Subdomain Switching Reveals Regions That Harbor Substrate Specificity and Regulatory Properties of Protein Tyrosine Kinases†

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ABSTRACT: Csk and Src are two protein tyrosine kinases that share a similar overall multidomain structural organization and a high degree of sequence homology but have different substrate specificities and regulatory properties. In this study, we generated chimeric kinases of Csk and Src by switching the C-terminal lobes of their catalytic domains, and we characterized their substrate specificity and regulatory properties. First, both Csk and Src phosphorylate Src as a common substrate, but on different Tyr residues. The C-terminal lobes of the kinase catalytic domain determined the site of phosphorylation on Src. Furthermore, toward several physiological substrates of Src, the substrate specificity was also determined by the C-terminal lobe of the catalytic domain regardless of the regulatory domains and the N-terminal lobe of the catalytic domain. Second, Csk and Src represent two general regulatory strategies for protein tyrosine kinases. Csk catalytic domain is inactive and is positively regulated by the regulatory domains, while Src catalytic domain is active and suppressed by its interactions with the regulatory domains. The regulatory properties of the chimeric kinases were more complicated. The regulatory domains and the N-lobe did not fully determine the response to a regulatory ligand, suggesting that the C-lobe also contributes to such responses. On the other hand, the intrinsic kinase activity of the catalytic domain correlates with the identity of the N-lobe. These results demonstrate that the chimeric strategy is useful for detailed dissection of the mechanistic basis of substrate specificity and regulation of protein tyrosine kinases.

Protein tyrosine kinases (PTKs)[1] mediate cellular signaling by responding to upstream signals and phosphorylating protein substrates on Tyr residues (*1*). To meet the complex needs of signal transduction, the human genome contains approximately 500 genes for protein kinases, including ~100 PTKs (*2*). What signals each PTK responds to is determined by its regulatory properties, and the selection of protein substrates is dictated by its substrate specificity. These two properties determine the signaling specificity and fidelity of a PTK and distinguish one PTK from another. The combination dictates the roles a PTK plays in the signaling network.

One of the best-understood PTK regulatory systems is the regulation of Src family protein tyrosine kinases (SFKs) (*3*, *4*). The Src family contains nine kinases, and each one is composed of, 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. SFKs are regulated by two major mechanisms. The first is autophosphorylation on Tyr416 (avian Src numbering) located in the activation loop, which activates the kinase (*5*−*7*). Autophosphorylation of this residue is catalyzed by the kinase itself through an intermolecular mechanism (*8*). The second mechanism is built on domain−domain interactions that are regulated by the phosphorylation of another Tyr residue located on the C-terminal tail, Tyr527 (*4*). Phosphorylation of Tyr527 is catalyzed by another family of protein tyrosine kinases, the C-terminal Src kinases Csk and Chk (*9*, *10*). PhosphoTyr527 (pTyr527) binds to the SH2 domain intramolecularly, which leads to other domain−domain interactions and a closed and inactive conformation of Src (*11*, *12*). This mechanism is dictated by the fact that the catalytic domain of Src is intrinsically active (*13*), and its activity can be suppressed by appropriate domain−domain interactions triggered by pTyr527 binding to the SH2 domain. A number of regulatory signals, such as a respective ligand to the SH3 (*14*) or the SH2 domain (*15*) or G-protein binding to the kinase domain (*16*), can alter the domain−domain interactions and activate Src. This enables Src kinase activity to respond to a number of signals from the cell surface, such as growth factors that activate receptor tyrosine kinases or G-protein coupled receptors.

The Csk family kinases, Csk and Chk, employ a contrasting regulatory setup to that of Src, despite the similarity in their structural organization. The Csk family kinases also contain regulatory SH3 and SH2 domains in addition to a catalytic domain (*17*), but the regulatory domains play

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[1] Abbreviations: CBP, Csk-binding protein; C-lobe, the C-terminal lobe of the catalytic domain of a protein tyrosine kinase; CSch, Csk−Src chimera; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; N-lobe, the N-terminal lobe of the catalytic domain of a protein tyrosine kinase; PTKs, protein tyrosine kinases; pTyr527, phosphotyrosine 527 of Src; SCch, Src−Csk chimera; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SFK, Src family kinases.

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different roles in Csk regulation. First, unlike the SFKs, Csk and Chk are not regulated by Tyr phosphorylation (*18*). Even though Csk and Chk also each contains an activation loop, their activation loops do not contain any Tyr residues. Second, in contrast to the Src catalytic domain that is intrinsically active, the catalytic domain of Csk is not active (*19*, *20*) and is activated by interactions with the regulatory domains. Furthermore, the domain−domain interactions in Csk are not regulated by Tyr phosphorylation (*21*, *22*). Ligands to the Csk SH2 domain, such as the Csk-binding protein (CBP), can further activate Csk (*23*). Thus the Csk regulatory setup is based on the fact that its catalytic domain is intrinsically inactive and needs to be activated by the regulatory domains. Consistent with these differences in the regulatory properties, the tertiary arrangement of the SH3, SH2, and catalytic domains is quite different in Csk (*24*) and Src (*11*). While the regulatory domains are located on the side of the catalytic domain in the inactive form of Src, the SH3 and SH2 domains are located on the top of the N-lobe. In these two arrangements, the interactions between the regulatory domains and the catalytic domain are completely different.

The Src and Csk family kinases also have contrasting properties in substrate specificity. The Src family kinases can phosphorylate more than 100 protein substrates on Tyr residues, sending regulatory signals to a large number of downstream targets (*25*). In contrast, Csk and Chk have extremely narrow substrate specificity and are known to phosphorylate only the Src family kinases on Tyr527 (*26*, *27*). The contrasting substrate specificity is crucial for the contrasting roles of Src and Csk in the Csk−Src regulatory system. The crucial importance of the contrasting substrate specificity between these two kinase families is also well illustrated by the phosphorylation of Src on different Tyr residues. Because Src serves as a substrate for both Csk, phosphorylating Tyr527, and Src, phosphorylating Tyr416, and phosphorylation of these two sites have opposite effects on Src activity (*28*), it is essential that the appropriate enzyme recognizes and phosphorylates the correct Tyr residue.

Delineating the structure−function relationships for PTK substrate specificity and regulation is a daunting task. While crystal structures of protein tyrosine kinases provide structural views of the kinases in certain states and a framework for understanding kinase function, they do not reveal what substructures are involved in what function during catalysis and regulation. Site-specific mutagenesis, combined with kinetic analysis and structural determination, can reveal the structure−function relationships for certain substructures and residues, but performing such studies on all the structural variations in kinases is time-consuming, and interpretation of mutagenic effects is often complicated by conformational changes associated with kinase regulation. It would be useful to develop approaches that can quickly relate the functional properties to substructures, providing a basis for further dissection by site-specific mutagenesis.

In this study, we explore the potential of using Csk and Src chimeras to help locate the structural basis for substrate specificity and certain regulatory properties. The study demonstrates that substrate specificity determinants are harbored in the C-terminal lobe (C-lobe) of the catalytic domain and that the intrinsic kinase activity level is determined by the N-terminal lobe (N-lobe) of the catalytic domain. Certain regulatory properties appear to require collaboration between the N- and C-lobes, because they cannot be transferred from one kinase to another by lobe switching. These results demonstrate that domain switching can be an effective strategy in locating the determinants for certain catalytic and regulatory properties of protein tyrosine kinases.

MATERIALS AND 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 Sephadex G25 were purchased from Sigma. Amylose resin for purifying maltose-binding protein was purchased from New England Biolabs. DNA primers were synthesized by Integrated DNA Technologies. [γ-^{32}P]-ATP (6000 Ci mol^{-1}) was purchased from Perkin-Elmer.

Recombinant Protein Expression. Wild-type human Csk was expressed in *Escherichia coli* (DH5α) by use of pGEX-Csk-st plasmid (*29*). Csk and Src chimeras were generated by standard molecular biology procedures and inserted into the expression vector pMal-c2x (*30*). The inserts were fully sequenced to ensure that they did not contain unintended mutations. The chicken Src mutant devoid of kinase activity (kdSrc) was coexpressed with GroEL and GroES chaperones in BL21(DE3) as previous described (*31*, *32*). Mutations were introduced into the kdSrc gene to generate various derivatives of kdSrc by Quickchange and confirmed by DNA sequencing. Protein concentration was determined by the Bradford method, and the purity was examined by SDS−PAGE and coomassie blue staining.

Kinase Activity Assays. Kinase activity was determined using polyE$_4$Y as the substrate as described previously (*18*). Phosphorylation reactions were performed in 50 μL volume 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 condition included 12 mM $MgCl_2$, 0.2 mM ATP ([γ-^{32}P]ATP, 1000 dpm pmol^{-1}) and 1 mg mL^{-1} polyE$_4$Y. After a 20 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$_4$Y was determined by liquid scintillation counting. All assays were performed in duplicate and at least three times with reproducible results.

To determine the catalytic parameters of the mutants, ATP (20−200 μM) was used as the variable substrate under otherwise standard conditions. The assays were performed as described above. Reactions without polyE$_4$Y were used as background controls. The background was under 2000 cpm, while the signals were in the range of 10 000−100 000 cpm. K_m and k_{cat} values were determined from double reciprocal plots.

Src Inactivation by Csk or SCch. The ability of Csk or kinase mutants to inactivate Src was determined as previously described (*7*). Src-Y416F (1 pmol) was incubated with different amounts of Csk or SCch in the presence of 0.2 mM [γ-^{32}P]ATP (1000 cpm pmol^{-1}) and 12 mM $MgCl_2$ at 30 °C for 30 min. At the end of the preincubation, the kinase activity in the reaction was determined by adding RCM-

lysozyme (0.8 mg mL^{-1}) as the phosphate-accepting substrate. The reaction proceeded for another 20 min; it was stopped by being spotted onto 2 × 1 cm filter paper, which was washed in warm 5% trichloroacetic acid. The phosphate incorporated into $polyE_4Y$ was determined by liquid scintillation counting. Preincubation reactions with Src without Csk are taken as 100% Src activity, and preincubations with varying amounts of Csk or SCch without Src were used as background. The residual Src kinase activity was plotted as a function of Csk used in the preincubation.

Phosphorylation Determination by Autoradiography. To determine the phosphorylation of various substrates by autoradiography, a fixed amount of the purified protein substrate was incubated with a certain amount of kinase activity (2.5 pmol min^{-1} toward 1 mg mL^{-1} $polyE_4Y$) under kinase assay conditions for 30 min at 30 °C, and the proteins were fractionated by SDS−PAGE. Phosphorylation of proteins was detected by autoradiography.

RESULTS

Design of the Chimera Strategy. It is intriguing that Csk and Src are highly conserved structurally but have very divergent substrate specificity and regulatory properties. There could be two extreme types of structure−function relationship to account for such a functional divergence. One extreme is that any functional property is determined by the global structure of the enzyme and thus could not be attributed to any specific structural motif(s). The other extreme is that functional properties are determined by specific motifs or a combination of motifs that are embedded in the general structural framework. In the latter view, the kinases can be seen as a general phosphorylation apparatus with a tunable substrate specificity and regulation conferred by structural motif(s) unique to each kinase. The fact that the general structural framework of protein tyrosine kinases is highly conserved appears to support the latter scenario. In this view, one should be able to transfer the structural motifs from one kinase to another and generate mutant kinases with combinatorial properties. Src and Csk appear to be ideal enzymes for exploring this potential, because they have similar primary domain organization but highly divergent biochemical properties. We decided to focus on the catalytic domains for testing this strategy because the catalytic domains of Csk and Src are highly conserved, yet they dictate dramatically different substrate specificities and regulatory properties.

The catalytic domain of a kinase is composed of two lobes. The N-terminal lobe (N-lobe) consists of approximately 80 residues (in Csk, Trp188 through Met269; in Src, Trp260 through Met341), and the C-lobe consists of approximately 180 residues (in Csk, Ala270 through Leu450; in Src, Ser342 through Phe520). There is a cleft between the two lobes where ATP binds and the transfer of the γ-phosphate from ATP to the Tyr hydroxyl group occurs. For this reason, the cleft is considered the active site. In crystal structures of protein kinases with peptide substrates, the peptide substrates invariably bind to the C-lobes of the kinases, so the C-lobe is often referred to as the peptide-binding lobe. To determine if certain catalytic and regulatory properties can be transferred from one kinase to another by switching subdomains or motifs, we switched the C-lobes of Csk and Src to generate

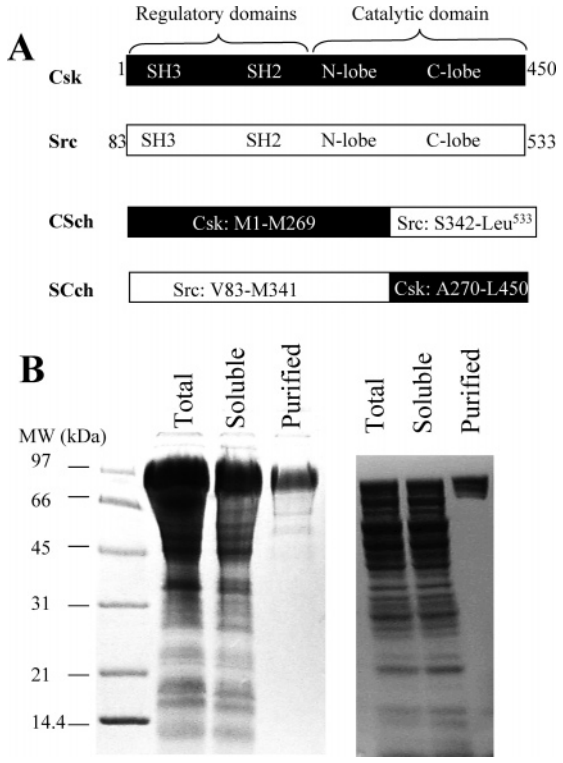

FIGURE 1: Design and purification of chimeric kinases. (A) Kinase constructs used in this study. The Src construct used in this study started with residue 83 and lacked the myristoylation motif and the unique region. Regulatory and catalytic domains of Csk and Src kinases are labeled. Chimeric kinases CSch and SCch were created by switching the C-lobes of the catalytic domains. (B) Chimeric kinases were expressed as fusion proteins with MBP and purified by amylose resin as previously described (*28*).

two chimeric kinases: the Csk−Src chimera (CSch) and the Src−Csk chimera (SCch). The switching points were the C-terminal peptide bonds of Met269 in Csk and Met342 in Src (Figure 1A). The choice of the switching points was based on two considerations: position and homology. Switching the C-lobes required that the switching points be located between the N- and C-lobes. Switching at a conserved residue ensured that equal structural portions were switched.

The chimeras were expressed as fusion proteins with maltose-binding protein (MBP) and purified on amylose resin. Figure 1B shows the purification of both fusion proteins from bacterial lysates. Both proteins were highly expressed in total cell lysates and in the soluble fractions, and could be purified to approximately 80% purity in a single step. With $polyE_4Y$ as a substrate, CSch had a catalytic activity of 0.8 min^{-1} and SCch had a catalytic activity of 35 min^{-1}. The fact both chimeras were active indicated the N- and C-lobes of Csk and Src could be recombined to make an active kinase (Table 1).

C-Lobe of Catalytic Domain Selects the Tyr Residue (Tyr416 versus Tyr527) of Src for Phosphorylation. The substrate specificity of the chimeras was first analyzed. Because Src serves as a substrate for both Csk (on Tyr527) and Src (on Tyr416), we determined whether the chimeric kinases could phosphorylate Src and, if so, on which Tyr residue. For this purpose, a kinase-defective mutant of Src (kdSrc, due to Lys295Met mutation) was used. Lacking intrinsic kinase activity, kdSrc could not autophosphorylate and thus could only serve as a substrate for another kinase

Table 1: Kinase Activity of Chimeras and Other Enzymes Used in This Study[a]

kinase	K_m (μM)	k_{cat} (min^{-1})
Src	70 ± 20	260 ± 10
Csk	126 ± 20	150 ± 27
SCch	140 ± 20	35 ± 7
CSch	400 ± 100	0.8 ± 0.3
Csk-cata	113 ± 9	0.14 ± 0.02
SCch-cata	131 ± 40	177 ± 50
CSch-cata	247 ± 6	0.42 ± 0.04

[a] The reported K_m and k_{cat} values are determined with ATP as the variable substrate and polyE$_4$Y at 1 mg mL^{-1} as the fixed phosphate-accepting substrate. The reported values are the average of two independent determinations (each in duplicate). The range of the two determinations is also reported.

(*31*, *32*). To determine which Tyr residue was phosphorylated, we mutated either Tyr416 or Tyr527 to Phe in kdSrc (kdSrc-Y416F and kdSrc-Y527F), leaving the other Tyr as the site of phosphorylation. The catalytic domain of kdSrc (kdSrc-cata), which contained both Tyr416 and Tyr527, was also included as a substrate.

The ability of each chimera to phosphorylate these kdSrc mutants was compared to that of Csk and Src. Because Csk, Src, and the chimeras had different specific kinase activities, we used equal amount of kinase activity (2.5 pmol min^{-1} under standard assay conditions) in each reaction. Under these conditions, Csk phosphorylated kdSrc, kdSrc-cata, and kdSrc-Y416F but did not phosphorylate kdSrc-Y527F, demonstrating that Csk phosphorylated kdSrc exclusively on Tyr527 (Figure 2A). Src phosphorylated kdSrc, kdSrc-cata, and kdSrc-Y527F but not kdSrc-Y416F, confirming that active Src phosphorylated kdSrc exclusively on Tyr416 (Figure 2B). In the absence of added kinase, the substrates did not autophosphorylate (Figure 2E). These control experiments demonstrated that the substrate specificity for Src and Csk was preserved in our assay design.

The Src−Csk chimera (SCch) exhibited an identical phosphorylation pattern toward these substrates as Csk did, phosphorylating kdSrc, kdSrc-cata, and kdSrc-Y416F but not kdSrc-Y527F (Figure 2C). This demonstrated that SCch retained the Csk substrate specificity in recognizing and phosphorylating Tyr527 of Src. The Csk−Src Chimera (CSch) exhibited an identical phosphorylation pattern as Src, phosphorylating kdSrc, kdSrc-cata, and kdSrc-Y527F but not kdSrc-Y416F (Figure 2D). This demonstrated that CSch had an identical specificity as Src in phosphorylating kdSrc on Tyr416. These results compellingly demonstrated that the C-terminal lobe of Src or Csk catalytic domain determined their site specificity in phosphorylating kdSrc, and thus the SH3 and SH2 domains and the N-lobe of the catalytic domain did not contribute to the substrate specificity in this system. It was noted that kdSrc and derivatives were far more efficient substrates for Csk than for Src. The reason for this low activity in autophosphorylation by Src is not fully clear and under further investigation.

As an independent test for the above conclusion, the ability of SCch to inactivate active Src was determined. Because SCch was able to phosphorylate kdSrc, it was expected that it would be able to inactivate Src by phosphorylating Tyr527. Src was incubated with different amounts of Csk or SCch in the presence of ATP and $MgCl_2$. SCch was equally effective in phosphorylating and inactivating Src, confirming

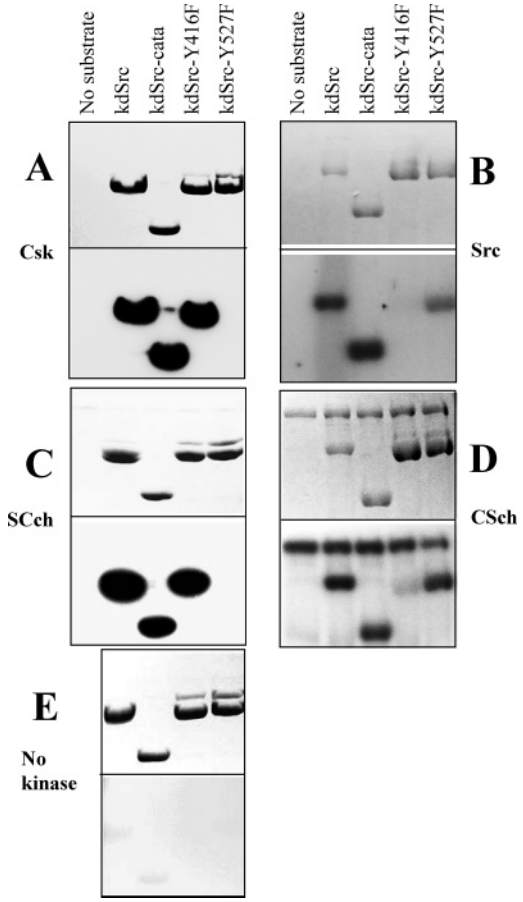

FIGURE 2: Phosphorylation of kdSrc and kdSrc derivatives by Csk, Src, and chimeric kinases. Kinase-defective Src (kdSrc) and derivatives (12 μg for kdSrc-cata or 18 μg for all others in each lane), labeled above each panel, were phosphorylated by Csk, Src, or chimeric kinases. Equal amount of kinase activity (2.5 pmol min^{-1} toward polyE$_4$Y at 1 mg mL^{-1}) was used for each reaction, and the kinase used is indicated on the side of each panel. The top half of each panel is a picture of a Coomassie-stained gel, and the bottom half is an autoradiogram of the same gel.

that the C-lobe of Csk carried the structural determinants for recognizing Src Tyr527. In contrast, CSch did not inactivate Src (data not shown). This experiment confirmed that SCch, containing the C-lobe as the only part from Csk, contained all necessary structural determinants to recognize Src on Tyr527.

C-Lobe of Src Determines Substrate Specificity toward Src Physiological Substrates. While Src and other Src family kinases are the exclusive physiological substrates for Csk, Src phosphorylates many other protein substrates (*25*). We tested whether the C-lobe in the chimeric kinases determined the substrate specificity toward other Src physiological substrates. Two established Src substrates, IκBα (*33*) and cortactin (*34*), were expressed and purified as fusion proteins with glutathione S-transferase (GST) and MBP, respectively. The ability of the chimeras to phosphorylate these substrates was compared to that of Csk, Src, and another Src mutant lacking the SH3 domain, Src-ΔSH3. Src-ΔSH3 was included in this analysis because previous studies had suggested that the Src SH3 domain might contribute to Src substrate recruitment (*35*). Csk and SCch, both carrying the C-lobe of Csk, did not phosphorylate either substrate (Figure 3). Src-ΔSH3, CSch, and Src, all containing a Src C-lobe, phosphorylated both substrates. These results confirmed that

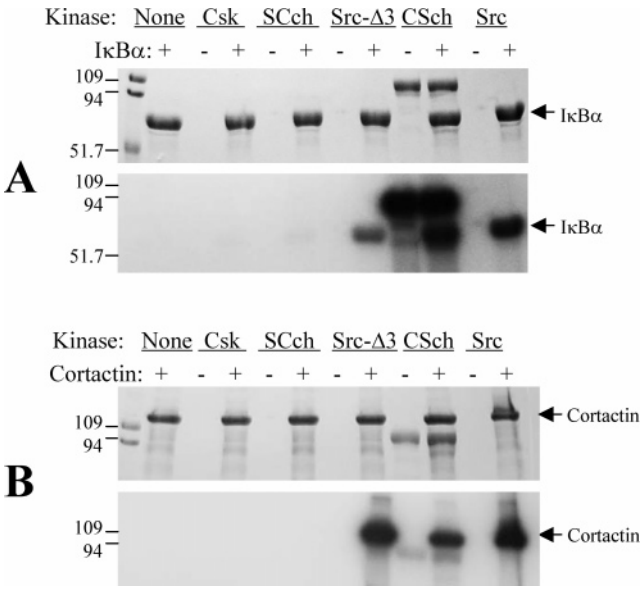

FIGURE 3: Phosphorylation of IκBα (A) and cortactin (B) by Csk, Src, and chimeric kinases. The top half of each panel shows a Coomassie-stained protein gel, and the bottom half is an autoradiogram. The substrate and kinase combinations are indicated on the top of each figure, and the positions of the substrates in both the protein gel and the autoradiogram are indicated on the right. IκBα (20 μg) or cortactin (30 μg) was used in each lane. The conditions of the experiments are presented in the text.

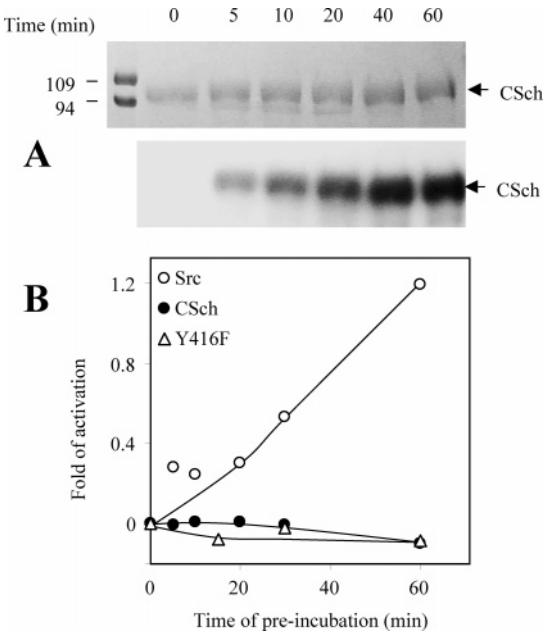

FIGURE 4: Time course of CSch autophosphorylation and effect on its activity. (A) CSch (16 μg for each lane) was incubated with 0.1 μM [^{32}P]ATP (1000 cpm pmol^{-1}) at 30 °C. At indicated timed, aliquots of the reaction were withdrawn and prepared for SDS−PAGE (top panel) and autoradiography (bottom panel). (B) Effect of autophosphorylation on CSch kinase activity. Src, Src-Y416F, and CSch (initial activity 11−20 pmol min^{-1}) were preincubated with ATP under the standard phosphorylation conditions. At the indicated times, aliquots of the autophosphorylation reaction was removed and mixed with 2 mg mL^{-1} polyE$_4$Y to initiate the kinase assay reaction. The kinase assay reactions went for 8 min and were stopped as described for the standard kinase assay. *x*-Fold activation = (activity at given time − initial activity)/initial activity.

the C-lobe of the Src catalytic domain contained the determinants for recognizing these physiological substrates of Src. These results collectively demonstrated that the C-lobe of Csk and Src determined the substrate specificity of the kinases toward these physiological substrates. The results also demonstrated that the Src SH3 domain was not required for Src to recognize and phosphorylate cortactin and IκBα.

Csk-Src Chimera Undergoes Autophosphorylation but Not Activation. Due to the amount of CSch used in the assays in the previous section, the ability of this chimera to autophosphorylate was clearly noted. This was not surprising because this chimera contained both the Src autophosphorylation site, Tyr416, and that C-lobe of Src, which carried the structural motifs for recognizing Tyr416. We determined the time course of CSch autophosphorylation and whether it was activated by autophosphorylation. CSch underwent a time-dependent autophosphorylation when incubated with [γ-^{32}P]-ATP under phosphorylation conditions (Figure 4A). While Src underwent rapid autophosphorylation (*30*) and was activated by this incubation, CSch also underwent autophosphorylation but was not activated over the 1 h time period (Figure 4B). This result indicated that the chimera was not activated by autophosphorylation. It appears that the autophosphorylation-induced activation requires collaboration between the N-lobe and possibly the regulatory domains.

Activity Level of Catalytic Domain Is Determined by N-Lobe. As noted earlier, Src and Csk represent two contrasting regulatory mechanisms. Csk has a catalytic domain that is intrinsically inactive and positively modulated by interactions with the regulatory domains; Src has a catalytic domain that is intrinsically active and suppressed by interactions with the regulatory domains. On the surface, the two strategies reflect two different setups in the domain−domain interaction, but at the foundation, the two strategies are dictated by and embedded in the catalytic domains. It is the catalytic domains and their intrinsic activity levels that dictate which strategy would be used. It is not clear what determines whether a catalytic domain of a kinase is by itself active. We tested whether the chimera strategy could be used to gain insights into this issue.

For this purpose, we constructed two chimeric catalytic domains, Csk−Src chimera catalytic domain (CSch-cata) and Src−Csk chimera catalytic domain (SCch-cata), and determined the activity of these constructs. As controls, we expressed the catalytic domains of Csk and Src in the same expression system. Csk-cata was well expressed and purified. Src-cata was not expressed in this system to any appreciable amount and could not be purified. This is consistent with the previous finding that Src expression is toxic to a bacterial cell and only constructs having an activity level below a certain threshold can be expressed and purified from *E. coli* (*36*).

The catalytic domain of Csk had a kinase activity of approximately 0.1 min^{-1}, as previously reported (*19*). The kinase activities of the two chimeric catalytic domains are shown in Table 1. CSch-cata had a kinase activity very similar to that of Csk-cata, while SCch-cata displayed a much higher kinase activity, close to that of wild-type Src. The fact that SCch-cata was highly active demonstrated the Src N-lobe made a catalytic domain active regardless of the C-lobe. Although the lack of activity for CSch-cata and Csk-cata could have other explanations, such as misfolding and instability, crystal structure of the catalytic domain of Csk

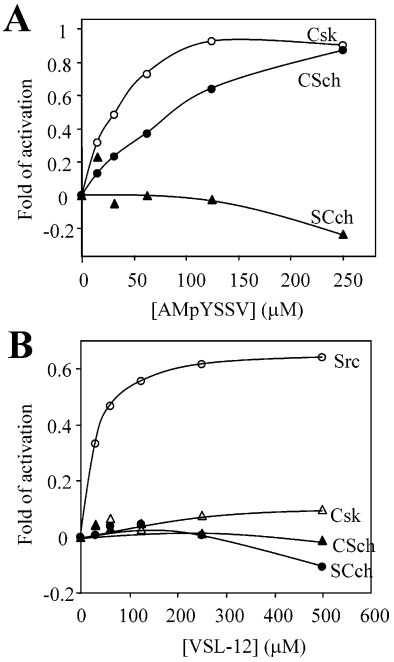

FIGURE 5: Response of CSch and SCch to Csk and Src regulators: Effects of Csk SH2 domain ligand (A) and Src SH3 domain ligand (B) on activity of chimeric kinases. *x*-Fold activation = (activity at a given concentration of ligand − activity without ligand)/activity without ligand.

(*37*) and the fact the catalytic domain can partially regain its activity by interaction with the regulatory domains (*19*) argue against these explanations. Thus, it is likely that the N-lobe of Csk catalytic domain carries the structural determinants that make a catalytic domain inactive. These results demonstrated that the N-lobe determined the kinase activity of a catalytic domain and likely dictated the regulatory strategy.

N-Lobe of Catalytic Domain Determines Some Regulatory Properties. As discussed earlier, Csk and Src are regulated by distinct strategies. The regulatory domains activate Csk but suppress Src, while both enzymes are also subject to activation by ligands to the regulatory domains. Because the regulatory domains interact with only the N-lobe, and the N-lobe and the regulatory domains in each chimera were from the same enzyme, we determined whether the regulation by domain−domain interaction was preserved in the chimeras.

We first tested whether the chimeras were activated by the Csk-binding protein, represented by a phosphopeptide mimicking the phosphorylation site of CBP, AMpYSSV (*21*, *23*). As shown in Figure 5A, both Csk and Csk−Src (data not shown) chimera were activated by the CBP peptide, while both Src and SCch were not activated by CBP. This indicated that the interactions between the Csk SH2 domain and the N-lobe of Csk remained in place in CSch. We then determined whether the chimeras responded to activation by a Src regulator, a peptide ligand to the SH3 domain with the sequence of VSLARRPLPPLP (*38*) (Figure 5B). Src was activated by the SH3 domain ligand about 60%, indicating that the SH3 domain, through its interaction with the linker between the SH2 domain and the catalytic domain, suppressed Src activity. Csk and CSch did not respond to this ligand, as expected because they did not contain an Src SH3 domain. Interestingly, SCch also did not respond to the SH3 domain ligand, indicating that the SH3 domain in this chimera was not involved in suppressing Src activity. This suggested that Src activity suppression by the regulatory domains required the cooperation by certain substructures in the C-lobe of Src. One of the key regulatory structures of Src is the activation loop and the autophosphorylation site located in this loop. The autophosphorylation loop and other substructures in the C-lobe of Src may be involved in Src suppression by the regulatory domains.

DISCUSSION

Chimera Strategy. Chimeras between different protein tyrosine kinases have been previously used to study signaling pathways (*39*−*41*). Most efforts take advantage of the fact that kinases are made up of multiple domains and these domains function largely independently. Chimeras combining regulatory domains or the extracellular domain from one kinase with the catalytic domain of another are often used to couple upstream regulatory signals of one kinase to the downstream targets of another kinase to "rewire" signaling pathways. In this study, we determined whether chimeras could be used to dissect the structure−function relationship of the catalytic domain. Although the catalytic domain of a kinase is composed of two lobes, they carry out one integrated function, that is, the phosphorylation reaction; thus, whether different aspects of the catalytic function can be attributed to specific lobes is an open question. The only two cases of catalytic chimeras to our knowledge are a study of Src/Abl chimera to determine the binding specificity to nucleotides (*42*) and a study of MAP kinases in their response to growth factors and stress signals (*43*). The function of a kinase consists of three essential components: substrate recognition, phosphoryl transfer, and appropriate response to regulatory signals. Because substrate recognition and regulation are unique to each kinase, we determined whether they could be attributed to a specific lobe in the catalytic domain by the chimera strategy.

Substrate Specificity. What enables a protein tyrosine kinase to phosphorylate one set of protein substrates but not others is not fully understood (*35*). For protein kinases in general, several models to account for substrate specificity have emerged. Some kinases, such as the cAMP-dependent protein kinase, recognize the peptide sequence surrounding the phosphorylation site (*44*). Such kinases use the P + 1 loop in the C-lobe as the main platform to interact with the peptide sequences (*45*). Other protein kinases, such as the MAP kinases, use a substrate-docking site that is located away from the active site to recognize a structural motif on the substrate that is far away from the phosphorylation site (*46*). Yet another Ser/Thr protein kinase, glycogen synthase kinase 3, uses a phosphate-binding site to recognize protein substrates already phosphorylated on priming phosphorylation sites (*47*). Recent mutagenic studies indicate that Csk uses a substrate-docking site that is located on the α-helix D to recognize Src in phosphorylating Tyr527 (*32*, *48*). All of these precedents use structural motifs located in the C-lobe of the catalytic domain for substrate recognition. However, Src family kinases recruit some of their protein substrates by the SH3 domain binding to polyproline motifs in the substrates (*49*−*52*). Mutation of either the polyproline motifs of the substrates or the SH3 domain decreases or abolishes the phosphorylation of these substrates. These data seem to

suggest an SH3-based model of Src substrate recognition. Several questions remain with this model. Is SH3 domain binding sufficient for Src substrate recognition? How does Src discriminate against different Tyr residues in the same substrate? What role, if any, does the Src catalytic domain play in substrate recognition? And does Src recognize all substrates by this mechanism? Characterization of the chimeric kinases provides some insights into these questions.

By use of chimeras between the Src and Csk catalytic domains, it was demonstrated that the C-lobe of Csk, which contained the α-helix D, harbored sufficient structural information for recognizing Src Tyr527. Furthermore, the C-lobe of Src carried sufficient structural determinants for recognizing three well-established Src substrates, Tyr416 of Src, cortactin, and IκBα. These results demonstrated that, at least for these substrates, the C-lobe of the catalytic domain of Src was the sole determinant of substrate specificity. It is also important to note that Src and CSch did not phosphorylate kdSrc on Tyr527, indicating that they did not indiscriminately phosphorylate whatever phosphorylation sites were available. Thus, the C-lobe contains a screening mechanism to select the Tyr residue to phosphorylate. We attempted to express several other Src substrates that reportedly depended on the Src SH3 domain for recognition but failed to establish recombinant expression systems to obtain those substrates. Thus we could not determine if the chimera with a Src C-lobe would be able to phosphorylate those substrates.

Strategy for Regulation. The difference between Csk and Src regulation rests in the different domain−domain organizations of the two enzymes. But at the core, the catalytic domains dictate the different regulatory strategies. For example, the catalytic domain of Csk is activated by extensive interactions with the SH2 domain and the linker between the SH3 and the SH2 domains (*21*, *22*). For this strategy to work, two structural elements have to be embedded in the catalytic domain. First, the catalytic domain needs to be inactive without interaction with the regulatory domains. Second, the catalytic domain needs to contain specific structures that can interact with the regulatory domains and transmit the signal of the interaction to certain parts of the catalytic structure to make it active. With such embedded structural determinants, the catalytic domains of Csk would be able to have proper interactions with the regulatory domains and be activated. In other words, the Csk catalytic domain contains an embedded "switch" that is in the off position and can be turned on by the interactions with the regulatory domains. For the same reason, the catalytic domain of Src would need to have an embedded switch that is intrinsically "on" and can be turned off by interactions with the regulatory domains. Previous studies from this and other laboratories have identified certain interactions between the catalytic domain and the regulatory domains that activate Csk (*21*, *22*), but the structural nature of a "switch" has not been determined.

The results in this study indicated that the switch was likely embedded in the N-lobe of the catalytic domain. The N-lobe of Csk dictates an inactive catalytic domain, whether it is attached to the C-lobe of Csk or Src. In contrast, the N-lobe of Src dictates an active catalytic domain, whether the C-lobe is from Csk or Src. The gain of function when the Src N-lobe replaces the Csk N-lobe in Csk-cata is particularly surprising and compellingly demonstrates that the activity level of a catalytic domain is mainly determined by the N-lobe. This result is an important step toward protein engineering efforts to design protein kinases with specific regulatory properties. Further mutagenic studies, such as switching a secondary structure or certain residues, will be needed to detail the structural basis of the properties and substrate specificity.

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