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Solution structure of the Shc SH2 domain complexed with a tyrosine-phosphorylated peptide from the T-cell receptor

(NMR/signal transduction)

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Communicated by Alfred G. Redfield, Brandeis University, Waltham, MA, May 25, 1995

ABSTRACT

Shc is a widely expressed adapter protein that plays an important role in signaling via a variety of cell surface receptors and has been implicated in coupling the stimulation of growth factor, cytokine, and antigen receptors to the Ras signaling pathway. Shc interacts with several tyrosine-phosphorylated receptors through its C-terminal SH2 domain, and one of the mechanisms of T-cell receptor-mediated Ras activation involves the interaction of the Shc SH2 domain with the tyrosine-phosphorylated chain of the T-cell receptor. Here we describe a high-resolution NMR structure of the Shc SH2 domain complexed to a phosphopeptide (GHDGLpYQGLSTATK) corresponding to a portion of the ζ chain of the T-cell receptor. Although the overall architecture of the protein is similar to other SH2 domains, distinct structural differences were observed in the smaller β-sheet, BG loop, (pY + 3) phosphopeptide-binding site, and relative position of the bound phosphopeptide.
Although the SH2 domain of Shc is homologous to other SH2 domains, there are some distinct differences: (i) the βD6 residue, which is part of the phosphotyrosine-binding pocket, is a leucine in Shc rather than a lysine or arginine as in other SH2 domains; (ii) the βD5 residue, which has been implicated in regulating the binding specificity of SH2 domains (19), is a leucine in Shc, a residue not found at this position in any of the known SH2 domains; (iii) the lengths of the loops between regular elements of secondary structure, which could reflect differences in the tertiary structure and binding specificities, are different in Shc compared to other SH2 domains. To determine whether these differences in primary structure result in alterations in the tertiary structure of this SH2 domain and to gain a better understanding of the Shc's binding specificity, we sought to determine the solution structures of Shc SH2/peptide complexes. Here we describe a high-resolution NMR structure of the Shc SH2 domain complexed with a phosphotyrosine-containing peptide corresponding to a portion of the ζ chain of the T-cell receptor.

MATERIALS AND METHODS

Preparation of NMR Samples. The Shc SH2 domain, corresponding to residues 370-473 of the full Shc protein (2), was subcloned into the pET20b plasmid (Novagen) and expressed in Escherichia coli BL21(DE3) cells. The protein contained an additional Leu-Glu-(His)$_6$ sequence at the C terminus to aid in protein purification. The SH2 domain of Shc was purified by affinity chromatography on a nickel-IDA column (Invitrogen), followed by HPLC ion-exchange chromatography in 20 mM sodium phosphate buffer (pH 7.8). Uniformly $^{15}$N- and $^{15}$N-,$^{13}$C-labeled proteins were prepared from bacteria grown at 37°C in M9 minimal medium containing $^4$N-labeled ammonium chloride (Isotec, Miamisburg, OH) in the absence or presence of uniformly $^{13}$C-labeled glucose (Isotec). The phosphotyrosine-containing peptides (GHDGLpYQGLSTATK and ATKDTpYDALHMQA) corresponding to portions of the third tyrosine-based activation motif of the ζ chain of the T-cell receptor were synthesized by solid-phase methods and purified by reverse-phase HPLC. NMR samples contained uniformly $^{15}$N- or $^{15}$N-,$^{13}$C-labeled protein (2 mM) and unlabeled phosphopeptide (4 mM) in a $^2$H$_2$O or a $^2$H$_2$O/$^2$H$_2$O (9:1) buffer (pH 6.5) containing 50 mM Tris-d$_{11}$, 50 mM NaC$_1$, and 5 mM dithiothreitol-d$_{10}$.

NMR Spectroscopy. NMR experiments were performed at 20°C on a Bruker (Billerica, MA) AMX500 or AMX600 NMR spectrometer equipped with triple resonance probes and z gradients. Several heteronuclear multidimensional NMR experiments were used to assign the NMR signals. These include the following: $^{15}$N-TOCSY-HSQC (20), HNHA (21), HNHB (22), HNCA (23), HN(CO)CA (24), HNCO (25), HN(CA)CO (25), HACACO (26), (H,C(CO)NH)-TOCSY (27), HCCH-TOCSY (28), and CCH-COSY (29) experiments (where COSY is correlated spectroscopy, TOCSY is total correlated spectroscopy, and NOESY is nuclear Overhauser effect spectroscopy). The Val and Leu methyl groups were stereospecifically assigned using the approach described by Wüthrich and coworkers (30) from an analysis of the $^{13}$C-$^{13}$C one-bond couplings observed in a high-resolution $^1$H-$^{13}$C HSQC spectrum of a fractionally $^{13}$C-labeled (15%) sample of the Shc SH2 domain complexed to an unlabeled peptide. An HMQC-J experiment was used to determine the three-bond $^1$H-$^4$H$_a$ coupling constants (31). Slowly exchanging amide protons were identified in a two-dimensional (2D) $^1$H-$^{15}$N HSQC experiment recorded ~2 hr after dissolving the lyophilized protein—peptide complex in a $^2$H$_2$O buffer. Double-filtered 2D $^{15}$N/$^{13}$C-filtered COSY, TOCSY, and NOESY (32) experiments were used to assign the proton resonances and derive intramolecular distance restraints involving the phosphopeptide. Intermolecular NOEs were identified from a 3D $^{13}$C-filtered (F1), $^{13}$C-edited (F3) NOESY-HSQC experiment recorded using a mixing time of 150 ms. However, for structure calculations intramolecular protein—protein and intermolecular protein—peptide distance restraints were derived from $^4$N-edited 3D NOE$^{13}$C/HSQC and $^{13}$C-edited 3D HMQC/NOE$^{13}$C experiments acquired with mixing times of 60 ms and 80 ms, respectively.

Peptide Binding Affinity. The binding affinities of the two peptides corresponding to the third tyrosine-based activation motif of the ζ chain of the T-cell receptor were calculated from the changes in chemical shifts of uniformly $^{15}$N-labeled Shc as a function of added concentration of the phosphopeptide.
The chemical shift differences were measured from a series of 2D $^{15}$N/$^1$H HSQC spectra.

**Structure Calculations.** Structure calculations were performed using the X-PLOR (33) program following the distance geometry/simulated annealing protocol described in the X-PLOR manual (34). A total of 1995 intramolecular and 77 intermolecular NOE-derived distance restraints were used in the structure calculations. On the basis of the integrated NOE cross peak volumes, these NOE restraints were grouped into three ranges (1.8-3.0 Å, 1.8-4.0 Å, and 1.8-5.0 Å), corresponding to strong, medium, and weak NOEs, respectively. Upper limits for distances involving methyl protons, nonstereospecifically assigned methylene protons, and aromatic ring protons were corrected for center averaging (35). In addition to the NOE-derived restraints, 36 $\phi$ angle restraints derived from $^3$J$_{HH\alpha}$ coupling constants measured from HMQC-J spectra were included using bounds of -120 ± 40° for those angles with $^3$J$_{HH\alpha}$ coupling constants > 9.0 Hz and -60 ± 40° for $^3$J$_{HH\alpha}$ coupling constants <5.5 Hz. Restraints for the latter were applied only in $\alpha$-helical regions of the protein that were defined from $^{13}$C$^\alpha$ chemical shifts and characteristic NOEs. A total of 35 hydrogen bond restraints were also included in the structure calculations with bounds of 1.8-2.5 Å (H→O) and 2.5-3.3 Å (N→O). The amides involved in hydrogen bonds were identified based on the slowly exchanging amide protons observed in 2D $^1$H-$^{15}$N HSQC spectra of the complex in $^2$H$_2$O and the carbonyl donors by a visual inspection of preliminary structures of the complex derived solely from the NOE data.

**RESULTS AND DISCUSSION**

**Peptide Binding Affinity.** To determine which phosphopeptide to use in our NMR studies, the binding affinities of the Shc SH2 domain were measured for two phosphopeptides corresponding to portions of the third tyrosine-based activation motif of the $\zeta$ chain of the T-cell receptor. Since the N-terminal peptide of the third tyrosine-based activation motif (GHDGLpYQGLSTATK) was found to bind more tightly (50 $\mu$M) to the Shc SH2 domain compared to ATKDTpYDALHMQA (650 $\mu$M), the N-terminal peptide was chosen for the NMR studies.

**NMR Assignments and Structure Determination.** The $^1$H, $^{13}$C, and $^{15}$N chemical shifts of the Shc SH2 domain when bound to the phosphopeptide were assigned from an array of heteronuclear multidimensional NMR experiments (36, 37). Sequential backbone assignments were established by correlating the $^{15}$N and $^1$H signals of the amides to the $^{13}$C$^\alpha$ signals of $i$ and $i-1$ residues from HNCA and HN(CO)CA experiments. These assignments were confirmed by linking the amide signals using the carbonyl chemical shifts as the common frequency from an analysis of 3D HNCO, HN(CA)CO, and HACACO spectra. The $^1$H and $^{13}$C signals of the side chains were assigned by correlating these signals to the assigned $^1$H$^\alpha$/$^{13}$C$^\alpha$ signals of the backbone in a 3D HCCH-TOCSY experiment and with the adjacent amide signals in a (H)C(CO)NH-TOCSY experiment. The $^1$H and $^{13}$C resonances of the Leu and Val methyl groups were stereospecifically assigned using a fractionally $^{13}$C-labeled (15%) sample of Shc by the method of Wittlich and coworkers (30). The phosphopeptide resonances were assigned using 2D isotope-filtered TOCSY and NOESY experiments (32) in which the signals of the $^{15}$N- and $^{13}$C-attached protons of the labeled protein were suppressed.

On the basis of the resonance assignments, NOEs were interpreted to generate the interproton distance restraints. Intramolecular NOEs involving the bound peptide were obtained from a 2D $^{15}$N/$^{13}$C-filtered NOESY spectrum, whereas NOEs between the protons of the protein were obtained from 3D $^{15}$N- and $^{13}$C-edited NOESY spectra. Intermolecular NOEs between the protein and peptide were obtained from a $^{13}$C-filtered (F1), $^{13}$C-edited (F3) 3D NOESY spectrum. The 3D structure of the Shc SH2 domain/phosphate complex was determined using a distance geometry/simulated annealing protocol (33, 34) from a total of 2143 NMR-derived restraints. All of the structures satisfy the experimental distance restraints with no violations >0.1 Å and show good covalent geometry and nonbonded contacts (Table 1). The backbone is well-defined by the NMR data (Fig. 1) except for the first residue at the N terminus and the last eight residues (LEHHHHHH) at the C terminus, which contains the histidine tag (Table 1). The portion of the phosphopeptide that contacts the protein (residues 6-9) is constrained by the NMR data. However, the
remaining peptide residues (1-5, 10-14) are not well-defined by the limited number of NMR-derived restraints involving these residues. The atomic rmsd about the mean coordinate positions for the protein residues 2-104 and peptide residues 6-9 of the complex is 0.39 ± 0.05 Å for the backbone atoms and 0.82 ± 0.04 Å for all heavy atoms.

Structure of the Shc SH2 Domain/Peptide Complex. Fig. 2A shows a schematic ribbon diagram of the NMR-derived structure of the Shc SH2/peptide complex. The structure consists of a three-stranded antiparallel β-sheet composed of strands βB, βC, and βD that is flanked on each side by two α-helices. The central β-sheet is extended by two short parallel strands (βA and βG) located on the opposite side of the peptide-binding site. The protein also contains a second, smaller β-sheet-like structure composed of strands βD', βE, and βF. As shown in Fig. 2, the structure of the central β-sheet and flanking helices of the Shc SH2 domain is similar to that of other SH2 domains whose structures have been previously determined (15-18). However, the position of strands βD' and βE is different in the Shc SH2 domain due to a kink between βD and βD' (Fig. 2A). Furthermore, unlike in other SH2 domains, the βD'1 residue of Shc does not form hydrogen bonds to the βE strand. Instead, the NMR data suggest that the βD'2 residue hydrogen bonds to the βE strand. Another distinguishing feature of the Shc SH2 domain is the relatively long length of the BG loop that contacts the peptide. Although the Syt SH2 domain has a BG loop of similar size, it is more hydrophilic and is located in a different position compared to Shc (Fig. 2).

As in other SH2 domains, the phosphopeptide adopts an extended conformation and binds to the Shc SH2 domain almost perpendicular to the βD strand of the central sheet (Fig. 2A). However, the location of the phosphopeptide relative to the protein is different in Shc compared to other SH2 domains (Fig. 3). In Shc, the phosphopeptide binds between the DE and BG loops (Fig. 2A), whereas, in Lck, PLC-γ1, and Syt, the phosphopeptide is located between the EF and BG loops (Fig. 2 B-D). The relatively long BG loop in Shc contacts the peptide and together with the DE loop forms a large cleft (Fig. 2A).

The phosphotyrosine-binding pocket in the Shc SH2 domain is formed by Arg αA2, Arg βB5, Val βC3, His βD4, and Leu βD6 (Fig. 4). The plane of the imidazole ring of the His βD4 is almost perpendicular to the plane of the aromatic ring of P-Tyr, forming aromatic-aromatic interactions, whereas the methyl groups of Val βC3 and Leu βD5 form hydrophobic interactions with the aromatic ring of the P-Tyr residue. Arg βB6 is located at the bottom of the P-Tyr-binding pocket. This residue is strictly conserved in the known SH2 domains and is part of the FLVR signature sequence of all SH2 domains (14). Since the NMR signals corresponding to the exchangeable Nη protons of Arg βB5 are observable in aqueous solution, these protons must be sequestered from solvent and are probably directly interacting with the negatively charged phosphate. Arg αA2 is also in close proximity to the P-Tyr residue of the peptide. However, unlike Arg βB5, the Nη protons were not observed and the linewidth of the Nε proton signal of Arg αA2 was quite broad, suggesting that this arginine residue may be more flexible than the Arg βB5 in the P-Tyr-binding pocket. In general, it appears that Arg αA2 residue is less critical than Arg βB5 for P-Tyr binding in SH2 domains. Mutation of Arg αA2 to Ala in the GTPase-activating protein SH2 domain only reduced the affinity but did not abolish P-Tyr binding (40). In addition, the Syt SH2 domain has a Gly residue at this position.

Compared to Syt, PLC-γ1, or Src-like SH2 domains in which a highly conserved positively charged residue is located at the βD6 position (a lysine in Src-like and Syt SH2 domains and an arginine in PLC-γ1 SH2 domain), the Shc SH2 domain contains a leucine at this position (Fig. 5). In the crystal structure of the Src SH2 domain/phosphopeptide complex, the Lys βD6 residue contributes to P-Tyr binding through hydrophobic and electrostatic interactions with P-Tyr. In addition, this Lys residue is hydrogen bonded to Thr BC3 in the phosphate-binding BC loop, resulting in the formation of an extensive hydrogen bonding network involving Glu BC1, Thr BC2, and the phosphate of the P-Tyr. The corresponding βD6 residue in Lck, Syt, and PLC-γ1 SH2 domains appears to have a similar function to that of the Src SH2 domain. In contrast, the Shc SH2 domain with a leucine βD6 residue lacks this network of hydrogen bonds involving the BC loop. Despite the loss of hydrogen bonds, however, the phosphate binding sites in Shc and the other SH2 domains are similar with the exception of a slight positional difference of the BC loop (Fig. 4). This phosphate-binding interaction loss in Shc may be at least partially compensated by additional hydrophobic interactions between the P-Tyr residue and Leu βD6 and Val βC3 residues of the Shc SH2 domain.
The \((pY + 1)\) Gln residue of the phosphopeptide is in close proximity to the side chains of Leu \(\beta D5\) and Ala BG7. In other SH2 domains, the \(\beta D5\) residue has been proposed to have a major influence on the binding specificity of phosphorylated peptides for SH2 domains. The hydrophobic Leu residue at the \(\beta D5\) position in Shc that interacts with the aliphatic side chain of Gln is consistent with the preference for hydrophobic residues (Ile, Tyr, and Leu) at \((pY + 1)\). Analogous to the interactions with Gln of the phosphorylated peptide, the Leu \(\beta D5\) residue could interact with the aliphatic portion of a glutamic acid that is also preferred at this site. In this regard, the C-terminal SH2 domain of Shc more closely resembles the SH2 domains of PLC-\(\gamma 1\) and Syp that have either a Cys or Ile at the \(\beta D5\) position rather than the Src-like SH2 domains that have a \(\beta D5\) Tyr or Phe.

The side chain of the \((pY + 2)\) Gly residue sits on the top of the binding site. Only one NOE was observed between the \(H^a\) protons of the \((pY + 2)\) Gly residue and the protein. The side chain of an L-amino acid, if present, would poke out toward solvent, which is consistent with the lack of selectivity at this position for binding to the Shc SH2 domain.

The Leu side chain at the \((pY + 3)\) position interacts with the protein in a hydrophobic pocket located on the other side of the central \(\beta\)-sheet (Fig. 2A and 4). The binding pocket is formed by hydrophobic residues from strands \(\beta D\) and \(\beta E\), the BG loop, and helix \(\alpha B\) at the bottom of the groove. Extensive contacts were observed between Leu at \((pY + 3)\) and the backbone of Arg \(\beta E3\) and the side chains of residues Leu \(\beta D5\), Leu \(\beta D7\), Val \(\beta E2\), Thr \(\beta E4\), Phe \(\beta F3\), Leu \(\alpha B5\), Ile BG4, Ser BG6, and Leu BG11 in the binding pocket of Shc (Fig. 4). The amino acid residues that make up this binding pocket in Shc are different from those of Src, Lck, Syp, and PLC-\(\gamma 1\). The \((pY + 3)\) Leu in Shc is much closer to the fo strand and N-terminal end of the pe strand, making extensive contacts with \(\beta D7\), \(\beta E2\), and \(\beta E3\) residues. In contrast, the corresponding residues in other SH2 domains were not part of the \((pY + 3)\) binding pocket. On the other hand, the EF1 and EF3 residues that interact extensively with the \((pY + 3)\) residue of the phosphopeptide in the other SH2 domains are far away from the peptide in Shc.

The other residues of the bound phosphopeptide are ill-defined by the NMR data. No intermolecular NOEs were observed involving these peptide residues except for three NOEs between the Ala residue at the \((pY + 4)\) position and Ile BG5 and Ser BG6. Thus, in contrast to the Syp and PLC-\(\gamma 1\) SH2 domain/peptide complexes, peptide residues beyond the \((pY + 4)\) position do not contact the protein.

In summary, we have determined a high-resolution NMR structure of the C-terminal SH2 domain of Shc complexed to a tyrosine-phosphorylated peptide from the \(\zeta\) chain of the T-cell receptor. Although the overall architecture of the protein is similar to other SH2 domains, distinct structural differences were observed in the smaller \(\beta\)-sheet, BG loop, relative position of the bound phosphopeptide, and \((pY + 3)\) binding site. In addition, the Shc SH2 domain contains a Leu residue at the \(\beta D6\) position that cannot form an extensive hydrogen-bonding network involving the BC loop as found in some of the other SH2 domains. This may help explain the relatively low binding affinity for phosphotyrosine-containing peptides displayed by the SH2 domain of Shc and other proteins containing a hydrophobic residue at this position (proteins such as GAP, p85 PI-3 kinase, and Zap70). To compensate for the low binding affinity, some proteins contain more than one SH2 domain, which can markedly increase the binding affinity and specificity of these proteins. Although Shc only contains one SH2 domain, the N terminus of Shc has recently been shown to bind to proteins containing phosphorylated tyrosines. Thus, the N-terminal phosphotyrosinebinding domain and C-terminal SH2 domain of Shc may bind to multiply phosphorylated proteins, increasing the affinity and selectivity of Shc for binding to its target proteins.

We thank Dr. J. D. Forman-Kay for sending us the structure coordinates of PLC-\(\gamma 1\) SH2 domain. K.S.R. is supported by a postdoctoral fellowship from the Medical Foundation of the Charles King Trust.
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Table 1    Structural statistics and rms deviation for 30 She SH2 domain structures
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Fig. 1  Stereoview showing the superposition of the $C^a$, $C'$, and N backbone atoms for the 30 solution structures of the Shc SH2/phosphopeptide complex. The N and C termini of the protein as well as the pY and (pY+3) residues of the phosphopeptide are indicated.

Fig. 2  Schematic diagrams showing comparison of SH2 domain/peptide complexes. (A) Shc. (B) Lck. (C) Phospholipase C-$\gamma$1 (PLC-$\gamma$1). (D) Syp. The view is from the peptide-binding surface and illustrates the secondary structural elements labeled using the nomenclature proposed by Harrison and coworkers (15). Side chains of the pY and (pY+3) residues of the phosphopeptide are shown in each complex. Figures were generated using the MOLSCRIPT program (39) with the coordinates of the averaged minimized NMR structure of the Shc SH2/peptide complex and the coordinates of the other SH2 domain complexes obtained from the Brookhaven Protein Data Bank.

Fig. 3  Ribbon plot of the Shc SH2 domain that has been superimposed with the structures of Lck, PLC-$\gamma$1, and Syp to illustrate the different locations of the bound peptides in the different SH2/peptide complexes. (A) Shc. (B) Lck. (C) PLC-$\gamma$1. (D) Syp.

Fig. 4  Schematic diagram depicting the NOEs observed between the phosphopeptide and Shc SH2 domain.

Fig. 5  Sequence alignment of the Shc SH2 domain with the Lck, PLC-$\gamma$1, and Syp SH2 domains based on a comparison of the secondary structural elements. The boundaries of the secondary structural elements are underlined, and the notation for these elements is shown at the top of the sequence alignment. Residues of Shc SH2 domain protein that have NOEs with the phosphopeptide are highlighted with asterisks.
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<td>$E_{tot}$ (kcal/mol$^{-1}$)</td>
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<td>$E_{repel}$ *</td>
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<td>Improper (degrees)</td>
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<tr>
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<tr>
<td>$(SA)$ vs. $(SA)$ *</td>
<td>0.37 ± 0.05</td>
<td>0.80 ± 0.05</td>
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<tr>
<td>$(SA)$ vs. $(\bar{SA})_r$ †</td>
<td>0.39 ± 0.05</td>
<td>0.82 ± 0.04</td>
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$(SA)$ is the ensemble of 30 final simulated annealing NMR structures; $(\bar{SA})_r$ is the mean atomic structure obtained by averaging the coordinates of the individual $(SA)$ structures following a least-squares superposition of the backbone heavy atoms (N, Cα, C') for residues 2-104; $(\bar{SA})_r$ is the energy minimized mean structure; 1 kcal = 4.18 kJ.

* The $x$-PLOR Frepl function was used to simulate van der Waals interactions with atomic radii set to 0.80 times their CHARMM (38) values.

† Torsional restraints were applied to 36 $\phi$ angles with bounds of $-120 \pm 40^\circ$ for those angles with $^{3}J_{HN-He}$ coupling constants > 9.0 Hz and $-60 \pm 40^\circ$ for coupling constants < 5.5 Hz. Restraints for the latter were applied only in the $\alpha$-helical regions. Force constants of 200 kcal-mol$^{-1}$-rad$^{-2}$ were employed on all torsional restraints.

‡ A total of 2072 nontrivial NOE-derived distance restraints were applied with a square well potential and a force constant of 50 kcal-mol$^{-1}$-Å$^2$. In addition, 35 hydrogen bonds were included and given distance bounds of 1.8-2.5 (H $\rightarrow$ O) Å and 2.5-3.3 (N $\rightarrow$ O) Å. No distance restraint was violated by >0.10 Å in any of the final structures.

§ RMSD for residues 2-104 of the protein.

¶ RMSD for residues 2-104 of the protein and residues 6-9 of the peptide.

Table 1.
Fig. 2
Fig. 5

shc  AEQLRGEPFHGLSRBREAALLQNN...GDFLYRESTTTP.QOYVLTPQ.S...G.
src  ...SQAEWVEEGKITEEERLLNPNPRGTRTFLVRESETTK.GAYCLSSDPRFDNAGL
lck  ...APEPWFLKNLSERDKAELVAPGNTQGSELIREESSTA.QSKLSYVDQGDQGE
plcC  ...IHESKEWNEALTRAQAEHMLMRVPRD.GAEVLRKRNPN..SYATRTEA.E...G.
syp  .....MRWFTHPNTGVEDAENLTTTRGVD.GSFLARPSKSNP.GDFTLSSRR.N...G.

shc  QPKHLLLVD.PEGYVVT.KHDFESYVHLLISYHMDNHLPIISA.GSELCLQOVYERKL
src  NVKHYKIRLQSGEXIT5RTQES5DOGVYAXYSKH...AGDL...CHKLTVEQT...
lck  YVKHYKIRLQDRGEYISPRITEVGLHDLIVREYHTNA...SDGL...CTRLSBEQGT...
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