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Determination of the substrate-docking site of protein tyrosine kinase C-terminal Src kinase

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Protein tyrosine kinases (PTK) are key enzymes of mammalian signal transduction. For the fidelity of signal transduction, each PTK phosphorylates only one or a few proteins on specific Tyr residues. Substrate specificity is thought to be mediated by PTK–substrate docking interactions and recognition of the phosphorylation site sequence by the kinase active site. However, a substrate-docking site has not been determined on any PTK. C-terminal Src kinase (Csk) is a PTK that specifically phosphorylates Src family kinases on a C-terminal Tyr. In this study, by sequence alignment and site-specific mutagenesis, we located a substrate-docking site on Csk. Mutations in the docking site disabled Csk to phosphorylate, regulate, and complex with Src but only moderately affected its general kinase activity. A peptide mimicking the docking site potently inhibited (IC50 = 21 μM) Csk phosphorylation of Src but only moderately inhibited (IC50 = 422 μM) its general kinase activity. Determination of the substrate-docking site provides the structural basis for understanding substrate specificity in other PTKs.

Early efforts to understand Csk substrate specificity used peptides mimicking this phosphorylation site (8, 16). However, such peptides are ∼1,000 times less efficient than SFKs as substrates for Csk (8, 16, 17). By screening a random peptide library, Cole and coworkers (17) identified an optimal peptide substrate for Csk with the sequence of EEEIYFF. The optimal peptide is 500 times better as a substrate than peptide mimicking the Src C-terminal tail and bears little resemblance to the physiological phosphorylation site (17). These studies demonstrate that the C-terminal tail does not contain sufficient determinants for Csk recognition. Recent mutagenic studies of Src indicated that, although the local sequence surrounding the phosphorylation site played important roles, additional determinants residing outside the C-terminal tail are required for efficient C-terminal phosphorylation by Csk (18). These observations suggest that Csk recognition of Ytail of SFKs involves two types of interactions: docking interactions between Csk and SFKs and local interactions between the active site of Csk and the tail peptide sequence. Such bivalent interactions would allow Csk to specifically recognize SFKs and position the Ytail into Csk active site for phosphorylation. The docking site on Csk and the docking determinants on SFKs have not been determined.

The tertiary structures of Csk (19, 20) and several SFKs (11, 12, 21) have been determined, but the structures do not provide clear clues to how Csk recognition of SFKs may be achieved. In this current study, using structure-guided site-specific mutagenesis, we determined a substrate-docking site on Csk that is critical for its ability to bind to, phosphorylate, and regulate SFKs but is not important for the general kinase activity. Furthermore, a peptide mimicking the substrate-docking site potently inhibited Csk phosphorylation of Src but only moderately inhibited the general kinase activity of Csk. To our knowledge, this is the first report of a substrate-docking site on any PTK.

Methods

Chemicals and Reagents. All reagents used for bacterial culture and protein expression were purchased from Fisher. Chromatographic resins, glutathione-agarose, iminodiacetic acid-agarose, and protein expression were purchased from Fisher. Chromatography of recombinant kinase was performed using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA). Peptide synthesis was performed using solid phase synthesis, purified by HPLC, and confirmed by electrospray mass spectrometry.

Recombinant Kinase and Substrate Expression and Site-Specific Mutagenesis. Wild type human Csk was expressed in E. coli (DH5α) by using pGEX-Csk-st plasmid (22, 23). Site-specific mutants were synthesized by Integrated DNA Technologies (Coralville, IA).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Csk, C-terminal Src kinase; Csk-homologous kinase; DM, double mutant; kds/C, kinase-defective src that contains a Lu255Met mutation; PTK, protein tyrosine kinase; SFK, Src family PTK; OM, quadruple mutant; SH, Src homology; Yres, the Tyr residue in the activation loop; Ytail, the Tyr residue in the C-terminal tail of Src family kinases.

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Csk: SLDDLDESLYVPLGLICLKSRCLECAEMFNEDYHEDLPLVLSVHLNVDKVLDFGLTQEDSGSAM--GLSETGKLWTAPEAIL
Chk: NFLNYFILKALEVQAPEGVESKLLTVLHVEILNLGTVILVCKLAFLRLSTGEDYTQAPQGKFFIKWTAPEAL
Src: SLDDLKSKDSGKQPLKLDFPSAQIGAEAGVAFQEIQQYNHRLDRAISSLVSLVCKIAFLGLARVIEQDSTEPGAKFEKIFKIIWTAPEAIL
Hck: FQSKT1K3DSVWLQELIMEVTGRYPGFNSMGEPLAIILRQVIAELITYVWYNMTCRWFPEQFPFLFYFQFLQFETYPYGLY6DVYAFTE3Q

Fig. 1. Alignment of the amino acid sequences of Csk, Chk, Src, and Hck in the peptide-binding lobe. Residues that are uniquely conserved in the Csk family are highlighted blue (polar or charged) or red (hydrophobic or Gly).

Kinase Activity Assays. Kinase activity of Csk and mutants was determined by using polyE4Y or kdSrc and [γ-32P]ATP (600 dpm pmol−1) as the substrates as described (26). Briefly, phosphorylation reactions were performed in 50-μl volumes at 30°C in the protein kinase assay buffer: 50 mM N-(2-hydroxyethyl)piperazine-N’-3-propanesulfonic acid (pH 8.0) containing 5% glycerol, 0.005% Triton X-100, and 0.05% 2-mercaptoethanol. The standard assay used 3 nM WT Csk, 12 mM MgCl2, 0.2 mM ATP, and 1 mg ml−1 polyE4Y or 10 μM kdSrc. After a 10-min reaction time, 35 μl of the reaction mixture was spotted onto Whatman filter paper chamber (2 × 1 cm), which were washed in 5% trichloroacetic acid at 65°C 3 times for 20 min each. The radioactivity incorporated into polyE4Y or kdSrc was determined by liquid scintillation counting. Assays were performed in duplicate, and each assay was repeated at least twice with reproducible results.

To determine the catalytic parameters of Csk by using kdSrc as a substrate, 0.71–7.1 μM kdSrc was used as the variable substrate. The assays were performed as described above. The reaction minus Csk was used as background controls. The background was <2,000 cpm, and the signals were in the range of 10,000–100,000 cpm. No autophosphorylation or phosphorylation of polyE4Y by kdSrc was detected under these conditions. The K_m and k_cat values were determined by using double reciprocal plot.

To determine the inhibition of Csk activity by peptides, Csk activity in the presence of various concentrations of the peptide was determined. The kinase activity as a function of the peptide concentration was fitted into a curve-fitting program (LABFIT) to determine the IC50.
observed when the uniquely conserved residues were mapped onto the tertiary structure of Csk.

To determine which of these residues may be part of a substrate-docking site, they were individually mutated to Ala and the mutant enzymes were purified to apparent or near homogeneity. For some reason, one of the mutants, Arg359Ala, was not produced. By functional nature of the hypothetical substrate-docking site, we reasoned that this site would be critical for Csk activity toward its physiological substrate but not important for phosphorylation of an artificial substrate. Thus, as the initial screening, the kinase activities of the mutants toward an artificial and a physiological substrate were determined (Fig. 2A). The artificial substrate used was polyE,Y, a random copolymer of Glu and Tyr in the ratio of 4:1. PolyE,Y lacks a defined phosphorylation site or higher orders of structure and is commonly used as a generic PTK substrate. The activity toward polyE,Y, therefore, is considered as general kinase activity. The physiological substrate used is a recombinantly expressed kinase-defective Src (kdSrc) that contains a Lys295Met mutation. The mutation inactivates Src but does not affect its ability to serve as a specific substrate for Csk or Chk (10, 18). The use of kdSrc instead of active Src eliminates interference to the assay by Src autophosphorylation.

If a residue is part of the substrate-docking site of Csk, its mutation to Ala will likely more dramatically decrease Csk activity toward kdSrc than toward polyE,Y. Three of the 10 mutants, Arg279Ala, Arg281Ala, and Arg283Ala, displayed this property, with the latter two exhibiting >80% of WT activity toward polyE,Y but <20% of WT activity toward kdSrc. Lys362Ala displayed the opposite effect, having a more dramatic effect on polyE,Y phosphorylation than kdSrc phosphorylation. This is representative of a group of Csk mutants that preferentially affect phosphorylation of polyE,Y over kdSrc, which were separately characterized (unpublished data). Mutation of the other residues had similar effects on polyE,Y and kdSrc phosphorylation. Overall, Ala scanning mutagenesis implicated Arg-281, Arg-283, and Arg-279 as part of the substrate-docking site.

**Additional Residues Are Identified to Be Critical for Src Phosphorylation.** Arg-279, Arg-281, and Arg-283 are located on α-helix D, a short helix located near the active site of Csk. In the tertiary structure, these three residues form a triangle (Fig. 2B). Each side of this triangle measures 15–16 Å. Two other residues, Ser-280 and Phe-382, although not uniquely conserved in Csk family, also fall within or near the area defined by the Arg triangle and could be part of the substrate-docking site. They were individually mutated to Ala, and the ability of the mutants to phosphorylate kdSrc and polyE,Y was determined. Ser280Ala and Phe382Ala displayed significantly less relative activity toward kdSrc than toward polyE,Y (Fig. 2C), indicating that these two residues are also part of the substrate-docking site. Ser-284 and Ser-381 are located just outside of the Arg triangle (Fig. 2B). The mutation of either one to Ala affected polyE,Y and kdSrc phosphorylation equally (Fig. 2C), indicating that these two residues were not specifically important for Src recognition.

Two residues, Ser-273 and Asp-276, are located on α-helix D, and between the Arg triangle and the active site (Fig. 2B). If the Arg triangle and the active site of Csk form a continuous binding surface for Src interaction, then these residues are also likely to be important for Src phosphorylation by Csk. To test this possibility, they were mutated to Ala and the mutants were purified and analyzed (Fig. 2C). Ser273Ala displayed WT level activity toward polyE,Y and ~70% WT activity toward kdSrc. Asp276Ala retained ~40% of WT activity toward either polyE,Y or kdSrc. This result suggests that Ser-273 is also part of the substrate-docking site.

The above Ala scanning study identified six residues (Ser-273, Arg-279, Ser-280, Arg-281, Arg-283, and Phe-382) as specifically important for kdSrc phosphorylation. The first five are located on α-helix D, and the last one is located next to the helix in the tertiary structure. Several residues immediately outside this region were not specifically important for kdSrc phosphorylation.
increased ~1.5-fold for DM and 3-fold for QM. Both mutants had $k_{cat}$ values <15% of that of WT, resulting in a 20-fold or more reduction in $k_{cat}/K_m$ ratio. In contrast, the mutants had similar $k_{cat}$ and moderately increased $K_m$ toward polyE$_4$Y, resulting in a reduction of $k_{cat}/K_m$ ratio by <3-fold. This result demonstrates that the mutated residues are specifically important for kdSrc phosphorylation.

Because the Csk mutants were defective in phosphorylating kdSrc, it was expected that they would be defective in regulating Src by phosphorylation. This possibility was tested by a Src inactivation assay (14) (Fig. 3B). Src expressed in and purified from insect cells (36, 37) was incubated with WT or mutant Csk (equal polyE$_4$Y kinase activity were used for WT or mutant Csk) in the presence of ATP and MgCl$_2$ for 10 min, and then the Src activity in the incubation was determined. If Csk or the mutants were able to inactivate Src, the preincubation would result in a decrease in Src activity. As expected, WT Csk inactivated Src, but the two mutants did not. This result confirmed that the mutations converted Csk into a generically active kinase without the ability to recognize and phosphorylate Src.

If the mutated residues in DM and QM are indeed the substrate-docking site, the mutants should have a much weaker interaction with kdSrc than WT Csk. Pull-down assays were performed, as described in Methods, to evaluate the interaction of Csk to kdSrc (Fig. 4). GST-wtCsk, but not GST, was able to pull down kdSrc close to 1:1 ratio, indicating that kdSrc was binding specifically to Csk. Similarly, Csk mutants S273, K393, and K401 were also able to pull down kdSrc, correlating to their significant residual kdSrc kinase activity. In contrast, DM, QM, R284, and F382 were not able to pull down kdSrc, in good agreement with their inactivity to phosphorylate kdSrc. This result further confirmed that the identified docking residues were specifically responsible for binding to Src. Because these residues are responsible for binding to Src and rendering Csk effective in phosphorylating Src, we conclude that these residues constitute the major determinants of the substrate-docking site.

Peptidic Mimic of the Substrate-Docking Site Specifically Inhibits the Phosphorylation of kdSrc by Csk. As another independent test for the identified substrate-docking site, the ability of peptides mimicking the docking site as inhibitors for Csk was determined. Peptides mimicking the substrate-docking site structure would be expected to compete against Csk in binding to Src. Thus, they should inhibit Csk phosphorylation of kdSrc but not that of polyE$_4$Y. To test this idea, three peptides were synthesized: VDYLR (P1), RSRRGS (P2), and RSVLGG (P3). They cover residues V275 through Ser-280 (P1), Arg-279 through Ser-284 (P2), and Arg-283 through Gly-288 (P3). Note that P2 contained four residues, Arg-279, Ser-280, Arg-281, and Arg-283, that are key residues of the substrate-docking site of Csk. In contrast, P1 or P3 contained only at most two residues important for SFK binding. P1 and P3 (up to 1 mM) did not inhibit Csk phosphorylation of polyE$_4$Y or kdSrc (data not shown). In contrast, P2 potently inhibited Csk phosphorylation of kdSrc ($IC_{50} = 21 \pm 3.8$ μM) but only moderately inhibited polyE$_4$Y phosphorylation ($IC_{50} = 422 \pm 46$ μM) (Fig. 5). The differential inhibition of

![Graph showing the effect of DM and QM on Csk kinase function.](image)

**Fig. 3.** Effect of DM and QM on Csk kinase function. The details and rationale of the mutations are described in the text. (A) Effect of docking site mutations on Csk phosphorylation of kdSrc. (B) Effect of docking site mutations Csk’s ability to inactivate Src.

**Table 1. Catalytic parameters of Csk mutants**

<table>
<thead>
<tr>
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<th>Activity with kdSrc</th>
<th>Activity with polyE$_4$Y</th>
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<tbody>
<tr>
<td>$k_{cat}$, min$^{-1}$</td>
<td>$K_m$, μM</td>
<td>$k_{cat}/K_m$</td>
</tr>
<tr>
<td>WT</td>
<td>53.8 ± 17.2</td>
<td>5.7 ± 2.2</td>
</tr>
<tr>
<td>DM</td>
<td>6.5 ± 1.2</td>
<td>13.9 ± 3.6</td>
</tr>
<tr>
<td>QM</td>
<td>7.6 ± 4.2</td>
<td>22.9 ± 9.4</td>
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Relative $k_{cat}/K_m = actual k_{cat}/K_m/actual k_{cat}/K_m$ of WT.
kdSrc and polyE4Y phosphorylation indicated that P2 specifically interfered with the interaction between Csk and kdSrc. This result further indicates that the residue cluster located on α-helix D and mimicked by P2 contained key determinants of the substrate-docking site.

Discussion

PTKs of the Csk family specifically phosphorylate SFKs on a C-terminal tail Tyr residue and regulate their activity. This system is chosen to investigate PTK substrate specificity because the exclusive PTK–substrate relationship is well established in vivo and in vitro, and extensive structural and biological data are available on Csk and SFKs to assist such studies. One unique feature of this system that was central to our strategy was that Csk and Chk shared functional identity but relatively low sequence identity (54%). This feature allowed us to locate some key residues in the docking site by evaluating uniquely conserved residues in Csk family.

The identified substrate-docking site of Csk is composed of six residues, Ser-273, Arg-279, Ser-280, Arg-281, Arg-283, and Phe-382. This identification of the substrate-docking site is supported

![Fig. 6. Structures of Csk catalytic domain and the substrate-docking site. (A) Ribbon structure of Csk with identified residues in the substrate-docking site shown in a ball-and-stick model. Several loop structures relevant to peptide substrate binding and catalysis are indicated by arrows. (B) Surface structure of Csk (yellow) and the substrate-docking site (colored by electrostatic potential). The active site cleft is indicated.](image)

Fig. 4. Pull-down assay to determine the interaction of Csk and mutants with kdSrc. (A) Purified GST, GST-Csk, GST-DM, and GST-QM. (B and C) kdSrc pull-down assay with various Csk variants. Each of the purified GST or fusion proteins (100 pmol) was incubated with purified kdSrc (200 pmol) and precipitated with glutathione-agarose. The proteins retained by the beads were analyzed by SDS/PAGE and Coomassie blue staining.
by several lines of evidence. First, several of the key residues of the docking site are uniquely conserved in Csk and Chk. The unique conservation is consistent with Csk and Chk being the only two kinases able to phosphorylate SFKs on Ytail. Second, mutations of the residues within the docking site abolished Csk activity toward Src without significantly affecting its general kinase activity. Mutation of many residues outside this region did not preferentially affect Csk’s ability to phosphorylate the physiological substrate. Third, Csk mutants containing multiple point mutations in the docking site resulted in a >95% loss of Csk activity toward kdSrc but only a modest decrease in activity toward artificial substrate, effectively converting Csk into a generic PTK unable to phosphorylate Src. Correspondingly, the Csk mutants were unable to regulate Src activity. Fourth, the loss of activity toward kdSrc correlates to the loss of ability to physically bind to kdSrc, indicating that the mutated site is indeed critical for Csk-Src interaction. Fifth, a peptide mimicking part of the docking site potently inhibited Csk phosphorylation of kdSrc but only moderately inhibited Csk activity toward kdSrc. This result suggests that the substructure consisting of the α-helix D and Phe-382 is indeed the docking site that specifically interacts with SFKs for efficient phosphorylation.

At present, it is difficult to assign quantitative contributions to the individual residues. The identified residues (Fig. 2B), containing three positively charged residues, two polar residues, and one hydrophobic residue, appear well suited to provide a complex surface for highly specific and unique interaction with the substrate. The docking site is located near the active site (Fig. 6) and appears well positioned for Src docking. Crystallized IRK-peptide substrate complexes (38) and mutagenic studies of other PTKs (39) indicate that the P + 1 loop provides the main platform for peptide substrate binding to PTKs. It is likely that the P + 1 loop in Csk performs the same function in binding to SFK C-terminal tail and presents Ytail to the active site. The docking site is located on the same side of the kinase molecule as the P + 1 loop. It can be envisioned that substrate-docking site would interact with the docking determinants on SFKs, which would bring the C-terminal tail peptide to Csk active site. Because Src binding and phosphorylation are both abolished by docking site mutations, it appears that the docking interaction may be primarily responsible for SFKs recognition by Csk.

This work raises several important questions that remain to be answered. First, what are the specificity determinants on SFKs that allow them to be specifically recognized by Csk and Chk? We reason that such determinants would be a surface area complementary to the Csk substrate-docking site. The identification of the docking site on Csk will facilitate the identification of such determinants on SFKs. Second, it is unclear whether other PTKs will use a similar mechanism for recognizing their physiological substrates. Csk family PTKs have unusually strict substrate specificity in that they only phosphorylate SFKs. Other PTKs may phosphorylate multiple families of protein substrates. Alignments of primary and tertiary structures of PTKs indicate that the structures of α-helix D are highly variable between PTK families, consistent with the possibility of its being a key structure for substrate specificity determination. Third, it is interesting that mutations of the docking site only moderately affected the $K_m$ of Csk for kdSrc but more dramatically affected the $k_{cat}$. We started this work with the expectation that the docking site would mainly affect the complementarity between the enzyme and the substrate, and thus perturbation to the docking site would more significantly affect the $K_m$. The large decrease in $k_{cat}$ caused by the mutations suggest that the docking interaction is critical for Csk transition state complementarity with SFKs instead.

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