Characterization of the Phosphotyrosine-binding Domain of the Drosophila Shc Protein*

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The phosphotyrosine-binding (PTB) domain of Drosophila Shc (dShc) binds in vitro to phosphopeptides containing the sequence motif NPXpY, and physically associates with the activated Drosophila epidermal growth factor receptor homologue (DER) in vivo. The structural elements, specificity and binding kinetics of the dShc PTB domain have now been characterized. The dShc PTB domain appeared similar to the insulin-like receptor substrate-1 PTB domain in secondary structure as suggested by Fourier transform infrared spectroscopy. Surface plasmon resonance measurements indicated that the dShc PTB domain bound with high affinity to phosphopeptides (Der) derived from the Tyr1228 site of the DER receptor. The kinetics of the dShc PTB domain-Der phosphopeptide interaction differed from those of a typical SH2 domain-ligand interaction, in that the PTB domain displayed slower on/off rates. Competition binding assays using truncated versions of the Der peptides revealed that high affinity binding to the dShc PTB domain requires, in addition to the NPXpY motif, the presence of hydrophobic residues at both positions −5 and −7 relative to phosphotyrosine. The dShc PTB domain showed a similar binding specificity to the human Shc (hShc) PTB domain, but subtle differences were noted; such that the hShc PTB domain bound preferentially to a phosphopeptide from the mammalian nerve growth factor receptor, whereas the dShc PTB domain bound preferentially to phosphopeptides from the Drosophila DER receptor. The invertebrate dShc PTB domain therefore possesses a binding specificity for tyrosine-phosphorylated peptides that is optimally suited for recognition of the activated DER receptor.

Interactions between signaling proteins are often mediated by protein modules such as Src homology (SH)1 or 2, SH3, and pleckstrin homology domains (1, 2). These domains represent common structural elements found in a diverse array of enzymes involved in signaling transduction, including protein kinases, protein phosphatases, lipid kinases, lipid phosphatases, phospholipases, cytoskeletal proteins, and transcription factors (2–4). A distinct group of molecules, termed adaptor proteins, possess such modules but lack catalytic domains, and therefore have a more specialized function in promoting inter-molecular interactions that control the activation of signaling pathways (5). Shc is a member of the adaptor protein family that apparently participates in multiple signal transduction pathways (6). The mammalian shc gene encodes three widely expressed isoforms with molecular masses of 46, 52, and 66 kDa (7). These Shc proteins become phosphorylated on both tyrosine and serine residues following exposure of cells to a wide spectrum of extracellular stimuli, including growth factors, antigens, and cytokines (8–12). Shc is also phosphorylated on tyrosine in cells transformed by oncogenic tyrosine kinases such as v-Src, v-Fps, and Bcr-Abl (13, 14). Moreover, it has been recently demonstrated that activation of G-protein coupled receptors can result in Shc phosphorylation (15–18). Phosphorylated Shc proteins may couple to the Ras signaling pathway through the formation of a Shc-Grb2-Sos complex (19–23).

The diverse functions of mammalian Shc can be rationalized, in part, from its unique structure. The 52 kDa Shc isoform contains three distinct domains: a C-terminal SH2 domain that preferentially recognizes pY(E/L)V/IXI/I/L/M motifs (24); a recently identified N-terminal phosphotyrosine-binding (PTB) domain (also termed the PI domain) (25–27), and a central collagen homology (CH1) region (7). In contrast to SH2 domains, which select residues C-terminal to phosphotyrosine (24), the PTB domain of Shc recognizes tyrosine phosphorylated sites in the consensus sequence ψXpY/IX/IX/I/L/I/M (where ψ signifies hydrophobic residues). PTB domain binding specificity is therefore determined by residues N-terminal to the phosphotyrosine (28–30). In vivo, the Shc PTB domain interacts with a range of activated receptor tyrosine kinases (8–10), the p145 coinstituent phosphatase (31), and polyoma virus middle T antigen (32). The coexistence of SH2 and PTB domains in a single molecule potentially allows Shc to interact with a wide range of tyrosine-phosphorylated proteins. The Shc CH1 region con-

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1 The abbreviations used are: SH, Src homology; MES, 2-(N-morpholino)ethanesulfonic acid; PTB, phosphotyrosine-binding; CH1, collagen homology 1; IRS, insulin-like receptor substrate; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; cRU, resonance unit; GST, glutathione S-transferase; FTIR, Fourier transform infrared; IL, interleukin.
tains a principle phosphorylation site at Tyr \(^{317}\), within the sequence YVYNV, which when phosphorylated binds the SH2 domain of Grb2. Multiple potential SH3 binding sites are also found in the CH1 region, together with a motif that mediates interactions with \(\alpha\)- and \(\beta\)-adaptors, and might therefore regulate endocytosis (7, 33).

To examine if Shc function is evolutionarily conserved, we have previously cloned a \(Drosophila\) shc gene homologue, dshc (34). This gene encodes a 45-kDa protein that is closely related in organization to mammalian p52 Shc and contains a C-terminal SH2 domain, an N-terminal PTB domain, and a central CH1 region. Sequence alignment revealed 51% identity between the PTB domains of p52 Shc and dShc, and the dShc PTB domain was found to interact with phosphopeptidates containing an NPXpY motif, including a phosphopeptide modeled after the Tyr\(^{329}\) site in the DER receptor tyrosine kinase. Autophosphorylation of Tyr\(^{329}\) in DER may therefore create a physiological binding site for the dShc PTB domain, based on the observation that the dShc protein associates physically with activated DER in vivo (34).

Functional PTB domains have been identified in Shc and its relatives ShcB and ShcC (35), as well as in the IRS-1 and IRS-2 proteins that serve as prominent substrates for the insulin receptor and the cytokine-activated tyrosine kinases (36). Binding studies and structural analysis have shown that both Shc and IRS-1 PTB domains recognize a \(\beta\)-turn structure adopted by the sequence NPXpY (28–30, 37–42). However, they differ from each other in that the Shc PTB domain prefers a hydrophobic residue at the \(-5\) position N-terminal to Tyr(P), while the IRS-1 PTB domain favors a patch of hydrophobic residues located at positions \(-6\) to \(-8\) (37–39). Here, we have investigated the specificity, binding kinetics, and structural composition of the dShc PTB domain. Our results suggest that the dShc PTB domain has many features in common with its mammalian counterparts, but shows a unique specificity, which is likely to be of physiological relevance.

**MATERIALS AND METHODS**

**Expression and Purification of the PTB Domains of dShc and hShc**

The N-terminal region of dShc (residues 1–203) containing the PTB domain was cloned into the PET-4b expression vector (Novagen). PCR was used to amplify the corresponding cDNA and to generate restriction endonuclease sites. Specifically, BamHI (5′) and EcoRI (3′) sites were added to the ends of the DNA fragment to facilitate its cloning. The resulting PET-4b-dShc plasmid was transformed into \(E\). coli DH5α (Novagen). PCR, followed by restriction analysis, demonstrated the correct size of the cloned fragment. The cell pellets were resuspended in a lysis buffer containing 50 mM sodium phosphate, pH 7.5, 150 mM sodium chloride, 0.5 mM EDTA, 5 mM dithiothreitol, and 0.5 mM benzamidine, and sonicated for five bursts of 15 s at 0°C. The lysate was cleared by spinning at 10,000 rpm for 15 min at 4°C.

For purification of dShc PTB, the clear lysate was diluted with two equal volumes of 20 mM Tris buffer containing 5 mM dithiothreitol, 0.5 mM EDTA, and 0.5 mM benzamidine, pH 7.5. The diluted lysate was then loaded onto a TyroBlue affinity column equilibrated with the same buffer. The TyroBlueagarose beads were washed using the protocol provided by the manufacturer (Pharmacia Biotech Inc.). Proteins bound to the beads were eluted using a linear gradient of 0–0.6 M NaCl. Fractions containing dShc PTB were confirmed by SDS-PAGE and pooled. The protein purified using the TyroBlueagarose affinity column was essentially pure as indicated by SDS-PAGE (Fig. 1). For structural studies, the protein was further purified by passage through a Superdex-200 gel filtration FPLC column (Pharmacia). Pure dShc PTB fractions (Fig. 1) were collected and concentrated. It should be noted that all purification procedures were carried out at 4°C under reducing conditions to prevent denaturation and oxidation of the protein. GST-fused hShc PTB protein was purified according to published procedures (27, 28).

**Peptide Synthesis and Purification**

Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer using standard 9-fluorenylemethoxy carbonyl (Fmoc) solid phase chemistry. Phosphotyrosine was directly incorporated as its N\(^{2}\)-fluorenylemethoxy carbonyl-O-phosphate-1-tyrosine derivative. Amino acids were activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and the peptide chain was elongated on a \(p\)-hydroxyphosphonylmethyl polystyrene type resin (Applied Biosystems). Peptides were cleaved from the resin using a mixture of trifluoroacetic acid/H\(_2\)O/1,2-ethanedithiol/thioglycolic acid (90/5/2.5, v/v). Crude peptides were precipitated with tert-butyl methyl ether cooled on dry ice. After lyophilization, the crude peptide was separated directly on a Primosphere 10 microm (Pharmacia) C18 column (250 × 10 mm, Phenomenex). Pure peptides were obtained after eluting with a linear gradient of acetonitrile (0.1% trifluoroacetic acid was added to the solvent). The identities of the peptides were confirmed by mass spectroscopy and amino acid analysis. The concentrations of peptide stock solutions were determined by triple amino acid analysis.

**Surface Plasmon Resonance (SPR) Analysis of dShc PTB Binding to Phosphopeptides**

Surface plasmon resonance measurements were conducted on a Bia-core apparatus (Pharmacia Biosensor). Immobilization of the Der phosphopeptide on a Biosensor chip was accomplished following essentially the same protocol described earlier (28). Specifically, the peptide IGPVPVSVDNPepYLLNAQRK was injected across the surface of the chip at 1 μM in 50 mM HEPES, 1 mM NaCl, pH 7.5. The immobilized peptide typically gave resonance signals in the range of 4000–5000 resonance units when saturated with the dShc PTB protein.

For competition assays, either 0.5 μM dShc PTB protein or 1 μM hShc PTB-GST fusion protein was mixed with the peptides of interest in aqueous buffer (containing 20 mM MES, pH 6.5, 1 mM dithiothreitol, 0.5 mM benzamidine, and 0.5 mM EDTA) before injecting across the surface of the sensor chip immobilized with the Der peptide. Signals obtained using different concentrations of the competing peptide were monitored and used for calculating the IC\(_{50}\) values. Kinetic analysis of the binding data were carried out using the BIAevaluation software (Pharmacia, Version 2.1).

**In Vitro Peptide Competition Assay**

A \(Drosophila\) strain carrying the transgene encoding the torso-DER fusion protein (with fusion of the extracellular domain of torso\(^{304}\) to the intracellular kinase domain of DER) under the control of the hsp70 promoter in the pWB transformation vector was stimulated at 37°C for 45 min and then allowed to recover at room temperature. \(Drosophila\) lysates prepared after heat shock were probed by GST-dShc PTB fusion protein with or without the presence of the Der phosphopeptides. The protein complexes were washed three times with HNTG (20 mM HEPES, pH 7.5, 150 mM sodium chloride, 10% glycerol, 0.1% Triton X-100, and 1 mM orthovanadate) boiled for 5 min in SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were blocked in 5% bovine serum albumin, 1% ovalbumin in TBS-T and probed with affinity-purified anti-Tyr(P) antibodies (1 μg/ml). Immunooblots were developed using an ECL kit (Amersham).

**RESULTS**

**Kinetics of dShc PTB Binding to a Phosphopeptide Derived from the DER Receptor**

A polypeptide containing residues 1–203 of dShc, and therefore encompassing the PTB domain, was expressed in \(E\). coli. This protein, which did not contain any fused sequences from another source, was purified to homogeneity (Fig. 1) as described under “Materials and Methods.” To test the ability of the purified PTB domain to recognize the Tyr\(^{329}\) site of the DER receptor in vitro, an 18-mer phosphopeptide (Ile-Gly-Val-Pro-Val-Ser-Val-Asp-Asn-Pro-Glu-Tyr(P)-Leu-Leu-Asn-Ala-Gln-Lys, designated Der1) was synthesized corresponding to the amino acids flanking Tyr\(^{328}\). This peptide contains an 11-residue extension N-terminal, and 5 residues C-terminal to the phosphotyrosine. The C terminus...
was extended to include a Lys residue to facilitate immobilization of the peptide to a Biosensor chip.

Binding of the dShc PTB domain to peptide Der1 was studied by flowing various concentrations of the purified PTB protein over a Biosensor chip to which the phosphopeptide was coupled. The PTB protein was found to interact with immobilized Der1 in a concentration-dependent manner as shown in the corresponding SPR sensorgrams (Fig. 2A). The interaction was specific, since the binding signals were completely eliminated by addition of excess amount of free Der1 peptide. A Scatchard plot of the SPR response (cRU) at equilibrium versus response/PTB concentration (cRU/[PTB]) yielded a linear line (Fig. 2B), suggesting that binding of the dShc PTB domain to the Der1 peptide followed a bimolecular process. The corresponding dissociation constant, $K_d$, was determined at $-0.43 \mu M$ from the Scatchard analysis. The same data could also be used for kinetic analysis (see “Materials and Methods”). Assuming a simple A + B $\rightarrow$ AB model of association and dissociation, the kinetic analysis yielded an association rate constant, $k_{on}$ of $3.48 \pm 0.38 \times 10^4 M^{-1} s^{-1}$ and a dissociation rate constant, $k_{off}$ of $1.74 \pm 0.03 \times 10^{-2} s^{-1}$. The $K_d$ value could then be calculated as $K_d = k_{off}/k_{on} = 0.50 \pm 0.06 \mu M$. This value agrees essentially with that determined using the Scatchard analysis.

It is interesting to compare the kinetics of phosphopeptide binding of the PTB domain and the SH2 domain. High affinity phosphopeptides bind to SH2 domain very rapidly with $k_{on}$ rate constants in the range of $-1 \times 10^6 M^{-1} s^{-1}$ to $2 \times 10^6 M^{-1} s^{-1}$. Dissociation of the peptide ligand is also relatively fast with typical $k_{off}$ values of $0.1 s^{-1}$ (44). In comparison, the $k_{on}$ and $k_{off}$ values for the interaction of Der1 with the PTB domain were approximately 1 order of magnitude slower than those of a typical peptide-SH2 interaction.

**High Affinity Binding of dShc PTB to the Der Phosphopeptides Requires Hydrophobic Residues at Both −7 and −5 Positions**—Since hydrophobic residues N-terminal to the Tyr(P) have been implicated in the interactions of the Shc and IRS-1 PTB domains with their respective phosphopeptide ligands, we investigated the roles of the hydrophobic residues present at positions −5, −7, −9, and −11 of the Der1 peptide in binding to the dShc PTB domain. For this purpose, we synthesized a series of truncated peptides based on the sequence of peptide Der1 (Table I). We then tested these peptides for their ability to compete for dShc PTB binding to full-length Der1 using surface plasmon resonance techniques.

As shown in Fig. 3A, solubilized Der1 peptide inhibited binding of dShc PTB to immobilized Der1 at nanomolar concentrations. Complete inhibition was achieved at a peptide concentration of $\sim 150 \mu M$. The peptide Der2 lacks the C-terminal 4 residues of Der1. This peptide inhibited PTB-binding with a similar efficiency to that of the free, full-length Der1 peptide, indicating that these 4 C-terminal residues do not contribute significantly to PTB-binding. Deletion of the 4 N-terminal amino acids, 2 residues at a time, yielded peptides Der3 and Der4 containing 9 and 7 residues N-terminal to the phosphotyrosine, respectively. These two peptides also displayed full binding activity, suggesting that the hydrophobic residues Ile$^{−11}$ and Val$^{−9}$ are not required for efficient PTB binding. However, over 80% of the binding affinity was lost when a single additional residue, Val$^{−7}$, was deleted in peptide Der5, suggesting that the hydrophobic residue at position −7 relative to phosphotyrosine plays a significant role in dShc PTB-binding. Deletion of Ser at position −6 in peptide Der6 had no further effect. As shown in Fig. 3B, a residual signal was still detectable when 715 nM of Der6 was used to compete for binding of dShc PTB to the full-length Der1 peptide. However, deletion of Val$^{−5}$ rendered the resulting Der7 peptide completely inactive (Table I). To probe the role of Leu-1, we replaced this hydrophobic residue by the hydrophilic amino acid Ser. The binding affinity of the resulting peptide, Der8, was approximately 60% lower than that of Der1. In contrast, replacement of the negatively charged Glu residue within the NPEpY motif by the neutral amino acid Thr had a negligible effect on PTB binding. The pivotal role played by Tyr(P) is demonstrated by peptides Der10 and Der11, in which the presence of non-phosphorylated Tyr resulted in a total loss
immobilized Der1 phosphopeptide. The concentration of the dPTB PTB domain used in these assays was 0.5 μM. IC\textsubscript{50} values of these peptides in inhibiting dShc PTB binding to immobilized Der1 peptide were determined and compared to those of peptide Der4 to yield their relative affinities. Peptide Der4 was chosen as the reference peptide, since it contains the minimum sequence required for high affinity binding to dShc PTB.

As shown in Table II, the TrkA peptide that binds strongly to the mammalian Shc PTB (45) did not interact with dShc PTB as favorably as peptide Der4. The TrkA peptide contains hydrophobic Ile residues at both −5 and −6 positions but lacks a hydrophobic amino acid at the −7 position, consistent with the view that the −7 position is important for high affinity binding to dShc PTB. Substitution studies on the NP\textsubscript{XY} motif of the TrkA peptide reinforced the conclusion from previous studies (27, 28, 37–39) that Asn\textsuperscript{−3} is critical, and Pro\textsuperscript{−2} plays an auxiliary role in high affinity PTB binding. Interestingly, the mT phosphopeptide, which was shown to associate tightly with the human Shc PTB domain (28, 39), competed poorly for binding to the dShc PTB domain (Fig. 3C). The lack of a hydrophobic residue at the −7 position in conjunction with the presence of a hydrophilic Ser residue at the +1 position may account for the inability of the mT phosphopeptide to bind strongly to the dShc PTB domain.

In contrast to its rather low affinity for the human Shc PTB domain (28, 39), the insulin receptor peptide IR(pY960) was found to effectively inhibit dShc PTB binding to Der1 with an IC\textsubscript{50} of 0.62 μM. The IR(pY960) peptide contains a relatively hydrophobic residue at the −7 position relative to Tyr(P), which may explain its favorable interaction with the dShc PTB domain. As the peptide also contains a Ser residue at the −5 position, which might offset its binding affinity, it was expected that replacing this residue by a bulky, hydrophobic residue would generate a high affinity ligand for dShc PTB. This proved to be the case as the resulting peptide IR(Ala at −5) competed effectively against Der1 for dShc PTB binding at nanomolar concentrations (Table II). The relatively low affinity exhibited by the phosphopeptide derived from the IL4 receptor for binding to the dShc PTB domain can be accounted for by the fact that it lacks bulky, hydrophobic residues at both −5 and +1 positions. Finally, a phosphopeptide representing a high affinity binding sequence for the SH2 domain of Grb2 was found to be completely inactive in PTB binding, consistent with the notion that the binding specificities of the PTB domain and SH2 domain do not overlap outside their common recognition of phosphotyrosine.

**Binding of hShc PTB Domain to Der Phosphopeptides**—Direct Bia-core comparison of the binding specificity of the human Shc (hShc) PTB domain with that of the dShc PTB domain was conducted using the same set of Der and Trk phosphopeptides (Table III). The hShc PTB domain bound to immobilized peptide Der1 with a typical dissociation constant (K\textsubscript{d}) of −6.5

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**Table I**

| Peptides | Amino acid sequence | IC\textsubscript{50} \( \mu M \) | Relative affinity % |
|----------|---------------------|-----------------|-------------------|
| Der1     | I G V P V S V D N P E pY L L N A Q K | 0.092 | 100 |
| Der2     | I G V P V S V D N P E pY L L | 0.105 | 87.6 |
| Der3     | V P V S V D N P E pY L L | 0.088 | 104 |
| Der4     | V S V D N P E pY L L | 0.090 | 102 |
| Der5     | S V D N P E pY L L | 0.580 | 15.8 |
| Der6     | V D N P E pY L L | 0.450 | 20.4 |
| Der7     | D N P E pY L L | >50 | ND |
| Der8     | V P V S V D N P E pY S L | 0.220 | 41.8 |
| Der9     | V P V S V D N P T C Y L L | 0.085 | 108 |
| Der10    | V S V D N P E Y L L | >50 | ND |
| Der11    | I G V P V S V D N P E Y L L | >50 | ND |

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**Plot**

**Fig. 3.** Inhibition of dShc PTB-binding to immobilized Der1 phosphopeptide by soluble peptides Der1 (A), Der6 (B), and mT(pY250) (C). Concentrations of the competing peptides are shown on the right of the corresponding set of sensorgrams.

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**Specificity of the dShc PTB Domain**—These assays using the truncated series of Der peptides showed that high affinity binding of the Der peptides to the dShc PTB domain requires the presence of hydrophobic residues at both −5 and −7 positions relative to Tyr(P). In addition, a hydrophobic residue at the +1 position appeared to be favored. The binding specificity of the dShc PTB domain was further tested using phosphopeptides derived from the PTB binding sites in various mammalian phosphoproteins, including the insulin receptor (IR), the nerve growth factor receptor (TrkA), the IL4 receptor (mIL4R), and the polyomavirus middle T antigen (mT) (Table II). The IC\textsubscript{50} values of these peptides in inhibiting dShc PTB binding to immobilized Der1 peptide were determined and compared to that of peptide Der4 to yield their relative affinities. Peptide Der4 was chosen as the reference peptide, since it contains the minimum sequence required for high affinity binding to dShc PTB.

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**TABLE II**  
Specificity of the PTB domain of dShc

| Peptide source | Amino acid sequence | IC$_{50}$$^a$ | Relative affinity |
|----------------|---------------------|---------------|------------------|
| Der4$^b$       | VSVDNPEpYLLNAQK     | 0.090         | 100              |
| TrkA(pY490)    | HIENPGpYFSD         | 0.135         | 66.7             |
| TrkA (Pro$^{-2} \rightarrow$ Ala) | HIENAGpYFSD       | 0.460         | 29.0             |
| TrkA (Asn$^{-3} \rightarrow$ Ala) | HIIEAPGpYFSD      | 2.75          | 3.2              |
| mTg(pY250)     | LSLSNPtpYVSVMRSK    | 2.40          | 3.8              |
| Ir(pY960)      | YASSNPepYLLSA       | 0.620         | 14.5             |
| IR (Ser$^{-3} \rightarrow$ Ile) | YIAISNPepYLLSA  | 0.065         | 13.8             |
| mIL4R(pY475)   | VLDNPApYRSF         | 1.35          | 6.7              |
| Shc(pY317)     | DPSpYVNVQNLDK       | >100          | ND               |

$^a$ IC$_{50}$ values were determined using the same procedures as described in the legends to Table I.  
$^b$ Der4 was chosen as the reference peptide due to its similar size to most of the other peptides listed in the table. As well, it contained the minimal elements for high affinity dShc PTB binding. ND, not determined.

**TABLE III**  
Relative affinities of the DER phosphopeptides to hShc PTB

| Peptides | Amino acid sequence | IC$_{50}$$^a$ | Relative affinity$^b$ |
|----------|---------------------|---------------|-----------------------|
| Der1     | IGVPSVDNPEpYLLNAQK | 1.45          | 2.8                   |
| Der2     | IGVPSVDNPEpYLL     | 1.25          | 3.2                   |
| Der3     | VPVSVDNPEpYLL     | 0.42          | 9.5                   |
| Der4     | VPSVDNPEpYLL     | 0.04          | 100                   |
| Der5     | SVDNPEpYLL       | 1.55          | 2.6                   |
| Der6     | VDNPEpYLL       | 2.00          | 2.0                   |
| Der7     | DNPepYLL       | >50           | ND                    |
| TrkA(pY490) | HIEENPGpYFS       | 0.025         | 160                   |

$^a$ IC$_{50}$ values were measured similarly as in Table I except that 1 μM hShc PTB (GST fused) was used.  
$^b$ Relative affinity of the phosphopeptides to hShc PTB calculated according to their respective IC$_{50}$ values. The relative affinity of peptide Der4 was set at 100%. ND, not determined.

μM (data not shown), which is approximately 1 order of magnitude greater than that of the dShc PTB-Der1 interaction. Of interest, the same group of Der phosphopeptides were shown to inhibit the hShc PTB-Der1 interaction in a significantly different fashion from that observed for the dShc PTB domain. In particular, peptide Der4, which extends only to the −7 residue, inhibited the binding of hShc PTB domain to immobilized Der1 at nanomolar concentrations, whereas addition of residues to the N terminus of peptide Der4 to include Val$^{-9}$ and Ile$^{-11}$, as in peptides Der1, Der2, and Der3, greatly decreased the affinities of these peptides for the hShc PTB domain. Similarly, deletion of Val$^{-7}$ in peptide Der4 also greatly reduced the affinity of the resulting peptides Der5 and Der6. Peptide Der7, which lacks Val$^{-7}$, showed no detectable binding to the hShc PTB domain. It thus appears that optimal binding of the Der site to the human Shc PTB domain is achieved only when the Der peptide contains residues from the −7 to +2 positions. Interestingly, the TrkA(pY490) peptide was found to be more effective than peptide Der4 in inhibiting hShc PTB binding to the Der1 chip. This is in distinction to the dShc PTB domain, which binds preferentially to Der4 compared to TrkA(pY490) (Tables I and II). Thus, while the binding properties of the human and Drosophila Shc PTB domains are similar, a subtle difference exists between these two PTB domains in binding to the −6/−7 region of the peptide. It is likely that the dShc PTB domain favors a hydrophobic residue at the −6 position, whereas the human Shc PTB domain favors more strongly to a hydrophobic amino acid at the −7 position. Moreover, hydrophobic residues upstream of the −7 position may play an important but different role in modulating phosphopeptide binding to the hShc PTB domain versus the dShc PTB domains. As a consequence, the dShc PTB domain binds preferentially to a phosphopeptide derived from the Drosophila epidermal growth factor receptor (DER), while the human Shc PTB domain prefers a phosphopeptide from TrkA, the mammalian nerve growth factor receptor.

**Phosphopeptides Compete for Binding of Activated DER to the dShc PTB Domain**—The relative affinities of the Der phosphopeptides were also examined in a competition study using a GST-fused dShc PTB domain, and an activated DER receptor variant, in which the extracellular domain of a torso 4021 mutant receptor is fused to the DER cytoplasmic region (34). As shown in Fig. 4A, the GST-PTB protein bound to the activated DER receptor in a Drosophila lysate while GST alone did not, suggesting that the binding was mediated by the dShc PTB domain. Addition of free peptide Der1 to the lysate was found to inhibit the binding of the DER receptor to dShc PTB, with complete inhibition observed at a peptide concentration of ~10 μM (Fig. 4A). In contrast, the non-phosphorylated peptide Der 11 was not able to compete for binding at the same concentration. At a 5 μM peptide concentration, peptides Der1–Der4 competed effectively for dShc PTB binding to the activated DER receptor (Fig. 4B). The efficiency of competition decreased significantly for peptides Der5 and Der6, which lack the hydrophobic residue at the −7 position. Peptide Der7, which lacks hydrophobic residues at positions −5 and −7, was found to be essentially inactive in competition for dShc PTB-binding to the DER receptor. These results are in agreement with those obtained from the Bia-core studies.

**Secondary Structure of the PTB Domain of dShc**—The structure of the PTB domain was investigated by FTIR. Deconvolution of the corresponding IR spectrum in the amide I region produced five discrete bands centered at 1620, 1633, 1652, 1673, and 1682 cm$^{-1}$, respectively (Fig. 5). The major band at 1652 cm$^{-1}$ is assigned to α-helix, while absorbances at 1620 cm$^{-1}$, 1633 cm$^{-1}$, and 1682 cm$^{-1}$ are attributed to β-sheets. The band at 1673 cm$^{-1}$ may have originated from turn structure elements in the protein (46). Quantitative analysis of the IR bands provided an estimation of the secondary structure of the dShc PTB domain. The secondary structure contents of the dShc PTB domain based on its IR spectra were then compared...
both their structures and binding affinities (40–42, 45). Our phosphotyrosine, the PTB domain and SH2 domain differ in motifs (27, 28, 36–42). Apart from their common recognition of the PTB domain from that of the human Shc PTB domain and either the human Shc PTB or the IRS-1 PTB domain, as the dShc PTB construct used in the present study (encompassing residues 1–203 of dShc) is significantly longer than the PTB proteins used in the NMR and the x-ray crystallographic studies (Table IV), it is nonetheless interesting to note that the dShc PTB domain contains a large proportion of β-sheet, similar to that found in the IRS-1 PTB domain (Table IV). In contrast, the secondary structure elements of the PTB domains of human Shc and dShc differ significantly from each other. As sequence alignment has indicated that residues important for the tertiary structure of the protein as well as residues critical for phosphopeptide binding are well conserved between these two domains (41), it is likely that these differences arise from local structural re-adjustments. The dShc PTB domain contains a deletion of several residues that correspond to the loop connecting the second α-helix (α2) and the second β-strand (β2) in human Shc PTB (41). The same loop region and the entire α2-helix are also absent in the IRS-1 PTB domain (42). It remains to be seen whether the observed difference in the binding specificity of the dShc PTB domain from that of the human Shc PTB domain stems from subtle structural alterations in these loop regions.

DISCUSSION

It is now established that the PTB domain is a protein module that can mediate the formation of protein complex through its recognition of specific phosphotyrosine-containing motifs (27, 28, 36–42). Apart from their common recognition of phosphotyrosine, the PTB domain and SH2 domain differ in both their structures and binding affinities (40–42, 45). Our

![Image](https://example.com/image.png)

**Fig. 4. In vitro competition of dShc PTB binding to the DER receptor by tyrosine-phosphorylated peptides.** Lysates containing activated torso-DER were incubated with immobilized GST-PTB or GST, in the presence of various concentrations of peptide Der1 and 10 μM Der11 (A) or in the presence of 5 μM of different Der phosphopeptides (B). The associated torso-DER was identified by immunoblotting with anti-Tyr(P) (α-pY) antibodies.

with those of the hShc PTB and of the IRS-1 PTB derived from their NMR and x-ray structures, respectively (Table III) (41, 42). Although it is difficult to make a direct comparison between the dShc PTB domain and either the human Shc PTB or the IRS-1 PTB domain, as the dShc PTB construct used in the present study (encompassing residues 1–203 of dShc) is significantly longer than the PTB proteins used in the NMR and the x-ray crystallographic studies (Table IV), it is nonetheless interesting to note that the dShc PTB domain contains a large proportion of β-sheet, similar to that found in the IRS-1 PTB domain (Table IV). In contrast, the secondary structure elements of the PTB domains of human Shc and dShc differ significantly from each other. As sequence alignment has indicated that residues important for the tertiary structure of the protein as well as residues critical for phosphopeptide binding are well conserved between these two domains (41), it is likely that these differences arise from local structural re-adjustments. The dShc PTB domain contains a deletion of several residues that correspond to the loop connecting the second α-helix (α2) and the second β-strand (β2) in human Shc PTB (41). The same loop region and the entire α2-helix are also absent in the IRS-1 PTB domain (42). It remains to be seen whether the observed difference in the binding specificity of the dShc PTB domain from that of the human Shc PTB domain stems from subtle structural alterations in these loop regions.

**Fig. 5. FTIR spectrum of dShc PTB in the amide I region.** Dashed lines represent deconvoluted peaks. The wavelengths of the peak centers are labeled on the diagram. See “Materials and Methods” for experimental details.

**Table IV**

| PTB domain   | Secondary structure content | %   |
|--------------|----------------------------|-----|
|              | α                  | β   | Others² |
| dShc (1–203) | 31.5               | 45.4| 24.1    |
| hShc (40–207)| 24.4               | 30.4| 45.2    |
| IRS-1 (161–264) | 25.9               | 42.3| 31.8    |

² Secondary structure content of the dShc PTB domain was estimated from its IR spectra using Spectra Calc (43). The NMR structure of the hShc PTB domain (41) and the crystal structure of the IRS-1 PTB domain (42) were used to derive the secondary structure compositions of these two domains. α, α-helix; β, β-sheet; others, secondary structural elements other than the α-helix and β-sheets, including random coil, loops, and turn structures.

Bia-core analysis of the interaction of the dShc PTB domain with the 18-mer phosphorylated Der1, derived from the Tyr1228 site in the DER receptor tyrosine kinase, suggests that the kinetics of PTB domain binding may also differ from those of the SH2 domain. The PTB domain of dShc has a significantly slower association rate constant (kₐ = 3.48 ± 0.38 × 10⁴ M⁻¹ s⁻¹) than the SH2 domain of the 85-kDa subunit of the phosphotyrosine protein 3′-kinase (kₐ = 1.6–3.3 × 10⁶ M⁻¹ s⁻¹) (44). Moreover, the observed dissociation rate constant for the dShc PTB-Der1 peptide interaction (kₒ = 1.74 ± 0.03 × 10⁻² s⁻¹) is approximately 1 order of magnitude slower than a typical SH2-ligand interaction (~0.1 s⁻¹) (3, 44). In a similar study, Laminet et al. (47) measured the kinetics of human Shc PTB binding to a tyrosine phosphorylated peptide derived from the c-ErbB2 receptor tyrosine kinase. This study yielded a similar association rate constant (4.7 ± 0.56 × 10⁹ M⁻¹ s⁻¹) and an even slower dissociation rate constant (2.5 ± 0.21 × 10⁻³ s⁻¹). It is therefore likely that an SH2 domain binds to its ligand much more rapidly than does a PTB domain. However, once the complex between the PTB domain and its ligand is formed, its dissociation may be much slower than for a typical SH2-ligand complex. The differences in binding kinetics between the SH2 and PTB domains may provide another level of regulation in signal transduction, aside from the different phosphopeptide motifs recognized by these two domains. While the rapid association and dissociation rates allow an SH2 domain to sample a series of candidate phosphorysine-containing sites for opti-
Binding Specificity of the PTB Domain of dShc

Tyr(P)-6 and Tyr(P)-7 positions of the peptide are found to be more solvent-exposed.

Such structural distinctions between the hShc and IRS-1 PTB domains may be used to explain our finding that peptides Der1, Der2, and Der3, which contain hydrophobic residues at the −11 and −9 positions, displayed significantly reduced affinities for the hShc PTB domain than their shorter counterpart, peptide Der4. It is possible that the −9/−11 hydrophobic residues in the Der peptides cannot be accommodated efficiently by the relatively limited hydrophobic surface in the binding site of hShc PTB domain, and consequently interfere with peptide binding. It is interesting to note that the same residues are tolerated by the dShc PTB domain as peptides Der1, Der2, and Der3 displayed similar affinities to the dShc PTB domain as peptide Der4. The subtle difference between these two closely related PTB domains is also reflected in the observation that peptide Der4, which contains a hydrophobic residue at the −7 position, is superior in binding to the dShc PTB domain as compared with peptide TrkA(pY490), which lacks a −7 hydrophobic amino acid but contains an Ile at the −6 position. In contrast, the latter peptide displayed greater affinity to the hShc PTB domain than the former. These results suggest that the Drosophila and human Shc PTB domains have developed to bind optimally to receptor sites of physiological relevance.

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