transcript distribution of *dshc* throughout *Drosophila* development. A ³²P-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.

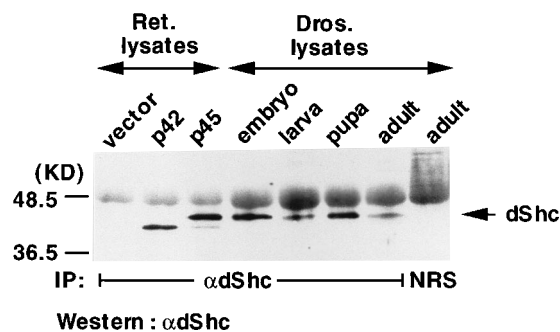


FIG. 3. Identification and expression of dShc proteins. Protein lysates isolated from *Drosophila* embryos, larvae, pupae, and adults (Dros. lysates) were immunoprecipitated with anti-dShcC antibodies (α dShc). As a control, the adult *Drosophila* lysate was also immunoprecipitated with nonimmune rabbit 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 torso⁴⁰²¹-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 torso⁴⁰²¹-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 torso⁴⁰²¹-DER tyrosine kinase is directly responsible for dShc association with pTyr-containing proteins, including autophosphorylated torso⁴⁰²¹-DER itself, and for dShc tyrosine phosphorylation.

The ability of dShc to associate with the sev receptor ty-

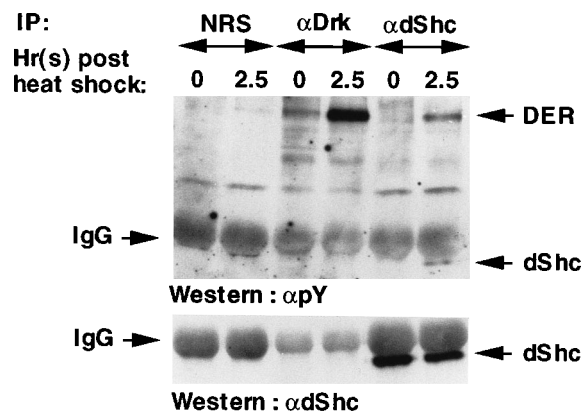


FIG. 4. dShc associates with the activated DER receptor tyrosine kinase. Transgenic flies carrying the *torso*⁴⁰²¹-DER minigene were lysed prior to heat shock or were heat shocked for 2.5 h, and lysates were immunoprecipitated (IP) with anti-dShcC (α dShc) or anti-Drk (α Drk) antibodies or with nonimmune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

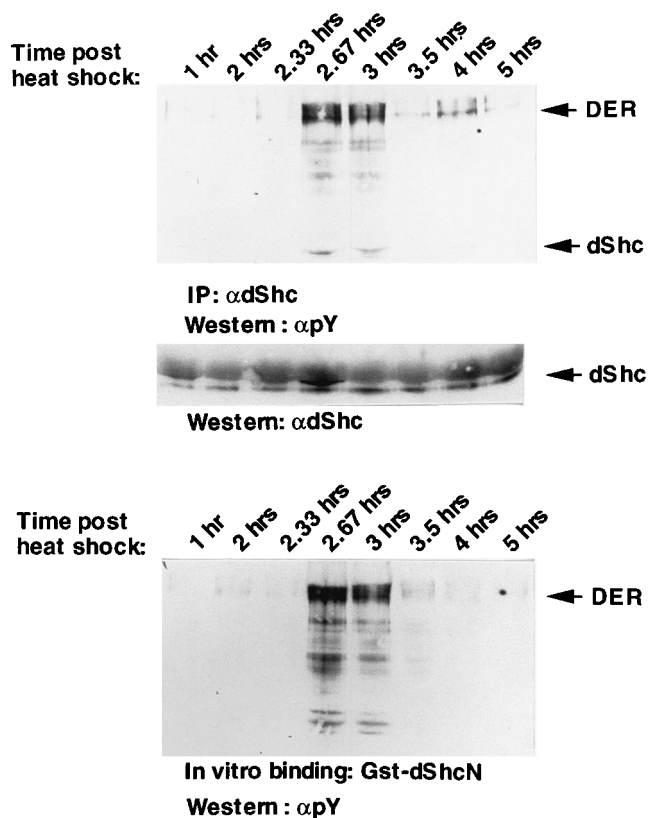


FIG. 5. In vivo and in vitro association of dShc with activated DER following heat shock. Flies carrying the *torso-DER* receptor transgene were heat shocked for the indicated time periods, and protein lysates were incubated with anti-dShcC (α dShc) antibodies (upper panel) or the GST-dShcN fusion protein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

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-

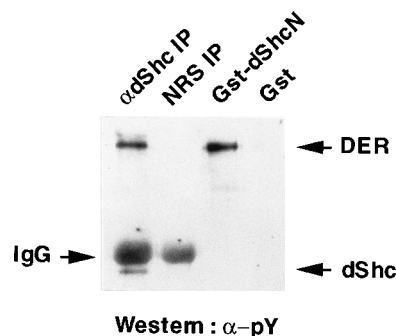


FIG. 6. The dShc PTB domain binds DER in vitro. The PTB domain of dShc was expressed as a GST fusion protein (GST-dShcN) containing dShc residues 1 to 203. Lysates containing activated *torso*-DER were incubated with immobilized GST or GST-dShcN, and associated *torso*-DER was identified by immunoblotting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

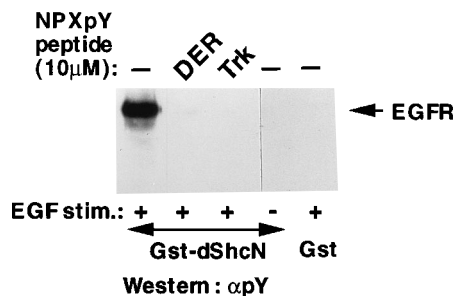


FIG. 7. The dShc PTB domain binds the activated human EGF receptor and phosphopeptides with NPXpY motifs. HER 14 cells, expressing the human EGF receptor, were stimulated (stim.) with EGF (+) or left unstimulated (-), as indicated. Cells were lysed and incubated with GST or the GST-dShcN fusion protein in the absence or presence of phosphopeptides (10 μ M) corresponding to the Tyr-1228 site (PVSVDNPEpYLLNAQK) of DER (DER peptide) or the Tyr-490 autophosphorylation site (HIIENPOpYFSD) of Trk (Trk peptide), as indicated. Autophosphorylated EGF receptor (EGFR) was identified by immunoblotting with anti-pTyr (α pY) 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 (HIENPQpYFSD) 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 (VDNPEY¹²²⁸LLNAQ) and that a very similar motif is found in members of the EGF receptor family in other species, including the human EGF receptor (VGNPEY¹¹¹⁴LNTVQ), human ErbB2 (VENPEY¹¹⁹⁶LTPO), and the avian EGF receptor (VDNPEY¹¹⁴⁹LNTNQ). 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.

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K.-M.V.L. and J.P.O. made equal contributions to this work.

REFERENCES

- Ashburner, M. 1989. *Drosophila: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Batzer, A. G., D. Rotin, J. M. Urena, E. Y. Skolnik, and J. Schlessinger. 1994. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol. Biol. Cell* **14**:5192-5201.
- Blaikie, P., D. Immanuel, J. Wu, N. Li, V. Yajnik, and B. Margolis. 1994. A region in Shc distinct from the SH2 domain can bind tyrosine phosphorylated growth factor receptors. *J. Biol. Chem.* **269**:32031-32034.
- Bonfini, L., C. A. Karlovich, C. Dasgupta, and V. Banerjee. 1992. The *Son of sevenless* gene product: a putative activator of Ras. *Science* **255**:603-606.
- Buday, L., and J. Downward. 1993. Epidermal growth factor regulates P21ras through the formation of a complex of receptor, Grb2 adaptor protein, and Sos nucleotide exchange factor. *Cell* **73**:611-620.
- Burns, L. A., L. M. Karnitz, S. L. Sutor, and R. T. Abraham. 1993. Interleukin-2-induced tyrosine phosphorylation of p52^{shc} in T lymphocytes. *J. Biol. Chem.* **268**:17659-17661.
- Campbell, K. S., E. Ogris, B. Burke, W. Su, K. R. Auger, B. J. Druker, B. S. Schaffhausen, T. M. Roberts, and D. C. Pallas. 1994. Polyoma middle tumor antigen interacts with SHC protein via the NPTY (Asn-Pro-Thr-Tyr) motif in middle tumor antigen. *Proc. Natl. Acad. Sci. USA* **91**:6344-6348.
- Crowe, A. J., J. McGlade, T. Pawson, and M. G. Hayman. 1994. Phosphorylation of the SHC proteins on tyrosine correlates with the transformation of fibroblasts and erythroblasts by the v-Sea tyrosine kinase. *Oncogene* **9**:537-544.
- Cutler, R. L., L. Liu, J. E. Damen, and G. Krystal. 1993. Multiple cytokines induce the tyrosine phosphorylation of Shc and its association with Grb2 in hematopoietic cells. *J. Biol. Chem.* **268**:21463-21465.
- Dickson, B., and E. Hafen. 1994. Genetics of signal transduction in invertebrates. *Curr. Opin. Genet. Dev.* **4**:64-70.
- Dickson, B., F. Sprenger, and E. Hafen. 1992. Prepattern in the developing *Drosophila* eye revealed by an activated torso-sevenless chimeric receptor. *Genes Dev.* **6**:2327-2339.
- Dilworth, S. M., C. E. Brewster, M. D. Jones, L. Lanfranccone, G. Pelicci, and P. G. Pelicci. 1994. Transformation by polyoma virus middle T-antigen involves the binding and tyrosine phosphorylation of Shc. *Nature (London)* **367**:87-90.
- Doyle, H. J., and J. M. Bishop. 1993. Torso, a receptor tyrosine kinase required for embryonic pattern formation, shares substrates with the sevenless and EGF-R pathways in *Drosophila*. *Genes Dev.* **7**:633-646.
- Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Sizeland, and R. Weinberg. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature (London)* **363**:45-51.
- Gale, W. N., D. Kaplan, E. J. Lowenstein, J. Schlessinger, and D. Bar-Sagi. 1993. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature (London)* **363**:88-92.
- Hafen, E., K. Basler, J. E. Edstroem, and G. M. Rubin. 1987. Sevenless, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236**:55-63.
- Hirai, H., Y. Maru, K. Hagiwara, J. Nishida, and F. Takaku. 1987. A novel putative tyrosine kinase receptor encoded by the eph gene. *Science* **238**:1717-1719.
- Kavanaugh, W. M., and L. T. Williams. 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* **266**:1862-1865.
- Lai, K.-M. V. Unpublished data.
- Lanfranccone, L., G. Pelicci, M. F. Brizzi, M. G. Arouica, C. Casciari, S. Giuli, L. Pegoraro, T. Pawson, and P. G. Pelicci. 1995. Overexpression of Shc proteins potentiates the proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of Grb2/Sos and Grb2/p140 complexes to the β receptor subunit. *Oncogene* **10**:905-917.
- Leicht, B., and J. Bonner. 1988. Genetic analysis of chromosome region 67A-D of *Drosophila*. *Genetics* **119**:579-593.
- Li, N., A. Batzer, R. Daly, E. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger. 1993. Guanine nucleotide releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. *Nature (London)* **363**:85-88.
- Livneh, E., L. Glazer, D. Segal, J. Schlessinger, and B.-Z. Shilo. 1985. The *Drosophila* EGF receptor gene homolog: conservation of both hormone binding and kinase domains. *Cell* **40**:599-607.
- McGlade, J., A. Cheng, G. Pelicci, P. G. Pelicci, and T. Pawson. 1992. Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **89**:8869-8873.
- Migliaccio, E., S. Mele, A. E. Salcini, G. Pelicci, K.-M. V. Lai, P. P. Di Fiore, T. Pawson, L. Lanfranccone, and P. G. Pelicci. Submitted for publication.
- Obermeier, A., R. Lammers, K. H. Wiesmuller, G. Jung, J. Schlessinger, and A. Ullrich. 1993. Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. *J. Biol. Chem.* **268**:22963-22966.
- O'Bryan, J., T. Saxton, and T. Pawson. Unpublished data.
- O'Connell, P. O., and M. Rosbash. 1984. Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**:5495-5513.
- Olivier, J. P., T. Raabe, M. Henkemeyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen, and T. Pawson. 1993. A *Drosophila* SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73**:179-191.
- Pelicci, G., L. Lanfranccone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, T. Pawson, and P. G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**:93-104.
- Prigent, S. A., and W. J. Gullick. 1994. Identification of C-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF-receptor/C-erbB-3 chimera. *EMBO J.* **13**:2831-2841.
- Pronk, G. J., J. McGlade, G. Pelicci, T. Pawson, and J. L. Bos. 1993. Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins. *J. Biol. Chem.* **268**:5748-5753.
- Puil, L., X. Liu, G. Gish, G. Mbamalu, D. Bowtell, P. G. Pelicci, R. Arlinghaus, and T. Pawson. 1994. BCR-ABL oncoproteins bind directly to activators of the ras signalling pathway. *EMBO J.* **13**:764-773.
- Raabe, T., J. P. Olivier, B. Dickson, X. Liu, G. D. Gish, T. Pawson, and E.

- Hafen.** Biochemical and genetic analysis of Drk SH2/SH3 adaptor protein of *Drosophila*. *EMBO J.* **14**:2509–2518.
34. **Ravichandran, K. S., and S. J. Burakoff.** 1994. The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. *J. Biol. Chem.* **269**:1599–1602.
 35. **Ravichandran, K. S., K. K. Lee, Z. Songyang, L. C. Cantley, P. Burn, and S. J. Burakoff.** 1993. Interaction of Shc with the ζ chain of the T cell receptor upon T cell activation. *Science* **262**:902–905.
 36. **Reichman-Fried, M., B. Dickson, E. Hafen, and B.-Z. Shilo.** 1994. Elucidation of the role of breathless, a *Drosophila* FGF receptor homolog, in tracheal cell migration. *Genes Dev.* **8**:428–439.
 37. **Rozakis-Adcock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell.** 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF-receptor to mSos1, an activator of Ras. *Nature (London)* **363**:83–85.
 38. **Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, P. G. Pelicci, J. Schlessinger, and T. Pawson.** 1992. Association of the Shc and Grb2/Sem-5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature (London)* **360**:689–692.
 39. **Ruff-Jamison, S., J. McGlade, T. Pawson, K. Chen, and S. Cohen.** 1993. Epidermal growth factor stimulates the tyrosine phosphorylation of SHC in the mouse. *J. Biol. Chem.* **268**:7610–7612.
 40. **Salcini, A. E., J. McGlade, G. Pelicci, I. Nicoletti, T. Pawson, and P. G. Pelicci.** 1994. Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* **9**:2827–2836.
 41. **Saxton, T. M., I. V. Oostveen, D. Bowtell, T. Aebersold, and M. R. Gold.** 1994. B cell antigen receptor cross-linking induces phosphorylation of the p21 ras oncoprotein activators SHC and mSOS1 as well as assembly of complexes containing SHC, GRB-2, mSOS1, and a 145-kDa tyrosine-phosphorylated protein. *J. Immunol.* **153**:623–636.
 42. **Segatto, O., G. Pelicci, S. Giuli, G. Digiesi, P. O. DiFiore, J. McGlade, T. Pawson, and P. G. Pelicci.** 1993. Shc products are substrates of Erb-2 kinase. *Oncogene* **8**:2105–2112.
 43. **Simon, L. A., G. S. Dodson, and G. M. Rubin.** 1993. An SH3-SH2-SH3 protein is required for p21^{Ras1} activation and binds to sevenless and Sos proteins in vitro. *Cell* **73**:169–177.
 44. **Simon, M. A., D. D. L. Bowtell, G. S. Dodson, T. R. Lavery, and G. M. Rubin.** 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* **67**:701–716.
 45. **Songyang, Z., S. E. Shoelson, M. Chadhuri, G. Gish, T. Pawson, F. King, T. Roberts, S. Ratnofsky, B. Schaffhausen, and L. C. Cantley.** 1993. Identification of phosphotyrosine peptide motifs which bind to SH2 domains. *Cell* **72**:767–778.
 46. **Songyang, Z., S. E. Shoelson, J. McGlade, P. Olivier, T. Pawson, R. X. Bustelo, H. Hanafusa, T. Yi, R. Ren, D. Baltimore, S. Ratnofsky, R. A. Feldman, and L. C. Cantley.** 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fes/fps, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* **14**:2777–2785.
 47. **Stephens, R. M., D. M. Loebe, T. D. Copeland, T. Pawson, L. A. Greene, and D. R. Kaplan.** 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma1 to mediate NGF responses. *Neuron* **12**:691–705.
 48. **Tantz, D., and C. Pfeiffe.** 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**:81–85.
 49. **van der Geer, P., S. Wiley, V. K.-M. Lai, J. P. Olivier, G. D. Gish, T. Stephens, D. Kaplan, S. Shoelson, and T. Pawson.** 1995. A conserved amino-terminal SHC domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Curr. Biol.* **5**:404–412.
 50. **Yokote, K., S. Mori, K. Hansen, J. McGlade, T. Pawson, C.-H. Heldin, and L. Claesson-Welsh.** 1994. Direct interaction between Shc and the platelet-derived growth factor β -receptor. *J. Biol. Chem.* **269**:15337–15343.
 51. **Yun, B., R. Farkas, K. Lee, and L. Rabinow.** 1994. The Doa locus encodes a member of a new protein kinase family and is essential for eye and embryonic development in *Drosophila melanogaster*. *Genes Dev.* **8**:1160–1173.