Probing the Communication between the Regulatory and Catalytic Domains of a Protein Tyrosine Kinase, Csk†

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Received August 27, 2004; Revised Manuscript Received November 8, 2004

ABSTRACT: Protein tyrosine kinases (PTKs) are important regulators of mammalian cell function and their own activities are tightly regulated. Underlying their tight regulation, all PTKs contain multiple regulatory domains in addition to a catalytic domain. C-terminal Src kinase (Csk) contains a catalytic domain and a regulatory region, consisting of an SH3 and an SH2 domain. In this study, we probed the communication between the regulatory and catalytic domains of Csk. First, kinetic characterization of SH3 and SH2 domain deletion mutants demonstrated that the SH3 and SH2 domains were crucial in maintaining the full activity of Csk, but were not directly involved in Csk recognition of its physiological substrate, Src. Second, highly conserved Trp188, corresponding to a key residue in domain−domain communication in other PTKs, was found to be important for maintaining the active structure of Csk by the presence of the regulatory region, but not required for Csk activation triggered by a phosphopeptide binding to the SH2 domain. Third, structural alignment indicated that the presence of the regulatory domains modulated the conformation of multiple substructures in the catalytic domain, some directly and others remotely. Mutagenic and kinetic studies supported this assignment. This report extended previous studies of Csk domain−domain communication, and provided a foundation for further detailed investigation of this communication.

Protein tyrosine kinases (PTK)[1] are a large family of enzymes that transfer the γ-phosphate of ATP to Tyr hydroxyl groups in proteins. By phosphorylation, PTKs regulate the conformation and function of their protein substrates. This covalent modification is a fundamental mechanism of signal transduction in mammalian cells. For fidelity and specificity of signal transduction, it is crucial that the activity of each PTK responds to appropriate regulatory signals and phosphorylates specific protein substrates. All PTKs have evolved elaborate mechanisms for regulating their activities. Crucial for their regulation, all PTKs contain multiple regulatory domains in addition to a catalytic domain, and the regulatory domains help a PTK respond to upstream regulatory signals and control the kinase activity. The communication between the regulatory and catalytic domains provides a foundation for PTK regulation.

C-terminal Src kinase (Csk) is a cytoplasmic PTK (*1*) that phosphorylates Src family kinases (SFKs) and down-regulates their kinase activity (*1*, *2*). Csk contains an SH3 and an SH2 domain at the N-terminus and a catalytic domain at the C-terminus (*3*). Unlike SFKs, in which the SH2 domain at the N-terminal portion binds to a phosphotyrosine residue located in the C-terminal tail (*4*, *5*), there are no binding ligands in the C-terminal portion of Csk for the SH3 or SH2 domain. Thus, how Csk SH3 and SH2 domains communicate with the catalytic domain is an intriguing question. Determination of the crystal structure of a full-length Csk provides a structural framework for addressing this question (*6*). In Csk structure (Figure 1A), the SH3 and SH2 domains are located on top of the catalytic domain, and form several direct interactions with the ATP-binding lobe. This domain arrangement is in direct contrast with the SFKs (Figure 1B), which also contain an SH3, SH2, and a catalytic domain in a similar primary structure arrangement, but the three domains are arranged in a different configuration in tertiary structure.

Numerous studies have suggested the importance of the SH3 and SH2 domains in regulating the function of Csk. First, the SH2 domain is proposed to recruit Tyr-phosphorylated proteins as substrates (*7*). Second, the SH2 domain binds to a number of phosphotyrosine-containing proteins (*8*−*11*), and such binding activates Csk activity (*12*). Third, Csk binding to Csk-binding protein (CBP) recruits Csk from the cytosol to the lipid rafts, where its substrates, SFKs, are located (*8*). Finally, deletion and site-specific mutagenesis studies indicate that the SH3 and SH2 domains are important for positively modulating the kinase activity, since the catalytic domain alone retains only approximately 1% of the wild-type (wt) activity (*13*, *14*). This conclusion is further supported by reconstitution studies in which incubation of the SH3 domain or SH3−SH2 fragment with the Csk catalytic domain activates the kinase activity of the catalytic domain (*14*). These studies demonstrate at least two

† This work was supported by grants from NIH (1 P20 RR16457) and the American Cancer Society (RSG-04-247-01-CDD).

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[1] Abbreviations: Csk, C-terminal Src kinase; ΔSH2, Csk mutant in which SH2 domain (Trp82 through Tyr156) is deleted; ΔSH3, Csk mutant in which SH3 domain (Met1 through Pro81) is deleted; GST, glutathione S-transferase; kdSrc, kinase-defective Src; PTK, protein tyrosine kinase; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFKs, Src family kinases; wt, wild type.

10.1021/bi048142j CCC: $30.25
Published on Web 01/14/2005

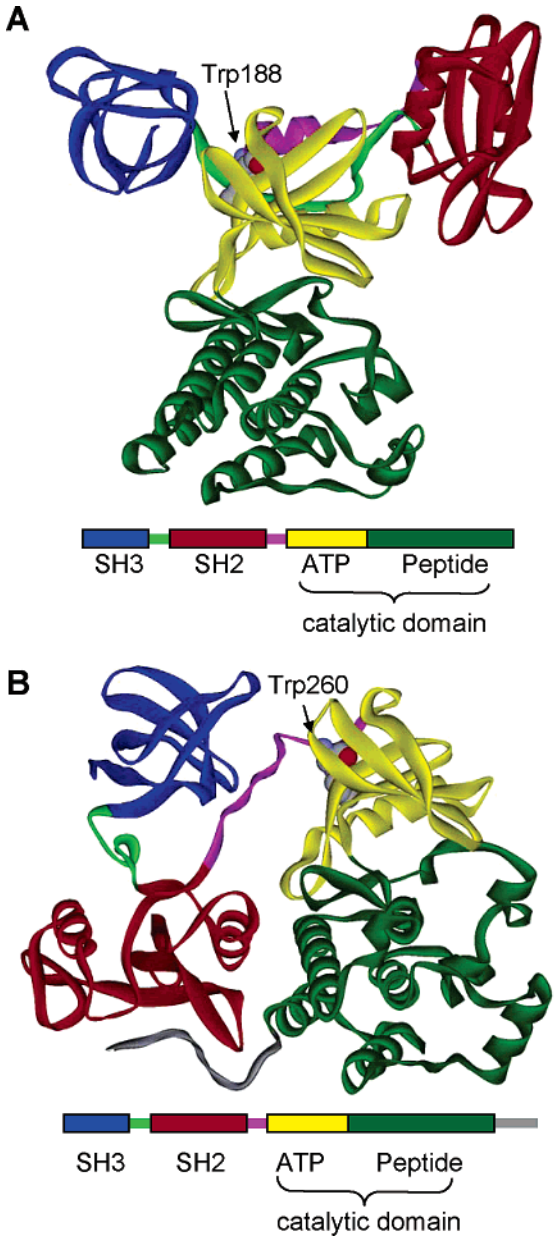

FIGURE 1: Structural comparison between Csk (A, PDB code 1K9A) and Hck (B, PDB code 1QCF). The domains and linker regions are similarly color-coded. Although Hck contains a unique region and an myristoylation motif at the N-terminus; these regions are deleted in the crystallized Hck and not shown in the figure. The residues Trp188 in Csk and Trp260 in Hck are shown in space-filling mode. Residues in each region of Hck are as follows: SH3, Ser80−Arg139; SH3-2 linker, Val140−Trp148; SH2, Ser149−Met246; SH2-kinase linker, Ser247−Ile262; ATP-binding lobe, Pro263−Ala342; peptide binding lobe, Lys343−Asp518; C-terminal tail, Phe519−Pro531. Residues in each region of Csk are: SH3, Ala6−Val65; SH3−2 linker, Gln66−Phe83; SH2, His84−Lys169; SH2-kinase linker, Pro170−Leu190; ATP-binding lobe, Asn191−Ala270; the peptide-binding lobe, Lys271−Leu450.

modes of communication between the regulatory and catalytic domains in Csk. First, the presence of the SH3 and SH2 domains is crucial in maintaining the active conformation of Csk. Second, binding of the SH2 domain to a phosphotyrosine-containing protein further activates Csk activity.

Communication between the regulatory and catalytic domains has been extensively studied in other PTKs, such as SFKs. In SFKs, the SH3 and SH2 domains suppress the kinase activity of the catalytic domain. The communication between the regulatory and catalytic domains appears to be mediated by interactions of a highly conserved Trp residue, Trp260 in Hck (*15*, *16*). Crystal structure of Hck reveals that the interaction of Trp260 with a number of residues in the ATP-binding lobe and SH2-catalytic domain linker (*5*, *16*, *17*) helps stabilize its inactive conformation. Mutation of Trp260 to Ala activates Hck activity by abolishing the suppression of the kinase activity by the SH3 and SH2 domains. Furthermore, wt Hck can be activated by SH3 and SH2 domain ligands, but the communication is abolished by Trp260Ala mutation (*16*). In contrast to the situation in Hck, the equivalent residue, Trp342, in c-Raf plays a distinctively different role, as mutation of this residue to Ala abolishes c-Raf activity (*18*). Both studies suggest that the Trp residue preceding the catalytic domain of a kinase is a key mediator of the communication between the regulatory and catalytic domains. The corresponding residue in Csk is Trp188.

In the current study, we examined the role of the SH3 and SH2 domains in Csk substrate recruiting, evaluated the contribution of Trp188 to Csk domain−domain communication, and located and characterized substructures in the catalytic domain that were regulated by this communication. These studies provided further evidence for the crucial role of the conserved Trp residue in protein kinase regulation, and a foundation for further elucidation of the detailed mechanisms of domain−domain communication in Csk.

EXPERIMENTAL PROCEDURES

Generation of Mutants. Glutathione S transferase (GST)−Csk fusion proteins were generated and purified as previously described (*13*). Csk point mutants were generated using QuikChange (Stratagene) and were confirmed by DNA sequencing. Kinase-defective Src (kdSrc) was produced as described previously (*19*).

Enzyme Purification. Bacteria harboring appropriate plasmids were cultured in LB medium at 37 °C with shaking at 250 rpm overnight. The overnight culture was then mixed with an equal volume of fresh LB medium, cooled to about 20 °C. Isopropyl-β-d-thiogalactopyranoside (0.2 mM) was added to the culture to induce recombinant protein expression at 20 °C for 4−6 h. The GST fusion proteins were purified by glutathione affinity chromatography as previously described (*20*, *21*). The purified enzymes were desalted on a Sephadex G25 column equilibrated with the storage buffer (100 mM Tris-Cl, pH 8.0, and 0.1% β-mercaptoethanol). Glycerol was added to the purified fractions to 30% and the enzymes were stored at −20 °C. Protein concentration was determined by the Bradford assay and the purity of purified proteins was assessed by SDS−PAGE with coomassie blue staining.

Kinase Activity Assay. For assaying PTK activity, phosphorylation of polyE$_4$Y (1 mg mL^{-1}) and kdSrc (10 μM) was measured using the acid precipitation assay as previously described (*19*, *22*). KdSrc was derived by introducing a Lys295Met mutation into chicken Src, and expressed as described previously (*23*, *24*). Reaction time for the assays was 10 min. When K_m and k_{cat} were determined with regard to one substrate, the kinase activity was determined at various concentrations of that substrate in the range of 20−200 μg mL^{-1} for polyE$_4$Y, and 1 to 10 μM for kdSrc. The k_{cat} and

Table 1: Catalytic Parameters of wt and Mutant Csk Enzymes

Csk	kdSrc as the substrate			polyE4Y as the substrate		
	k_{cat}	K_m	relative k_{cat}/K_m	k_{cat}	K_m	relative k_{cat}/K_m
wt	177 ± 67	15 ± 7.8	1	85 ± 23	150 ± 31	1
DSH3	30 ± 17	27 ± 12	0.094	1.8 ± 0.7	328 ± 97	0.01
DSH2	15 ± 14	42 ± 24	0.03	0.2 ± 0.02	164 ± 27	0.002
W188 F	33 ± 2	17 ± 1	0.16	3.1 ± 0.2	299 ± 21	0.02
W188 A	6 ± 0.1	68 ± 1	0.008	0.2 ± 0.03	204 ± 21	0.0017
K361 G/ K362 A	16 ± 5	32 ± 15	0.042	1.6 ± 0.8	659 ± 216	0.004

K_m values were determined by Lineweaver−Burk plot with linear regression using Microsoft Excel. All steady-state kinetic assays were performed in duplicate, and repeated at least three times. Standard errors were calculated from three independent experiments.

Csk Activation by CBP-Based Phosphopeptide. Activation of Csk by a CBP-based phosphopeptide, AMpYSSV, was determined by assaying Csk activity toward polyE$_4$Y in the presence and absence of a series of concentrations of the peptide. The enzyme concentration in the assay (in the range of 1 to 100 nM) was chosen to produce a good signal-to-noise ratio, but still in the linear range for each enzyme variant. The concentration of polyE$_4$Y used in the activation assays was 1 mg mL^{-1}, which was severalfold higher than the K_m for most Csk mutants. Dose-dependent activation data was fitted into the following equation to obtain maximal activation and K_d: relative activation = maximal activation x [phosphopeptide]/(K_d + [phosphopeptide]). Curve fitting was performed with Lab Fit (*25*).

RESULTS

The SH3 and SH2 Domains Are Not Involved in Csk Recognition of Physiological Substrate, Src. The SH3 and/or SH2 domains are involved in substrate recruitment in several PTK families (*26*), and the SH2 domain of Csk has been suggested to be involved in binding to its physiological substrates (*7*). This possibility has not been examined kinetically with a physiological substrate. To determine if the SH3 and SH2 domains contribute to Csk recognition of physiological substrate Src, two Csk deletion mutants lacking the SH3 or the SH2 domain were characterized (Table 1). The activities of these mutants were analyzed with two substrates: kdSrc and polyE$_4$Y. KdSrc is defective in kinase activity, but retains the specificity and efficiency as a physiological Csk substrate (*22*, *23*). PolyE$_4$Y is a random copolymer of Glu and Tyr in the ratio of 4 to 1. It does not contain defined phosphorylation sites or higher order structures. It is an excellent substrate for all PTKs so far tested. Since various tested PTKs contain different regulatory domains, or no regulatory domain in some cases, polyE$_4$Y phosphorylation does not rely on interactions with any regulatory domains (*22*). Thus, a comparison of relative effects of a mutation on kdSrc and polyE$_4$Y phosphorylation will reveal if the mutated structure is specifically important for phosphorylation of the physiological substrate, Src. Deletion of the SH3 or SH2 domain significantly decreased Csk activity toward both kdSrc and polyE$_4$Y, but the deletions did not affect kdSrc phosphorylation more significantly than polyE$_4$Y phosphorylation. This suggested that the SH3 and SH2 domains were not part of the machinery important for Csk recognition of Src. In contrast, both deletion mutants displayed higher relative k_{cat}/K_m ratio toward kdSrc than toward polyE$_4$Y, indicating that the presence of SH3 and SH2 domains is more important for the phosphorylation of polyE$_4$Y than kdSrc.

Characterization of Trp188 as a Key Residue for Csk Domain−Domain Communication. Csk and Src family kinases have a similar overall primary structure organization (Figure 1). They all contain an SH3, an SH2, and a catalytic domain. The catalytic domains of both families, as well as those of the overwhelming majority of PTKs, follow a highly conserved signature Trp residue, Trp260 in Hck, and Trp188 in Csk. Trp260 is not required for the catalytic activity of Hck, but mediates communication between the SH3/SH2 domains and the catalytic domain (*16*). In full-length Csk, Trp188 is located in a pocket created by Arg68 (from SH3 domain−SH2 linker), Phe183 and Ser186 (from the SH2-catalytic domain linker), Thr241 (from α-helix C), and four other residues from the ATP-binding lobe (Gln250, Leu251, Leu252, and Val254) (Figure 2A). This structural arrangement supports the possibility that Trp188 may also be a key residue in mediating domain−domain communication in Csk.

To test the role of Trp188 in Csk domain−domain communication, it was mutated to Ala and Phe. Steady-state kinetic analysis (Table 1) revealed that both mutants displayed significantly reduced activity toward both kdSrc and polyE$_4$Y, mostly due to decreases in k_{cat}. However, mutation to Ala had a 10−20-fold more severe effect on Csk activity than Phe mutation, indicating that Phe could partially fulfill the function of Trp188. The inactivating effects are the opposite of the result in Hck, where the mutation of Trp260 to Ala activates Hck. Assuming Trp188 is not directly involved in Csk catalysis, as demonstrated in Hck, this result suggests that Trp188 is a key residue for the regulatory domains to positively modulate the kinase activity in Csk. It was noted that Trp188 mutations affected polyE$_4$Y phosphorylation more severely than kdSrc phosphorylation. The substrate-dependent effects were similar to those of the SH3 and SH2 deletions.

Mutations of Trp188 Do Not Disrupt SH2-Mediated Activation of Csk by a Phosphotyrosine-Containing Peptide. Two modes of communication between the regulatory domains and the catalytic domain have been noted in Csk: positive modulation of the activity by the presence of the regulatory domains, and further activation of the activity by the SH2 domain binding to a phosphopeptide. The second mode of activation is presumably due to a conformational change in the SH2 domain caused by ligand binding, which can be communicated to the catalytic domain (*12*). The experiment in the previous section suggested that Trp188 was important for the first mode of activation, and we then determined if Trp188 was also important for the second. This

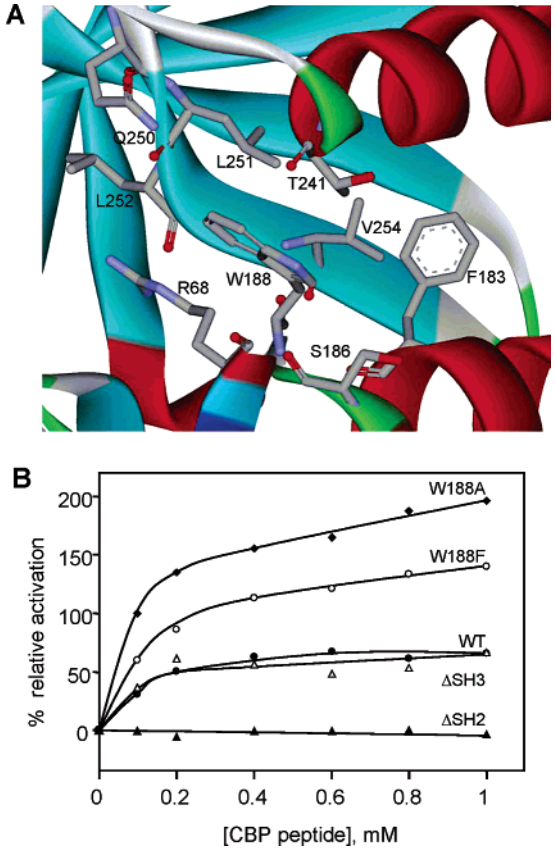

FIGURE 2: Tertiary environment of Trp188 in Csk (A) and activation of wt and Trp188 mutants by a phosphopeptide based on Csk-binding protein (B). The residues surrounding Trp188 are shown and labeled. The phosphopeptide sequence used was AMpYSSV. Assay conditions are given in Experimental Procedures. The data in this graph was fitted to a hyperbolic binding equation to obtain the activation parameters reported in Table 2.

possibility was tested by comparing the effects of an SH2 domain ligand (AMpYSSV) on the kinase activity of wt and Trp188 mutants (Figure 2B). This phosphopeptide mimicked the amino acid sequence surrounding pTyr314 of CBP, and was previously shown to bind to Csk SH2 domain and activate Csk (*12*). Consistent with the previous report, the phosphopeptide activated wt Csk in a concentration-dependent manner, reaching approximately 70% activation by 1 mM phosphopeptide. As a control, the phosphopeptide did not activate ΔSH2, indicating that the activation of the wt Csk was through the phosphopeptide binding to Csk SH2 domain and not to the catalytic domain. Unexpectedly, the relative activation of the Trp mutants by the phosphopeptide was significantly greater than for the wt Csk, with activation of Trp188Ala reaching approximately 200% by 1 mM phosphopeptide. Fitting the activation data into a hyperbolic binding equation revealed that the phosphopeptide bound to the two mutants and wt Csk with similar affinity, but maximal activation was 160% for Trp188Phe and 210% for Trp188Ala, respectively, much higher than that for wt Csk at 75% (Table 2). These binding parameters indicated that the mutations did not directly affect Csk SH2 domain binding to the phosphopeptide, but made the catalytic domain more responsive to conformational changes in the SH2 domain as a result of ligand binding. The response of ΔSH3 to the phosphopeptide was similar to that of wt Csk, indicating that the second mode of communication between the SH2 domain

Table 2: Parameters of AMpYSSV Activation of wt and Trp188 Mutants of Csk[a]

Csk	maximal relative activation (%)	K_d (μM)
wt	75 ± 4	117 ± 29
W188F	160 ± 3	172 ± 10
W188A	210 ± 8	119 ± 18

[a] The activation parameters were obtained by fitting the activation data reported in Figure 2B into a hyperbolic binding curve as described in Experimental Procedures.

and the catalytic domain did not require the presence of the SH3 domain. Together, these results demonstrated that Trp188 was crucial for maintaining the active conformation of Csk by the SH3 and SH2 domains (mode 1), but unimportant for the SH2-mediated activation of Csk by the phosphotyrosine-containing peptide (mode 2). These results indicated that there were at least two mechanisms of interdomain communication in Csk: one was mediated by the interactions of Trp188, and the other directly between the SH2 domain and the catalytic domain, likely mediated by direct interactions between these two domains.

Comparison of the Crystal Structures of Full-Length and Catalytic Domain of Csk Suggests Regulated Substructures in the Catalytic Domain. An intriguing observation from the above studies was that mutations in the regulatory region inactivated Csk in a substrate-dependent manner, with each mutation affecting polyE_4Y phosphorylation more severely than kdSrc phosphorylation (Table 1). Since it is unlikely that the regulatory domains are directly binding polyE_4Y, the substrate-dependent effects suggest that the SH3 and SH2 domains regulate the conformation of certain substructures in the catalytic domain that are more important for phosphorylation of polyE_4Y than kdSrc. To further investigate this possibility, we compared the structures of the catalytic domain in two crystallized Csk constructs: one construct contained only the catalytic domain (Protein Data Bank ID 1BYG) (*27*), and the other a full-length Csk (Protein Data Bank ID 1K9A, chain A) (*6*). The catalytic domain without the regulatory domains represents the conformation of an unregulated catalytic domain. The catalytic domain in full length Csk, in contrast, would represent a catalytic domain under the regulation of the SH3 and SH2 domains. Therefore, the substructures whose conformations are different in these two constructs are likely under the regulation of the regulatory domains. We are aware of two potential caveats for the strategy. First, in both crystal structures, part of the activation loop (Ser341 through Gly346 for 1K9A, and Thr336 though Lys347 for 1BYG) was missing, suggesting that this region was flexible and lacks a defined conformation. Thus the activation loop was not considered further. Second, 1BYG-was crystallized with an ATP-competitive inhibitor, staurosporine, which may also influence the structure of the catalytic domain. The structural comparison was performed using K2 structural alignment program (*28*). This program first aligns the proteins' secondary structure elements and then extends the alignment to include any equivalent residues found in loops and turns. Alignment of residues is based on the position of the α-carbon. The alignment of secondary structure elements is based on an optimization of a scoring function based on intramolecular distance matrix (*28*). For residues not located in secondary structure elements, the

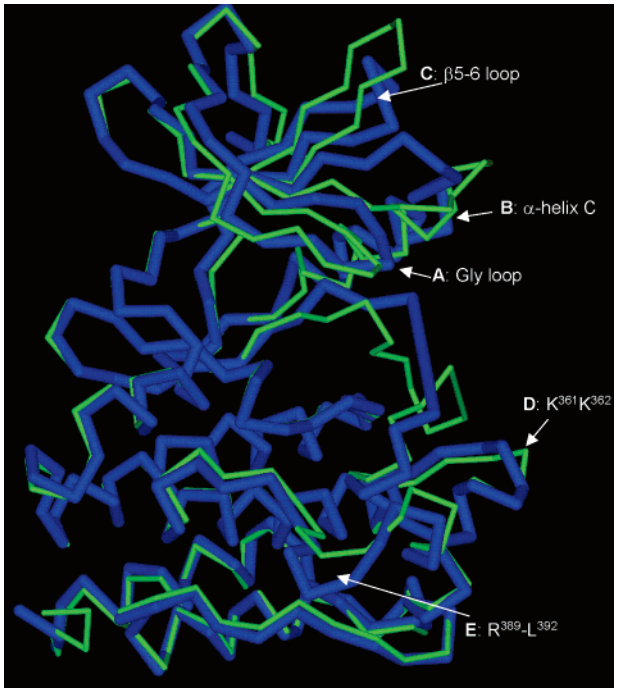

FIGURE 3: Structure alignment in the catalytic domain of full-length Csk and a Csk mutant without the SH3 and SH2 domains. The thick blue line is the catalytic domain without the regulatory domains (PDB code 1BYG), and the thin green line is the catalytic domain of a full length Csk (PDB code 1K9A). The regions that are different in the two crystal structures are indicated.

α-carbons of pairing residues need to be within 5Å of each other to be considered aligned.

The alignment identified five substructures in the catalytic domain that displayed dramatically different conformations (Figure 3): A, Gly204−Phe206; B, Ile224−Met240; C, Glu257−Gly261; D, Lys361−Lys362; and E, Arg389−Leu392. Regions A−C are located in the ATP-binding lobe, while D and E are located in the peptide-binding lobe. Region A is in the Gly-rich loop (GXGXXG loop) conserved in all protein kinases, and region B includes α-helix C and a loop immediately N-terminal to α-helix C. Both the Gly-rich loop (*29*, *30*) and α-helix C (*31*) are critical for the activity of various protein kinases, and the conformational differences would explain the differences in activity between the full-length and the regulatory domain mutants of Csk (*32*). Region C is a short loop between β4 and β5 in the ATP-binding lobe. Both region C and part of the region B (the loop before α-helix C) directly interact with the SH2 domain in full-length Csk. This structural alignment has two implications. First, since the two loops in B and C directly interact with the SH2 domain and their conformations are different in the presence or absence of the SH2 domain, they likely mediate the direct communication between the SH2 domain and the catalytic domain. Second, since regions A−C are located in the ATP-binding lobe, their conformational changes are unlikely to mediate the substrate-dependent effects (kdSrc vs polyE$_4$Y) of regulatory domain mutations. Instead, conformational changes in the peptide-binding lobe are likely responsible for the substrate-dependent effects. The first implication is currently being investigated and will be reported elsewhere. We next focused on regions D and E to determine if their conformational changes could mediate the substrate-dependent effects of mutations in the regulatory domains.

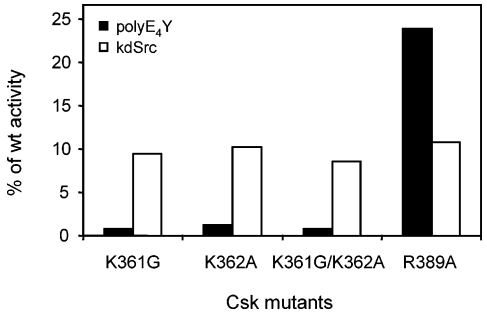

FIGURE 4: Effects of mutations in regions D and E on Csk activity. Csk activity was assayed with polyE$_4$Y (1 mg mL^{-1}) and kdSrc (10 μM) following methods described in Experimental Procedures. Assays were performed in duplicates and repeated at least twice with comparable results. The reported values were the average of two independent assays.

Mutations in Region D, but Not in E, Result in More Severe Loss of Activity toward PolyE$_4$Y than toward kdSrc. Region D is a loop harboring two Lys residues, Lys361 and Lys362. This loop is located next to the activation loop, and immediately outside of the active site cleft in Csk tertiary structure. To assess the role of this substructure, Csk mutants containing single (Lys361Gly or Lys362Ala) or double (Lys361Gly/Lys362Ala) point mutations were generated. Region E is a loop at the base of the peptide-binding lobe around Arg389. To test the role of this loop, Arg389 was mutated to Ala. Relative activities of these mutants are presented in Figure 4. Mutants involving Lys361 and Lys362 displayed 0.8 to 1.2% of wt activity toward polyE$_4$Y but about 9wt % activity toward kdSrc, suggesting that this substructure is much more important for polyE$_4$Y phosphorylation than kdSrc phosphorylation. Mutation of either one or both Lys residues had similar effects on Csk activity, suggesting that both Lys residues were required for full activity. The mutation of Arg389Ala affected kdSrc phosphorylation slightly more severely than polyE$_4$Y phosphorylation, opposite of the effects of the regulatory domain mutations. This observation was inconsistent with the possibility that Arg389 region mediated the polyE$_4$Y-specific effects by the regulatory domains. Steady-state kinetics were performed on the double mutant of Lys361Gly/Lys362Ala using both polyE$_4$Y and kdSrc as the variable substrate. The loss of activity in both cases was due to a combination of decreases in k_{cat} and increases in K_m (Table 1). The relative k_{cat}/K_m ratio again showed about a 10-fold difference, with the mutation more severely affecting the activity toward polyE$_4$Y. Overall the above data indicated that mutations in region D had similar substrate-dependent effects on Csk activity as mutations in the regulatory domains. These data support the proposition that substrate-dependent effects of the regulatory domain mutations are mediated by conformational changes in region D.

DISCUSSION

Since all PTKs contain multiple regulatory domains in addition to a catalytic domain, how the regulatory domains communicate with the catalytic domain to modulate its function is an important aspect of PTK structure−function relationship. Using crystal structures as a guide and site-

specific mutagenesis and steady state kinetics as tools, the current study probed mechanisms of communication between regulatory and catalytic domains in a cytosolic PTK, Csk. The studies led to the following findings. First, the SH3 and SH2 domains played a major role in positively modulating the kinase activity, but they were not directly involved in recognizing the Csk physiological substrate, Src. Second, positive modulation of catalytic activity by the presence of SH3 and SH2 domains was mediated by interactions of a highly conserved Trp residue, Trp188. However, Trp188 and the SH3 domains were not required for the SH2-mediated activation of Csk by a phosphopeptide. This finding suggested that the SH2 domain could directly communicate with the catalytic domain independently of the SH3 domain and Trp188. This conclusion is in direct contrast to the situation in Hck, where the corresponding Trp260 residue mediates both the suppression of catalytic activity by the presence of the SH3 and SH2 domains and the activation of catalytic activity by SH3 or SH2 domain binding to respective ligands (*16*). Third, the Csk catalytic domain contained at least five substructures whose conformation was modulated by the presence of the regulatory domains. Two of these substructures directly interacted with the SH2 domain, and may be crucial in mediating SH2-catalytic domain communication. Two others were located in the peptide-binding lobe. One of the regulated substructures in the peptide-binding lobe, region D, was more important for phosphorylation of polyE$_4$Y than for the physiological substrate, and likely a key mediator of polyE$_4$Y-specific effects of regulatory domain mutations.

Csk SH3 and SH2 Domains in Substrate Binding and Specificity. Csk specifically phosphorylates Src family kinases on a C-terminal tail Tyr residue, which leads to SFK inactivation. How Csk specifically recognizes SFKs for phosphorylation has been an active topic of research (*23*, *33*, *34*). Previous studies have demonstrated that the SH3 and SH2 domains are not involved in Csk recognition of an artificial substrate, polyE$_4$Y (*13*, *14*). Mutagenic and kinetic studies in the current report suggested that Csk SH3 and SH2 domains were not directly involved in recruiting Src, as deletions of these domains did not affect kdSrc phosphorylation more than polyE$_4$Y phosphorylation. This conclusion is consistent with the recent determination of a substrate-docking site of Csk (*22*), which is centered on a short α-helix D in the peptide-binding lobe. Mutations of key determinants of the substrate-docking site only moderately decrease Csk activity toward polyE$_4$Y, but abolish its ability to complex with and phosphorylate kdSrc. These findings and the characterization of the SH3 and SH2 deletion mutants in the current report demonstrate that substrate recognition by Csk is a function of the catalytic domain, even though the regulatory domains could influence substrate specificity.

The employment of a substrate-docking site for recognition of the physiological substrate is also consistent with the observation that mutations in the regulatory domains and region D have a more severe effect on Csk phosphorylation of polyE$_4$Y than on the phosphorylation of kdSrc. Assuming that region D and possibly other substructures are involved in peptide substrate recognition, mutation or conformational changes due to regulation by the SH3 and SH2 domains would have severe consequences on phosphorylation of peptide substrates such as polyE$_4$Y. On the other hand, kdSrc recognition would depend on both the substrate-docking site and the peptide substrate recognition site (for recognizing the C-terminal tail containing Tyr527). Thus, mutations or conformational changes in substructures such as region D would have much less affect on kdSrc phosphorylation. This analysis suggests that even though the SH3 and SH2 domains are not directly involved in binding to the peptide or protein substrates, they can influence Csk substrate specificity by regulating the conformation of certain substructures in the catalytic domain.

Mechanisms of Domain−Domain Communication in Csk. Communication between the regulatory and catalytic domains of a PTK provides a foundation for regulation. The regulatory domains modulate the catalytic activity of Csk by at least two modes. First, the presence of the SH3 and SH2 domains positively modulates Csk activity, as demonstrated previously and by the characterization of ΔSH3 and ΔSH2 in this study. Second, the Csk SH2 domain binds to phosphotyrosine-containing proteins, such as CBP, and this binding activates Csk activity. Mutation of Trp188 to Ala apparently abolishes the positive modulation of Csk activity by the presence of the SH3 and SH2 domains, but does not abolish the second mode of communication. This differentiation demonstrates that the two modes of activation are mediated by separate mechanisms, with the former relying on interactions of Trp188, and the latter likely on direct interactions between the SH2 and the catalytic domains. This conclusion is consistent with a recent report of an important role of Phe183, one of the residues in the environment of Trp188, in stabilizing the active conformation of Csk (*35*).

It is not clear why Trp188Phe and Trp188Ala are more sensitive to activation by the phosphopeptide. It may be that the loss of Trp188 can be partially compensated for by a conformational change in the SH2 domain due to ligand binding. We suspect that optimal activation of Csk is achieved by numerous interactions between the catalytic domain and the regulatory region, such as interactions of Trp188 with its surrounding residues and direct interactions between the SH2 domain and regions in the catalytic domain, such as B and C. It is likely that these multiple interactions converge to regulate key catalytic substructures, such as the α-helix C, and/or the Gly-rich loop. Convergence of multiple channels of communication to a common regulated substructure would allow one interaction to compensate for the loss of another. The exact mechanism for the observed compensation awaits further investigation.

These findings are in direct contrast to the mechanism of communication in Hck and likely all Src family kinases. In Hck, the SH3 and SH2 domains suppress its kinase activity and the regulatory-catalytic domain−domain communication appears to be mediated by the linker between the SH2 and catalytic domains, which contains Trp260, corresponding to Trp188 in Csk (Figure 1B, 16). Mutation of Trp260 activates Hck by increasing its K_m for a peptide substrate and decreasing its k_{cat}, and renders Hck catalytic activity unresponsive to ligand binding to the SH3 or SH2 domain. The dramatically different roles of Trp188/Trp260 in domain−domain communication in Csk and Src family appear to reflect the different domain configurations in these enzymes (Figure 1). Continued investigation of the specific interactions involved in interdomain communications will likely reveal

the mechanistic basis for divergent tertiary configurations for kinases that have similar primary structures.

ACKNOWLEDGMENT

We wish to thank Dr. David Laux at URI for critically proofreading the manuscript, and Dr. Zhiping Weng and her lab at Boston University for providing access to K2 structural alignment program (http://zlab.bu.edu/k2/) and assistance for using the program. DNA sequencing was performed at URI Genomics and Sequencing Center

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BI048142J