2006

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Citation/Publisher Attribution

Lee, S., Ayrapetov, M. K., Kemble, D. J., Parang, K., & Sun, G. (2006). Docking-based Substrate Recognition by the Catalytic Domain of a Protein Tyrosine Kinase, C-terminal Src Kinase (Csk). *Journal of Biological Chemistry, 281*(12), 8183-8189. doi: 10.1074/jbc.M508120200
Available at: http://dx.doi.org/10.1074/jbc.M508120200

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 12, pp. 8183–8189, March 24, 2006

Docking-based Substrate Recognition by the Catalytic Domain of a Protein Tyrosine Kinase, C-terminal Src Kinase (Csk)*

Received for publication, July 25, 2005, and in revised form, January 23, 2006 Published, JBC Papers in Press, January 26, 2006, DOI 10.1074/jbc.M508120200

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Protein tyrosine kinases are key enzymes of mammalian signal transduction. Substrate specificity is a fundamental property that determines the specificity and fidelity of signaling by protein tyrosine kinases. However, how protein tyrosine kinases recognize the protein substrates is not well understood. C-terminal Src kinase (Csk) specifically phosphorylates Src family kinases on a C-terminal Tyr residue, which down-regulates their activities. We have previously determined that Csk recognizes Src using a substrate-docking site away from the active site. In the current study, we identified the docking determinants in Src recognized by the Csk substrate-docking site and demonstrated an interaction between the docking determinants of Src and the Csk substrate-docking site for this recognition. A similar mechanism was confirmed for Csk recognition of another Src family kinase, Yes. Although both Csk and MAP kinases used docking sites for substrate recognition, their docking sites consisted of different substructures in the catalytic domain. These results helped establish a docking-based substrate recognition mechanism for Csk. This model may provide a framework for understanding substrate recognition and specificity of other protein tyrosine kinases.

The human genome contains ~500 genes for protein kinases, including ~100 protein tyrosine kinases (PTKs)[3] (1). PTKs are important mediators of signal transduction and key targets of anticancer drug discovery (2). They mediate cellular signaling by responding to upstream signals and phosphorylating protein substrates on Tyr residues. To send a regulatory signal to specific protein targets, each PTK phosphorylates only one or a few protein substrates on specific tyrosine residues. Thus, substrate specificity determines signaling specificity and fidelity and distinguishes one PTK from another (3).

One of the best understood PTK regulatory systems is the regulation of Src family protein tyrosine kinases (SFKs) by phosphorylation of a Tyr on its C-terminal tail (4, 5). There are nine kinases in the Src family, and each one contains, from the N to the C terminus, a myristoylation motif, a unique region, an SH3 domain, an SH2 domain, a catalytic domain, and a regulatory C-terminal tail. The C-terminal tail contains a Tyr residue (Tyr-527 in avian Src) for phosphorylation by the C-terminal Src kinase (Csk) (6) and the Csk-homologous kinase (7). The phosphorylated tail Tyr binds to the SH2 domain intramolecularly (8, 9), which leads to inactivation of SFKs (10). Ever since the PTK-substrate relationship between Csk and Src families was established about 15 years ago, how Csk specifically recognizes Src family kinases and phosphorylates them on the C-terminal tail Tyr has been an intriguing question (11, 12). This exemplifies the largely unanswered question of how PTKs recognize protein substrates in general.

Although the Tyr residue for phosphorylation is located on the C-terminal tail of SFKs, SFKs are ~50,000-fold better substrates than peptides, mimicking the C-terminal tails (k_{cat}/K_m ratio comparison) (13). Mutagenic studies confirm that the C-terminal tail does not contain all necessary determinants for Csk recognition (14). These findings suggest that SFKs contain structural motifs located away from the C-terminal tail that are recognized by Csk, and by extension, that Csk contains a SFK recognition site away from the active site. These suggestions would imply that remote docking interactions are a key component of Csk-Src recognition. Since PTK substrate specificity can rarely be accounted for by local amino acid sequences surrounding the phosphorylation site, such a docking-based substrate recognition mechanism may apply to many, if not all, PTKs.

We have recently (15) determined that the Csk catalytic domain contains a substrate-docking site that is crucial for the recognition and phosphorylation of Src. Four residues located in a region centered on the α-helix D in the peptide-binding lobe are critical for Csk to bind to and phosphorylate Src. Csk mutants carrying multiple mutations at these positions are fully or nearly fully active toward a peptide substrate but unable to bind to, phosphorylate, or regulate Src. Furthermore, a peptide mimicking the sequence of the α-helix D inhibits Csk phosphorylation of Src with a 20-fold higher potency than Csk phosphorylation of a peptide substrate. These results compellingly demonstrate that these residues are key determinants of the Csk substrate-docking site for specifically recognizing Src family kinases.

Building on this foundation, the current study identified key residues in Src that are crucial for Csk recognition and examined their interactions with the substrate-docking site of Csk. The results demonstrated a docking-based mechanism for Csk-Src recognition. This finding should facilitate our effort to understand PTK substrate recognition and specificity in general.

MATERIALS AND METHODS

Chemicals and Reagents—All reagents used for bacterial culture and protein expression were purchased from Fisher. Chromatographic res-

* This work was supported by American Cancer Society Grant RSG-04-247-01-CDD and National Institutes of Health Grants 1RO1CA111687 and 1 P20 RR16457. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: PTK, protein tyrosine kinase; Csk, C-terminal Src kinase; GST, glutathione *S*-transferase; kdSrc, kinase-defective Src that contains a K295M mutation; SFK, Src family kinase; TM, triple mutant; WT, wild type; MAP, mitogen-activated protein; SH, Src homology; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; RCM, carboxymethylated-maleylated, reduced.

ins, glutathione-agarose, iminodiacetic acid-agarose, and Sephadex G25 were purchased from Sigma. DNA primers were synthesized by Integrated DNA Technologies. [γ-^{32}P]ATP (6,000 Ci mol^{-1}) was purchased from PerkinElmer Life Sciences.

Recombinant Protein Expression and Site-specific Mutagenesis—Wild type human Csk was expressed in *Escherichia coli* (DH5α) using pGEX-Csk-st plasmid (16). Site-specific mutants were generated using QuikChange (Stratagene) and confirmed by DNA sequencing. The chicken Src mutant devoid of kinase activity (kdSrc) was co-expressed with GroEL and GroES chaperone in BL21(DE3) (14). Full-length human *yes* gene was closed from a human fetal cDNA library (17) and inserted into the pRSET-A plasmid for expression of a His_6-Yes fusion protein. The fusion protein was purified as described for kdSrc. Yes-TM was generated by QuikChange. Protein concentration was determined by Bradford method and A_{280} in 6 M urea, and the purity was examined by SDS-PAGE and Coomassie Blue staining.

Kinase Activity Assays—Kinase activity of Csk and mutants was determined using polyE_4Y or kdSrc as the substrate as described previously (15). Briefly, phosphorylation reactions were performed in 50-μl volumes at 30 °C in the protein kinase assay buffer: 50 mM EPPS (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 $MgCl_2$, 0.2 mM ATP ([γ-^{32}P]ATP, 1,000 dpm pmol^{-1}), and 1 mg ml^{-1} polyE_4Y or 10 μM kdSrc. After a 10-min reaction time, 35 μl of the reaction mixture was spotted onto Whatman filter paper strips (2 × 1 cm), which were washed in 5% trichloroacetic acid at 65 °C three times for 20 min each. The radioactivity incorporated into polyE_4Y or kdSrc was determined by liquid scintillation counting. All assays were performed in duplicates and at least three times with reproducible results.

To determine the catalytic parameters of Csk using kdSrc as a substrate, 1–20 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 under 2,000 cpm, whereas the signals were in the range of 10,000–100,000 cpm. The K_m, and k_{cat} values were determined using double reciprocal plot.

Csk Inactivation of Yes—The ability of Csk to inactivate Yes and Yes-TM was determined as described previously (18). Yes and Yes-TM (250 ng) was incubated with Csk (3 nM) in the presence of 0.1 mM ATP and 12 mM $MgCl_2$ at 30 °C for 10 min. To determine Yes and Yes-TM phosphorylation, [γ-^{32}P]ATP (10,000 dpm pmol^{-1}) was used in the phosphorylation reaction, and a sample of 10 μl of the reaction mixture was withdrawn at indicated times. The reaction was mixed right away with SDS-PAGE sample buffer and heated at 90 °C for 10 min. The proteins were fractionated by SDS-PAGE, and phosphorylation was detected by autoradiography. To determine the effect of Csk phosphorylation on Yes kinase activity, non-radioactive ATP was used in the Csk phosphorylation reaction. At different times, 10-μl aliquots were withdrawn, and the Yes activity was determined using 0.4 mg/ml RCM-lysozyme and [γ-^{32}P]ATP (1000 dpm pmol^{-1}). Since RCM-lysozyme was preferentially phosphorylated by Yes, the residual Yes activity after the initial incubation could be accurately determined without removing Csk from the incubation. To minimize continued Csk phosphorylation of Yes during the activity assay, the reactions were allowed to proceed for 1 min. The reactions were stopped by spotting onto filter papers and washing with 5% warm trichloroacetic acid. The phosphate incorporated into RCM-lysozyme was determined by liquid scintillation counting. Similar reactions in the absence of Yes were carried out in parallel and used as background.

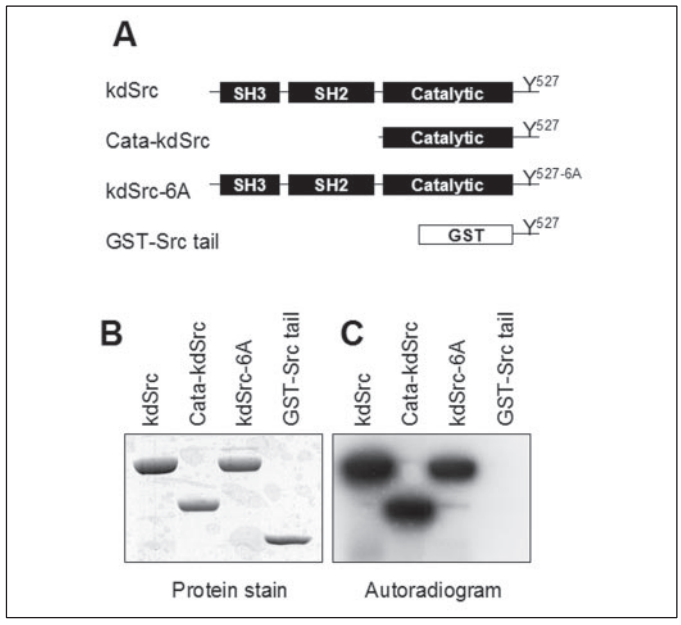


FIGURE 1. **Characterization of Src phosphorylation by Csk.** *A*, deletion mutants of kdSrc used to locate the recognition motifs in Src. KdSrc starts at Val-71, and cata-kdSrc starts at 248. KdSrc-6A is identical to kdSrc except containing six mutations of E524A, P525A, Q526A, Q528A, P529A, and G530A. GST-Src tail contains GST fused to the Src C-terminal tail starting with Thr-521. *B* and *C*, phosphorylation of the kdSrc mutants by Csk. The mutants (10 μM) were incubated with Csk (36 nM) in 12.5 μM kinase assay buffer containing 20 μM [γ-^{32}P]ATP (10,000 dpm pmol^{-1}) for 10 min. The proteins were fractionated by SDS-PAGE and stained by Coomassie Blue (*B*), and the phosphorylation of proteins was detected by autoradiography (*C*).

RESULTS

Analysis of Csk-Src Recognition Using a Kinase-defective Src Mutant—A kinase-defective Src mutant (kdSrc, containing the K295M mutation) is well expressed in bacteria (14), retains the specificity for phosphorylation by Csk on the C-terminal tail Tyr, and does not autophosphorylate; thus, it serves as a faithful and convenient substrate for kinetic and mutagenic analyses of Src recognition by Csk (15). To characterize the recognition of Src by Csk, we generated several deletion or site-specific mutants of kdSrc and determined their phosphorylation by Csk (Fig. 1, *A–C*). Src contains an SH3 and an SH2 domain in addition to the catalytic domain; however, the catalytic domain with the C-terminal tail (cata-kdSrc) is still readily phosphorylated by Csk (k_{cat}/K_m ratio of 3.7 × 10^5 for kdSrc *versus* 9.5 × 10^5 M^{-1} s^{-1} for the catalytic domain) (14) (also see Fig. 1*C*). Thus, the Src SH3 and SH2 domains do not contain the structural motifs recognized by Csk. To assess the relative contribution of local and remote docking interactions to Src-Csk recognition, two other constructs were generated. The first was a kdSrc mutant in which 3 residues on each side of the tail Tyr (Tyr-527) were mutated to Ala (kdSrc-6A), and the second was a fusion protein between GST and the Src C-terminal tail (GST-Src tail). A previous study demonstrated that the 3 residues on the amino side of Tyr-527 were important for Csk phosphorylation (14). However, kdSrc-6A was still readily phosphorylated by Csk. Initial velocity comparisons indicated that kdSrc-6A retained about 10% of the kdSrc ability to be phosphorylated by Csk. This indicated that remote docking interactions are sufficient for Src to be recognized by Csk, although the local sequences contribute significantly to this recognition. In contrast, Csk phosphorylation of GST-Src tail was non-detectable under identical conditions, consistent with reports that the C-terminal peptides are very poor substrate for Csk (13). These results confirmed the

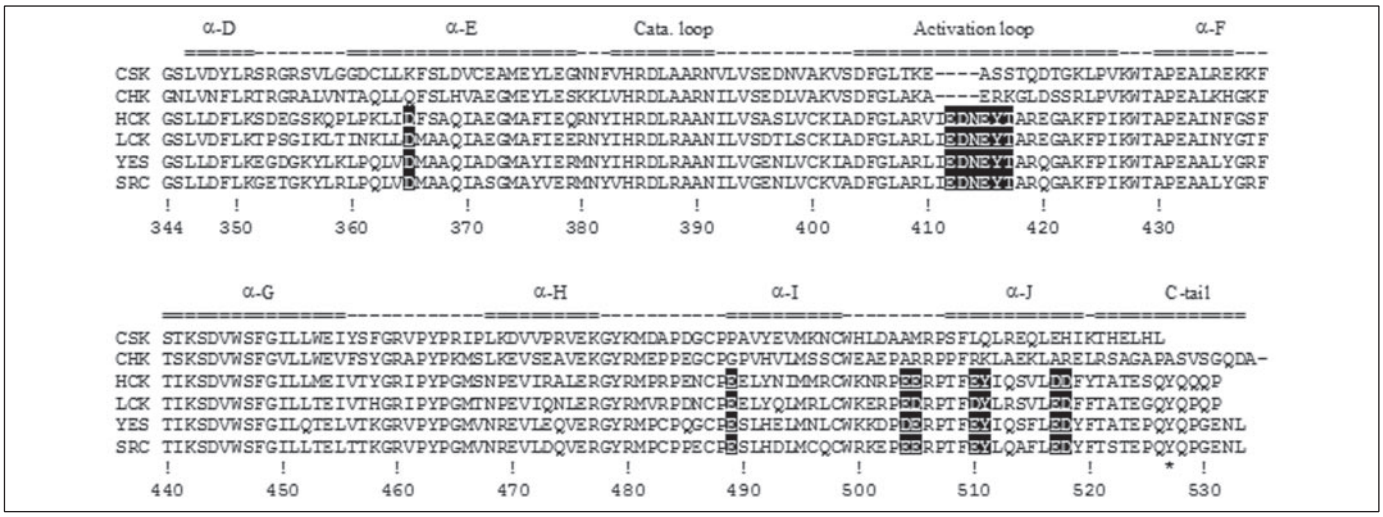


FIGURE 2. **Amino acid sequence alignment in the peptide-binding lobe for the Csk and Src family kinases.** The SFK residues that are potential determinants for Csk recognition are shown in *white letters* on *black background*. The secondary structure motifs are labeled on the *top* of the sequences. The residue numbering, based on chicken Src sequence, is shown at the *bottom* of the sequences. *Cata. loop*, catalytic loop; *C-tail*, C-terminal tail.

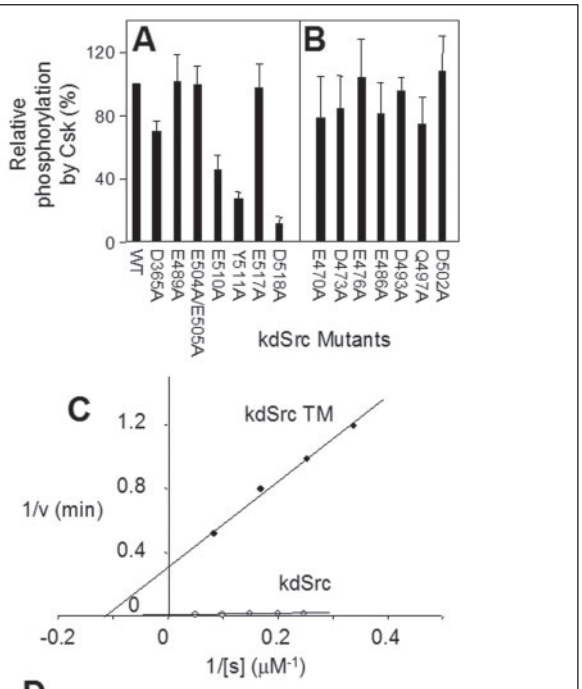
essential roles of remote docking interactions in Csk-Src recognition and located the docking determinants to the catalytic domain of Src.

Ala Scanning Identifies Three Src Residues Crucial for Csk Recognition—The Src catalytic domain is composed of an ATP-binding lobe and a peptide-binding lobe. Because the ATP-binding lobe is highly conserved in structure and function among PTKs, the docking determinants for Csk recognition are likely to be located on the peptide-binding lobe. We used the following criteria to identify the putative residues as potential docking determinants. First, because all SFKs are substrates for Csk, the docking determinants are likely conserved in SFKs but not in other PTKs. Second, the residues that interact with Csk would need to be surface-exposed. Third, because the Csk substrate-docking site is dominated by 3 Arg residues (15), the Src determinants are likely dominated by residues either negatively charged or otherwise attracted to positively charged residues. We aligned the primary sequences of SFKs and Csk family kinases in the peptide-binding lobe and identified the residues that met these criteria (Fig. 2). In the alignment, the 2 residues with a negative charge, Asp and Glu, were not differentiated. A total of 14 residues met these criteria. Six of the residues were located in the activation loop. Because the function of the activation loop in Src regulation was well defined, the residues from the activation loop were not included for further studies. The other 8 residues were selected for further studies: Asp-365, Asp-489, Glu-504, Glu-505, Glu-510, Tyr-511, Glu-517, and Asp-518.

These residues were individually mutated to Ala, except Glu-504 and Glu-505, both of which were mutated to Ala in one mutant. The ability of the purified mutants to serve as substrates for Csk was determined (Fig. 3*A*). Three mutants displayed phosphorylation by Csk less than 50% of that for kdSrc: E510A, Y511A, and D518A. All 3 residues are located on the α-helix J immediately preceding the C-terminal tail. Upon identification of the 3 α-helix J residues, we observed that 7 other residues (Glu-470, Asp-473, Glu-476, Glu-486, Asp-493, Gln-497, and Asp-502) located nearby had the potential to interact with the Arg residues of Csk. Although these residues are not strictly uniquely conserved in the Src family, they can also be part of the docking determinants. These were also analyzed by Ala scanning (Fig. 3*B*), but none of the mutants displayed significantly decreased phosphorylation by Csk. These results suggest that Glu-510, Tyr-511, and Asp-518 are the major determinants in Src for Csk recognition.

Mutation of Glu-510/Tyr-511/Asp-518 Disables kdSrc as a Substrate for Csk—We next kinetically characterized the effects of mutating all 3 residues to Ala individually or in combination (kdSrc-TM for triple mutation) on kdSrc phosphorylation by Csk (Fig. 3, *C* and *D*). Mutating each residue did not dramatically alter the k_{cat} but significantly increased the K_m, indicating that each mutation significantly affected Csk-Src recognition. The kdSrc-TM had a similar K_m to kdSrc but a dramatically decreased k_{cat}. The surprising recovery of the low K_m and the dramatic decrease in k_{cat} for kdSrc-TM contradicted the k_{cat}-K_m trend for the individual mutations. Currently, we do not have a ready explanation for this observation and continue to investigate the molecular basis for these kinetic observations. Whatever the detailed binding mechanism is for kdSrc-TM, mutation of the 3 residues resulted in a greater than 98% decrease in kdSrc phosphorylation by Csk. These results indicate that Glu-510, Tyr-511, and Asp-518 are the crucial determinants for Csk recognition.

Src Family Kinase Yes Uses Corresponding Residues for Csk Recognition—Since kdSrc is not active as a kinase, it has two drawbacks when used for analyzing Src phosphorylation by Csk. First, we cannot assess the effect of a mutation on Src kinase activity to determine whether a mutational effect is specific for Src recognition by Csk. Second, kdSrc cannot be used to determine whether a mutation affects Src inactivation by Csk. To overcome these drawbacks, we used another Src family kinase, Yes, which could be expressed as an active kinase in bacteria, to examine the mutational effects. Like Src, Yes is inactivated by phosphorylation on the C-terminal Tyr and activated by autophosphorylation on a Tyr in the activation loop (17, 18). To remove the interference of Yes autophosphorylation, the autophosphorylation site of Yes was mutated from Tyr to Phe. On this background (referred to as Yes), the 3 corresponding residues, Glu-520, Tyr-521, and Asp-528, were mutated to Ala to generate Yes-TM. Yes and Yes-TM were expressed with an N-terminal His_6 tag in bacteria and purified. Yes-TM (84.7 nmol min^{-1} mg^{-1}) had a comparable specific kinase activity as Yes (91.3 nmol min^{-1} mg^{-1}), indicating that the triple mutation did not disrupt the folding and activity of the enzyme. When incubated with Csk and ATP, Yes was readily phosphorylated by Csk (Fig. 4*A*, *top*), whereas the phosphorylation of Yes-TM by Csk was markedly reduced (Fig. 4*A*, *bottom*). In the absence of Csk, both Yes and Yes-TM underwent similar levels of autophosphorylation, most likely on the C-terminal tail Tyr (19). The



D

Substrate	k_{cat} (s^{-1})	K_m (μM)
kdSrc	258 ± 58.8	12.3 ± 1.2
kdSrc-E510A	232 ± 37.3	25.1 ± 3.6
kdSrc-Y511A	237 ±17.6	75.1 ± 5.0
kdSrc-D518A	163 ± 18.2	86.6 ± 9.5
kdSrc TM	3.6 ± 0.8	11.1 ± 2.6

FIGURE 3. **Identification of the Src residues crucial for Csk recognition.** *A* and *B*, Csk phosphorylation of kdSrc mutants. Details of the assay are described under "Materials and Methods." The assays were performed five times in duplicates. *C*, double reciprocal plot of kdSrc and kdSrc-TM by Csk. This shows a representative of three determinations with similar results. *D*, kinetic parameters of Csk phosphorylation of kdSrc and kdSrc mutants. Standard errors based on three sets of data are given.

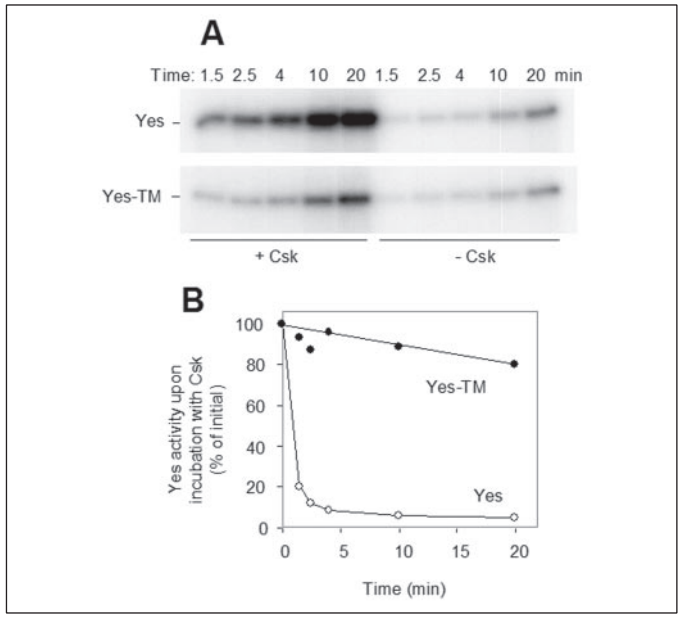


FIGURE 4. **Phosphorylation and inactivation of Yes and Yes-TM by Csk.** *A*, phosphorylation of Yes and Yes-TM in the presence and absence of Csk. The conditions of the phosphorylation reactions are described under "Materials and Methods." *B*, time course of inactivation of Yes and Yes-TM by Csk phosphorylation. Details of the assay are given under "Materials and Methods."

abilities of Csk to phosphorylate and inactivate Yes and Yes-TM were then compared. Upon incubation with ATP and Csk, Yes was rapidly inactivated, losing more than 80% of the initial activity within 1.5 min. However, Yes-TM retained more than 80% of the initial activity after 20 min of incubation with Csk and ATP (Fig. 4*B*). This result indicates that Glu-520, Tyr-521, and Asp-528 are crucial for Yes recognition and phosphorylation by Csk. Overall, these results and those with kdSrc demonstrate that the mutational effects of the 3 residues are specific for Csk recognition.

Glu-510 of Src Interacts with Arg-283 of Csk in Csk-Src Recognition—To identify potential interacting residue pairs between Csk and Src, a panel of kdSrc mutants (E510A, Y511A, and D518A) was analyzed against a panel of Csk point mutants (R279A, S280A, R281A, R283A). The mutated Csk residues are key determinants of the Csk substrate-docking site that could interact with the 3 Src docking residues. If 2 residues, one from Csk and the other from kdSrc, interact with each other, the effects of their mutations would not be additive. On the other hand, mutations of 2 residues that do not interact with each other would likely have additive effects. The result of this analysis is shown in Fig. 5*A*. Most of the mutant combinations were approximately additive with one exception, kdSrc mutant E510A and Csk mutant R283A. E510A exhibited 44% of kdSrc activity as a substrate for WT Csk, whereas Csk R283A was 16% as effective as WT Csk in phosphorylating kdSrc. If the two mutations have additive effects, 6.3% phosphorylation would be expected. However, Csk R283A exhibited almost identical activity as WT Csk in phosphorylating kdSrc-E510A. This indicated that although R283A mutation dramatically decreases the ability of CSK to phosphorylate kdSrc, this mutation has no effect on Csk phosphorylation of kdSrc-E510A. This lack of additivity suggests that Arg-283 of Csk interacts with Glu-510 of kdSrc in Csk-Src recognition.

To further investigate the possibility of Csk Arg-283 interacting with Src Glu-510, we determined how various combinations of positive and negative charges at these two positions affected Csk phosphorylation of kdSrc (Fig. 5*B*). Using kdSrc (with Glu at position 510) as a substrate, mutation of Csk Arg-283 to Lys, Ala, and Glu resulted in progressively more severe losses of activity. The least phosphorylation (6% of Csk phosphorylation of kdSrc) was observed when Glu replaced Arg-283 in Csk. However, using kdSrc E510A as a substrate, the activity trend for the Csk mutants was reversed, with WT Csk and Csk R283K displaying the lowest activity. The highest activity was observed with Csk R283E. These comparisons demonstrated that the identity of an optimal residue at Csk 283 depends on what residue is present at position 510 of kdSrc. When kdSrc contains a negatively charged Glu at position 510, a positively charged Arg is optimal for Csk at position 283. On the other hand, when kdSrc contains a positively charged Arg at position 510, a negatively charged Glu is optimal for Csk at position 283. This residue switching experiment confirmed that Glu-510 of Src interacts with Arg-283 of Csk, likely by a salt bridge, in the docking interactions between Csk and Src. More importantly, this identification of an interacting residue pair strengthened our conclusion of Glu-510, Tyr-511, and Asp-518 as the docking determinants of Src.

Characterization of Tyr-511 and Asp-518—To determine the structural attributes of Src Tyr-511 that were important for Csk recognition, it was mutated into several other residues sharing various structural similarity to Tyr (Fig. 5*C*). Both the Phe and the Trp mutants retained ~80% of WT kdSrc activity as a substrate for Csk, whereas Ala, Ile, or Leu mutants retained 20% or less ability to be phosphorylated by Csk. This result indicated that 1) the hydroxyl group of Tyr is not important

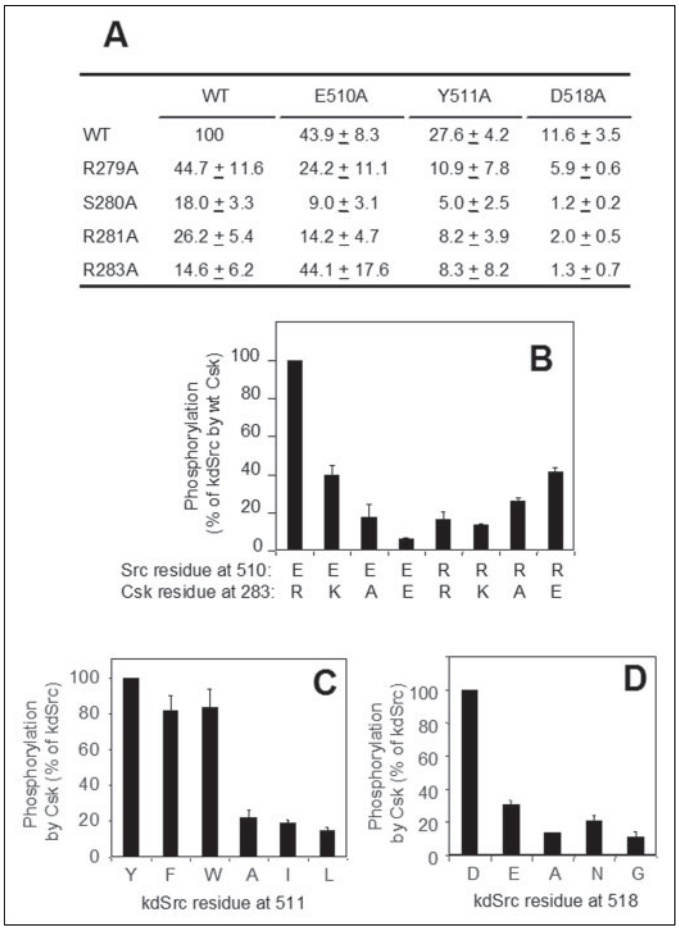


FIGURE 5. **Characterization of Src residues Glu-510, Tyr-511, and Asp-518 in Src-Csk recognition.** *A*, phosphorylation of a panel of kdSrc mutants by Csk mutants in the substrate-docking site. *B*, phosphorylation of kdSrc and kdSrc mutant E510A by Csk and Csk variants at position 283. *C*, phosphorylation of kdSrc variants at position 511 by Csk. *D*, phosphorylation of kdSrc variants at position 518 by Csk.

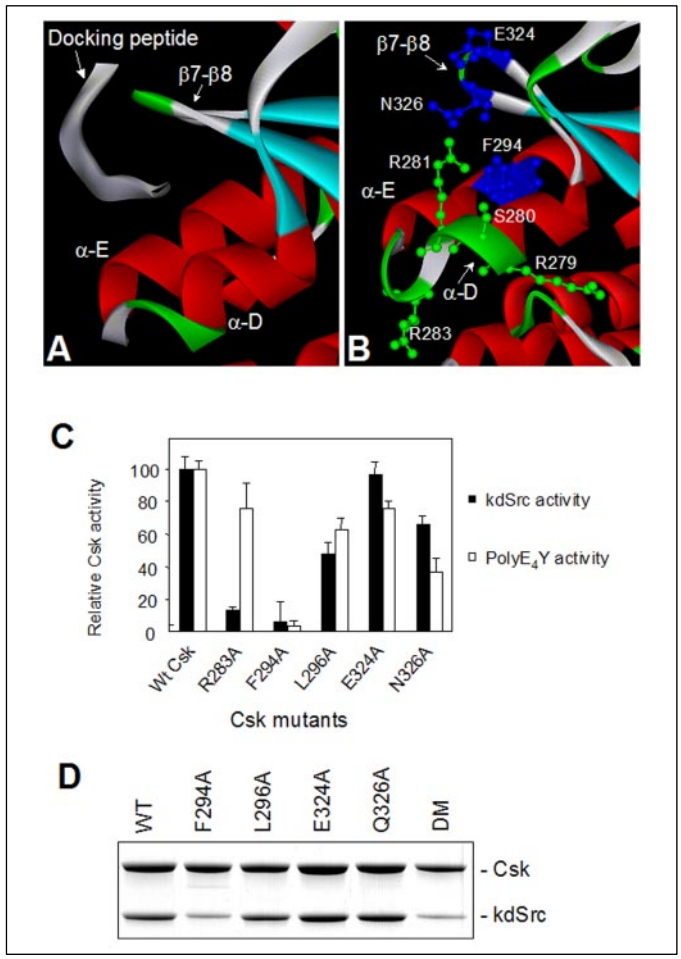


FIGURE 6. **Characterization of Csk β7-β8 reverse turn and α-helix E as parts of the substrate-docking site.** *A*, structure of the substrate-docking site of the p38 MAP kinase. The relevant substructures are labeled and colored by secondary type (α-helix in *red*, β-sheets in *light blue*, turn in *green*, and random coil in *gray*). This structure is based on the crystal structure of 1LEW in the Protein Data Bank. *B*, the substructures of Csk corresponding to the p38 MAP kinase substrate-docking site. The residues that have been previously shown to be part of the substrate-docking site of Csk are shown in *green*. The residues that correspond to p38 MAP substrate-docking residues in the β7-β8 reverse turn and the α-helix E are shown in *blue*. Leu-296 is hidden behind the α-helix E. *C*, phosphorylation of kdSrc and polyE$_4$Y by Csk mutants in the α-helix D (R283A), α-helix E (F294A and L296A), and β7-β8 reverse turn (E324A and N326A). *D*, binding of Csk mutants to kdSrc. The GST pull-down binding assay is described under "Materials and Methods." *DM*, a Csk double mutant containing the mutations of R281A and R283A, was used as a negative control.

and 2) hydrophobicity is not sufficient for the function of Tyr-511. Combination of these two considerations suggests that the aromatic ring is crucial in Src recognition by Csk. One type of interaction that is consistent with all these features is a cation-π interaction, in which the π electron cloud of the aromatic ring is attracted to the positively charged residues of Arg or Lys (20, 21). This possibility is further strengthened by the fact that the Csk substrate-docking site is dominated by 3 Arg residues. Definitive identification of the interacting partner for Tyr-511 awaits further investigation.

To determine the structural attributes of Asp-518 important for its function, it was mutated to Glu, Asn, and Gly. D518E mutant retained the highest activity, at about 30% of that of kdSrc as a substrate for Csk, whereas the other mutants retained 10–20% of kdSrc activity (Fig. 5*D*). This observation suggests that a negative charge is likely important but not sufficient for the function of this residue.

The Substrate-docking Sites Are Different in Csk and MAP Kinases—The MAP kinases, a family of Ser kinases, also use a substrate-docking site for substrate recognition (22). The substrate-docking site in p38 MAP kinase was revealed by the crystal structure of a complex of the kinase with a docking peptide from its substrate (23). The docking site in p38 MAP kinase is composed of three substructures in the peptide-binding lobe, the α-helixes D and E and the β7-β8 reverse turn (Fig. 6*A*). These three substructures form a clamp that holds the docking peptide of the substrate. Our previous study identified several residues in the α-helix D as crucial determinants of the Csk substrate-docking site (Fig. 6*B*) (15). Intrigued by this similarity, we determined whether the α-helix E and the β7-β8 reverse turn were also part of the substrate-docking site of Csk. Two residues each from the β7-β8 reverse turn and from the α-helix E that would form the clamp structure were mutated to Ala, and their effects on Csk phosphorylation of kdSrc and the artificial substrate polyE$_4$Y were determined (Fig. 6*C*). In a previous study, we demonstrated that mutations in the substrate-docking site dramatically decreased Csk activity toward kdSrc but had little effect on Csk activity toward the artificial substrate (as shown in R283A in Fig. 6*C*) (15). In contrast to mutants involving docking residues, such as R283A, the four mutants involving residues from the α-helix E and the β7-β8 reverse turn all displayed similar relative activity toward kdSrc and polyE$_4$Y, indicating that these residues were not specifically important for Csk recognition of kdSrc. Three of the four mutants also displayed similar a ability to complex with kdSrc (Fig. 6*D*), indicating that these residues were not important for Csk interaction with kdSrc. The only exception

was Phe-294 in the α-helix E, the mutation of which to Ala significantly decreased Csk binding to kdSrc. Since this mutant was virtually inactive as a kinase toward kdSrc or polyE$_4$Y, the effect was likely not specific for Src recognition. Overall, these results indicated that the α-helix E and the β7-β8 reverse turn are not part of the substrate-docking site in Csk. Thus, the substrate-docking sites in Csk and the MAP kinases have some overlap but are not identical. The comparison of clamp-shaped docking site in p38 MAP kinase *versus* the surface-like docking site in Csk would suggest dramatic differences in the mode of substrate binding for these two family of kinases.

DISCUSSION

Substrate specificity is one of the most fundamental properties that determine the function of a protein tyrosine kinase. Elucidating the mechanisms of PTK substrate recognition and specificity will provide a key mechanistic insight into cellular signaling mediated by this family of enzymes. In addition, many protein tyrosine kinases are targets for drug discovery. Understanding PTK substrate recognition and specificity may also have a profound impact on kinase-based drug discovery. Despite such central importance, the mechanisms by which protein tyrosine kinases recognize protein substrates are poorly understood.

Csk Serves as a Model in Understanding PTK Substrate Recognition—Csk family kinases, Csk and Csk-homologous kinase, exclusively phosphorylate Src family kinases on a C-terminal tail Tyr and down-regulate their activities. Numerous studies have demonstrated that Csk recognition of Src family kinases is not solely based on recognition of the local amino acid sequence of the C-terminal tail. Thus, remote docking interactions are likely a key part of the recognition mechanism. Our previous studies determined that Csk recognition of Src was not dependent on the Csk SH2 or SH3 domains (24); rather, Csk contained a substrate-docking site located in the peptide-binding lobe, centered on the α-helix D (15). In the current study, we identified several residues in the α-helix J of Src as the docking determinants recognized by the Csk substrate-docking site. Mutation of these residues to Ala reduced Src phosphorylation by Csk more than 98%. Using another Src family kinase, Yes, we demonstrated that these mutations do not alter the Yes kinase activity but specifically abolish its phosphorylation and regulation by Csk. Coordinated mutations in the Csk substrate-docking site and the Src docking determinants demonstrated that Glu-510 of Src interacts with Arg-283 of Csk, likely by a salt bridge, in the Csk-Src recognition. These results provided mechanistic insights into how Csk specifically and exclusively recognizes Src family kinases and phosphorylates them on a C-terminal Tyr residue.

The determination of the substrate-docking site on Csk (15) and the docking determinants of Src in this report compellingly demonstrate a docking-based recognition between the catalytic domain of Csk and its physiological substrates, the Src family kinases. Based on these studies, the Csk-Src recognition appears to consist of two components. First, docking interactions between the Csk substrate-docking site and the Src determinants on α-helix J are critical for the recognition. We propose that such docking interactions bring Csk and Src in an arrangement so that the C-terminal tail of Src is located near the active site of Csk. Second, the C-terminal tail of Src interacts with Csk to present Tyr-527 to Csk active site for phosphorylation. The detailed interactions await further structural determination.

Structural versus Kinetic Substrate Recognition—In this current study, the focus of our investigation is to identify physical interactions between Csk and Src that enable the specific Csk-Src recognition. However, it is important to note that the recognition of protein substrates by a kinase, although initially dependent on physical interactions between the kinase and the substrate, is also influenced by other factors. Lieser *et al.* (12) report that the kinetic affinity between Csk and Src measured by the K_m is strongly enhanced by a fast phosphoryl transfer step. It is proposed that the phosphoryl transfer step functions as a chemical clamp facilitating substrate recognition. Thus, any factors that affect the catalytic activity of Csk could potentially affect Csk-Src recognition, even if such factors do not directly affect the physical interaction between Csk and Src. For example, Csk catalytic activity is positively regulated by the presence of its SH3 and SH2 domains (24) and the SH2 domain ligand (26). Such regulations could potentially affect the substrate recognition as well. In this regard, the catalytic clamp mechanism likely affects the affinity of the kinase for protein substrates in a generic manner, enhancing Csk substrate recognition in general, whereas the physical interactions between Csk and the substrates determine the specificity of the recognition. The contribution of multiple factors to the K_m may also be responsible for the complicated k_{cat}-K_m patterns observed for Csk phosphorylation of various kdSrc mutants reported in Fig. 3.

Comparison with Substrate Recognition by Protein Ser/Thr Kinases—For protein kinases that phosphorylate their substrates on Ser/Thr residues, there are two main mechanisms for recognizing protein substrates. The first is based on interactions with the local amino acid sequence surrounding the phosphorylation site. Each kinase with this mechanism would have a consensus substrate sequence. This mechanism is best represented by the cAMP-dependent protein kinase, which phosphorylates a Ser residue preceded by 2 positively charged residues at the −3 and −2 positions (27). The structural context of the consensus sequence may hinder, but does not significantly enhance, the recognition (28). The second type of mechanism relies on remote docking interactions between the substrate and the kinase. For this mechanism, a substrate would contain two structural motifs to be recognized by two corresponding binding sites on the kinase (29). This mechanism is best represented by the MAP kinases. A MAP kinase contains a substrate-docking site that binds to the docking determinants distal to the phosphorylation site (30, 31). In addition, residues near the phosphorylation site also interact with the active site of the kinase.

Recently, the structure of p38 MAP kinase complexed with a docking determinant peptide is determined (23). The structure reveals that the docking site is composed of three structural motifs, the β7-β8 reverse turn and the α-helixes D and E. Together they form a clamp-like structure, holding the docking peptide. However, our data clearly demonstrated that the substrate-docking sites in Csk and p38 MAP kinase are distinct from each other, although they share some similarity in the secondary structures used.

Docking-based Substrate Recognition May Be Used by Other PTKs—The mechanisms for PTK substrate recognition and specificity are poorly understood in general. For virtually all PTKs, peptides are extremely inefficient substrates (13, 32), suggesting that some form of remote interaction may be responsible for substrate recognition. One line of emerging evidence suggests that regulatory domains, such as the SH3 or SH2 domains, may help with substrate recruiting for cytosolic PTKs (3). For example, Src phosphorylates STAT3 (33), p130Cas (34), and SAM68 (35). These substrates contain a Pro-rich motif recognized by the Src SH3 domain. Furthermore, the addition of the SH2 domain recognition motifs (phospho-Tyr-containing peptide sequences) enhances the phosphorylation of peptide substrates by SH2 domain-containing protein tyrosine kinases (25). These data implicate the SH3 and SH2 domains in helping recruit substrates for PTKs. However, it is not clear how such remote interdomain cooperation achieves sufficient specificity and precision and how the catalytic domain accurately recognizes the Tyr to be phosphorylated. Further-

more, receptor type protein tyrosine kinases do not contain regulatory domains like the SH3 and SH2 domains. They would need to use either the catalytic domain or the C-terminal tail for substrate recognition and ensure substrate specificity. Whether docking interactions in the catalytic domain similar to Csk-Src recognition are part of the substrate recognition mechanisms for PTKs in general awaits further investigation.

Acknowledgments—We thank P. S. Cohen for critically reading the manuscript, P. A. Cole for providing pREP4groESL plasmid, and R. Jove for providing pMcSrc Met-295 plasmid. DNA sequencing was performed at University of Rhode Island Genomics and Sequencing Center.

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Docking-based Substrate Recognition by the Catalytic Domain of a Protein Tyrosine Kinase, C-terminal Src Kinase (Csk)

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J. Biol. Chem. 2006, 281:8183-8189.
doi: 10.1074/jbc.M508120200 originally published online January 26, 2006
