Domain Interactions in Protein Tyrosine Kinase Csk†

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ABSTRACT: Csk (C-terminal Src kinase) is a protein tyrosine kinase that phosphorylates Src family member C-terminal tails, resulting in downregulation of Src family members. It is composed of three principal domains: an SH3 (Src homology 3) domain, an SH2 (Src homology 2) domain, and a catalytic domain. The impact of the noncatalytic domains on kinase catalysis was investigated. The Csk catalytic domain was expressed in *Escherichia coli* as a recombinant glutathione S-transferase-fusion protein and demonstrated to have 100-fold reduced catalytic efficiency. Production of the catalytic domain by proteolysis of full-length Csk afforded a similar rate reduction. This suggested that the reduction in catalytic efficiency of the recombinant catalytic domain was intrinsic to the sequence and not an artifact related to faulty expression. This rate reduction was similar for peptide and protein substrates and was due almost entirely to a reduced k_{cat} rather than to effects on substrate K_ms. Viscosity experiments on the catalytic fragment kinase reaction demonstrated that the chemical (phosphoryl transfer) step had a reduced rate. While the Csk SH2 domain had no intermolecular effect on the kinase activity of the Csk catalytic domain, the SH3 domain and SH3−SH2 fragment led to a partial rescue (4−5-fold) of the lost kinase activity. This rescue was not achieved with two other SH3 domains (lymphoid cell kinase, Abelson kinase). The extrapolated K_d of interaction for the Csk catalytic domain with the Csk SH3 domain was 2.2 μM and that of the Csk catalytic domain with the Csk SH3−SH2 fragment was 8.8 μM. Taken together, these findings suggest that there is likely an intramolecular interaction between the catalytic and SH3 domains in full-length Csk that is important for efficient catalysis. By employing a Csk SH3 specific type II polyproline helix peptide and carrying out site-directed mutagenesis, it was established that the SH3 surface that interacts with the catalytic domain was distinct from the surface that binds type II polyproline helix peptides. This finding suggests a novel mode of protein−protein interaction for an SH3 domain. The implications for Csk substrate selectivity, regulation, and function are discussed.

The majority of known protein tyrosine kinases are composed of multiple domains outside of the canonical ∼260 amino acid catalytic core. Depending on the protein tyrosine kinase, these noncatalytic domains have been suggested to be involved in substrate selection, regulation of catalytic activity, and cellular localization (*1*−*2*). In general, there is a poor understanding of domain interactions in protein tyrosine kinases, perhaps because of the dearth of studies on purified and well-characterized protein systems. Src[1], a prototypical multidomain protein tyrosine kinase, is made up of three well-characterized domains, an SH3 domain (Src homology 3 domain), an SH2 domain, and a catalytic domain (*3*−*6*). The SH3 and SH2 domains, which bind to proline-rich sequences and phosphotyrosine sequences, respectively, have been found in dozens of signal transduction proteins (*7*−*10*). In the case of Src (*11*−*14*), the SH2 and SH3 domains play key roles in regulating catalytic activity by binding to the tail phosphotyrosine and the SH2-catalytic linker region respectively, resulting in inactive kinase (Figure 1).

The protein tyrosine kinase Csk is responsible for site-specific tail phosphorylation of Src and Src family members in vivo (*15*−*17*). By inactivating Src and Src family members, Csk has been demonstrated to be important in the regulation of neural development, T cell development and regulation, and cytoskeletal organization. It has a modular layout similar to Src with SH3, SH2, and catalytic domains and is about 40% identical in overall amino acid sequence (*18*) (Figure 2). Recent studies on its catalytic mechanism have suggested that it involves a dissociative transition state (*19*, *20*), and details of metal ion preferences (*21*, *22*), substrate amino acid sequence selectivities (*23*, *24*), and rate-limiting step(s) have been reported for the full-length protein (*25*, *26*). The roles of the noncatalytic domains in Csk are not well understood. A proline-rich sequence from protein

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[1] Abbreviations: Csk, C-terminal Src kinase; Lck, lymphoid cell kinase; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Fmoc, fluorenylmethoxycarbonyl; PMSF, phenylmethanesulfonyl fluoride; PEP, hematopoietic protein tyrosine phosphatase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Src, protein tyrosine kinase of Rous sarcoma virus; SH2, Src homology 2;, SH3, Src homology 3; GST, glutathione S-transferase; Csk_{SH3}, Src homology 3 protein domain of C-terminal Src kinase; Csk_{SH2}, Src homology 2 protein domain of C-terminal Src kinase; Csk_{SH3SH2}, covalently joined C-terminal Src kinase Src homology 3 and 2 domains; $Csk_{full\ length}$, full-length C-terminal Src kinase protein; Abl_{SH3}, Src homology 3 protein domain of the Abelson kinase; Lck_{SH3} domain, Src homology 3 protein domain of lymphoid cell kinase).

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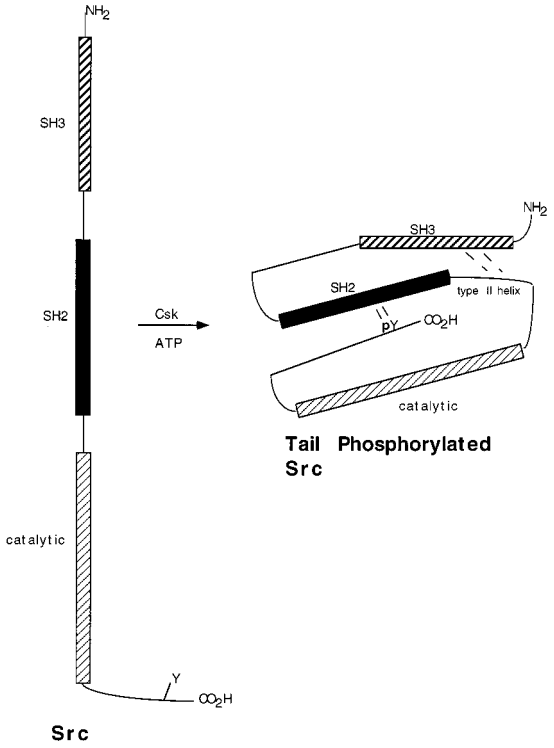

FIGURE 1: Tail tyrosine phosphorylation of Src by Csk leads to a conformational change. This conformational change involves intramolecular interactions between the Src SH3 domain and the SH2−catalytic connecting region (which adopts a type II polyproline helix conformation) and between the Src SH2 domain and the tail phosphotyrosine. The tail-phosphorylated form of Src and Src family members shows reduced tyrosine kinase activity compared to the tail-nonphosphorylated form.

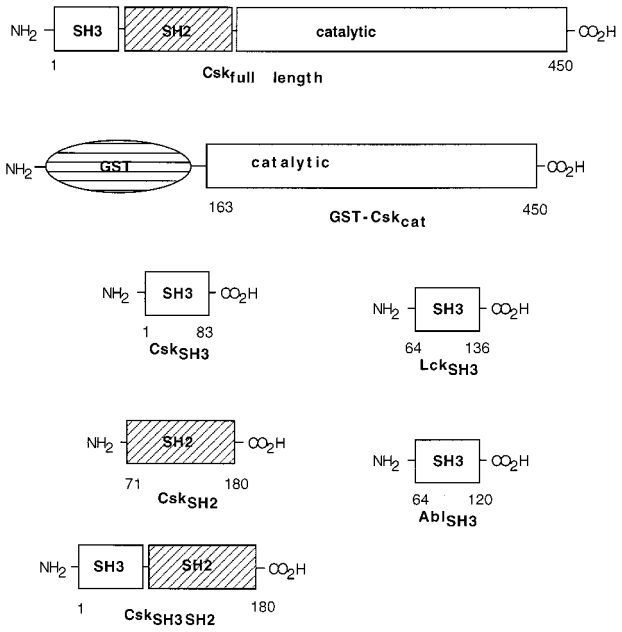

FIGURE 2: Protein constructs used in this work.

tyrosine phosphatase PEP has been reported to bind to the Csk SH3 domain and a phosphotyrosine-containing peptide sequence from Src to the Csk SH2 domain (*27*−*29*). The effects of mutations and deletions in the SH2 and SH3 domains on Csk kinase activity have been described and have provided apparently conflicting results (*30*−*33*). In some studies, such mutations were shown to have no effects whereas others were stated to lead to loss of catalytic activity. These previous investigations monitored Csk kinase function in crude cell extracts, immunoprecipitates, or in vivo, making them somewhat difficult to interpret.

Analogous to Src, ligation of a phosphotyrosine tail to Csk via expressed protein ligation appeared to induce a Csk conformational change involving intramolecular engagement of the phosphotyrosine tail by the Csk SH2 domain (*29*), but unlike Src, this conformation enhanced tail phosphorylation of Csk's physiologic protein substrate (Lck). To better understand the functional roles of the noncatalytic domains in Csk, we have undertaken a more complete analysis of the roles of SH2 and SH3 domains on Csk kinase activity using purified recombinant components and report the results here. An unexpected outcome of this work was the observation of a novel SH3 domain−catalytic domain interaction. This interaction appears to involve an SH3 surface distinct from the prototypical type II polyproline helix recognition pocket characteristic of SH3−protein interactions. Moreover, the interaction was shown to have functional consequences related to Csk kinase activity.

MATERIALS AND METHODS

General. Reagents not described below were obtained from commercially available sources except for Lck protein [prepared as described previously (*23*)], Abl SH3 domain (gift from David Cowburn, Rockefeller University), and peptides EDNEYTA (*26*) and KKKKEEIYFFF (*23*), which have been synthesized previously.

Preparation of GST−Csk$_{cat}$. Primers containing an *Nde*I site (upstream) and a *Hin*dIII site (downstream) were used to PCR-amplify the region of the Csk gene coding for the kinase domain (aa 163−450) of wild-type human Csk DNA. The PCR product was inserted in frame into the pGEX-3Xb vector (Pharmacia), and the resulting vector pGEX-3Xb-Csk$_{cat,}$ which was free of mutations in the coding region based on DNA sequencing, was transformed into DH5α strain of *E. coli* with ampicillin selection. Overproduction of the protein was carried out following similar procedures as for Csk$_{full\ length}$ protein. In brief, the cells were grown on a 6 L scale, induced with IPTG, and cells were resuspended in lysis buffer (25 mM Na-Hepes, pH 8.0, 150 mM NaCl, 1 mM DTT, 5% glycerol, 5% ethylene glycol, 1 mM $MgSO_4$, and 0.5 μg/mL DNase) and lysed by passage through a French pressure cell (*21*). The lysate was clarified by centrifugation and the supernatant was then incubated with 2 mL of glutathione−agarose (100 mg of dry resin swollen in water and preequilibrated with lysis buffer) for an hour at 4 °C with Nutator (Fisher Scientific) mixing. The suspension was centrifuged at 2000g for 5 min and the pellet was resuspended in and washed twice with 10 mL of high-salt buffer (lysis buffer with additional 200 mM NaCl) at 4 °C. The resultant resin was resuspended in 20 mL of glutathione buffer (lysis buffer with 50 mM glutathione and 200 mM additional NaCl, pH was adjusted to 7 with 8 N NaOH) for 45 min at 4 °C. The resin was pelleted and the recovered supernatant was dialyzed against storage buffer (20 mM Tris acetate, 10% glycerol, and 2 mM DTT, pH 8.0). The protein was concentrated by Centricon ultrafiltration to a concentration of 2 mg/mL. The GST−Csk$_{cat}$ prepared in this way was judged to be ∼90% pure by SDS−12%

PAGE stained with Coomassie and the protein yield was 0.5−1 mg/L of culture as determined by Bradford analysis. The protein was stored in aliquots at −80 °C.

Proteolysis of Csk and the Purification of the Catalytic Domain. $Csk_{full\ length}$ (300 μL, 5 mg/mL, in storage buffer) was treated with subtilisin Carlsberg (Sigma) (1.5 μg) for 30 min at 4 °C followed by quenching with 1 mM PMSF. The cleavage reaction mixture was then diluted with storage buffer so that protein concentration was 1 mg/mL (final volume = 1.5 mL). This solution was dialyzed against column buffer (25 mM Na-Hepes and 2 mM DTT, pH 7.7) and the resultant was loaded on a column of *O*-phospho-L-tyrosine−agarose (8 × 8 mm) over 5 min with the diluted cleavage reaction mixture and then eluted with low-salt column buffer (column buffer + 50 mM NaCl), which afforded pure catalytic domain (>95% by SDS−PAGE). The catalytic domain was determined to be 0.25 mg/mL by Bradford assay. N-Terminal sequencing of the protein revealed that cleavage occurred principally at three places affording proteins with the following N-termini: Ala-179, Asp-181, and Gly-187.

Preparation of Csk and Lck SH3 and SH2 Domains. Primers containing an *Nde*I restriction site (upstream) and a *Hin*dIII restriction site (downstream) were used to PCR-amplify various domains of Csk DNA as follows: Csk_{SH3} (residues 1−83), Csk_{SH2} (residues 71−180), and Csk_{SH3SH2} (residues 1−180). In an analogous fashion Lck_{SH3} (residues 64−136) was also prepared from an Lck DNA template. The genes amplified by PCR were then inserted in frame into the pGEX-3Xb vector (Pharmacia). The resulting expression plasmids (pGEX-3Xb-Csk_{SH3}, pGEX-3Xb-Csk_{SH2}, pGEX-3Xb-Csk_{SH3SH2}, and pGEX-3Xb-$Lck_{SH3)}$ were shown to be free of mutations in the coding region by DNA sequencing and were transformed into DH5α strain of *E. coli* using ampicillin selection. The cells were grown and lysed as described above for GST−Csk_{cat}. The supernatants were bound to glutathione resin and washed as described above, but instead of elution from the resin with glutathione-containing buffer, the pelleted resin was equilibrated with cleavage buffer (2 × 10 mL, 50 mM Tris acetate, 100 mM NaCl, 1 mM DTT, 1 mM $CaCl_2$, and 5% glycerol, pH 8.0). Following equilibration, the pelleted resin was incubated with 3 mL of cleavage buffer and 30 units of the protease factor Xa (Pharmacia) and mixed on a Nutator (Fisher Scientific) at 4 °C for 24 h in a 15 mL sealed plastic tube. The resin was then pelleted by centrifugation at 2000g for 5 min and the supernatant containing the cleaved protein was separated from the resin and quenched with 1 mM PMSF. The protein solutions were then dialyzed against storage buffer (20 mM Tris acetate, 10% glycerol and 2 mM DTT, pH 8.0). The cleaved proteins were of ∼90% purity as estimated by SDS−12% PAGE analysis. The concentrations of the proteins postdialysis were estimated by Bradford analysis and for a typical preparation were found to be approximately 1 mg/mL for $Csk_{SH3,}$ 0.25 mg/mL for $Csk_{SH2,}$ 1 mg/mL for Csk_{SH3SH2}, and 0.25 mg/mL for Lck_{SH3}. These preparations were stored in aliquots at −80 °C.

Preparation of $Csk_{SH3(W47V)}$. The W47V mutant of Csk_{SH3} was prepared by using the Stratagene QuikChange site-directed mutagenesis kit with pGEX-3Xb-Csk_{SH3} as the template and by following the suggested protocol. The resulting vector pGEX-3Xb-$Csk_{SH3(W47V)}$ was found to contain only the desired mutation, determined by DNA sequencing of the entire coding region. The protein $Csk_{SH3(W47V)}$ was then prepared following the procedure described above for the preparation of Csk and Lck domains. The final concentration of the purified mutant protein obtained was 0.4 mg/mL.

Synthesis of PEP Peptide. The protein tyrosine phosphatase PEP proline-rich peptide (PEP peptide, residues 605−629, NH_2-SRRTDDEIPPPLPERTPESFIVVEE-CO_2H) (*28*) was synthesized by automated Fmoc solid-phase chemistry on a Rainin PS3 machine on Wang resin (0.25 mmol). Cleavage from the resin and deprotection of side chains were carried out by stirring with 10 mL of reagent K (8.3 mL of trifluoroacetic acid, 0.48 mL of phenol, 0.48 mL of thioanisole, 0.48 mL of water, and 0.24 mL of ethanedithiol) for 5 h. After ether precipitation, the peptide was purified by HPLC on a C-18 reverse-phase column with a linear gradient [acetonitrile/water containing 0.05% v/v trifluoroacetic acid] and then lyophilized to dryness. The peptide was shown to have the correct mass by electrospray ionization mass spectrometry and the purity of the peptide was estimated to be >95% based on analytical HPLC analysis.

Biotinylation of PEP Peptide. PEP peptide (25 mg, 8.6 μmol) prepared as described above was taken prior to HPLC purification and dissolved in aqueous 100 mM Na-HEPES (pH 7.7, 1.4 mL) and subjected to reaction with (+)-biotin *N*-succinimidyl ester (Fluka) (15 mg, 43 μmol) in the presence of pyridine (15 μL, 190 μmol). The reaction was stirred at room temperature for 4 h. The biotinylated peptide was purified on a C-18 reverse phase column with a linear gradient [acetonitrile/water containing 0.05% (v/v) trifluoroacetic acid] and then lyophilized to dryness (14 mg). The biotinylated PEP peptide was shown to have the correct mass by electrospray ionization mass spectrometry and the purity of the peptide was estimated to be >90% based on analytical HPLC analysis.

Chromatography of SH3 Domains. (A) Preparation of PEP Peptide Resin. A column was prepared by pouring 1 mL of streptavidin−agarose solution (Sigma) into a plugged 3 mL plastic syringe. The column was equilibrated by washing with 15 mL of wash buffer (25 mM Na-Hepes, pH 7.4, 50 mM NaCl, 10% glycerol, 2 mM DTT, and 100 μM PMSF). The biotinylated PEP peptide (1 mL of 1 mg/mL, pH 7.0) was loaded on to the column and the flowthrough was collected and passed over the column again. This process was repeated two more times. The column was then washed with 5 mL of high-salt wash buffer (wash buffer containing a total of 500 mM NaCl). This column was further equilibrated with standard wash buffer (15 mL).

(B) Assessment of SH3 Domain−PEP Peptide Interaction. SH3 domain (1 mL of ∼0.5 mg/mL, 20 mM Tris acetate, 10% glycerol, and 2 mM DTT, pH 8.0) was eluted over the column and the flowthrough was reapplied twice again on the column. The column was then washed with 5 column volumes (5 mL) of wash buffer and the flowthrough was collected as 1 mL fractions. The column was then either eluted with wash buffer containing 1% SDS (5 mL) or with wash buffer containing the nonbiotinylated PEP peptide (1 mL of 2 mg/mL) and 1 mL fractions were collected. A control column was prepared and run following the exact same procedure with the exception that it was not treated with the biotinylated PEP peptide. All fractions collected

Table 1: Relative Steady-State Kinetic Parameters for $Csk_{full\ length}$ and Csk_{cat}

enzyme	K_m (μM) ATP	K_m (μg/mL) poly(Glu,Tyr)	k_{cat} (min^{-1}) poly(Glu,Tyr)	k_{cat}/Km (rel) poly(Glu,Tyr)	k_{cat}/Km (rel) Lck
$Csk_{full\ length}$[a]	12 ± 1	48 ± 2	40 ± 2	100	100
GST−Csk_{cat}[b]	17 ± 2	75 ± 11	0.4 ± 0.05	0.64	1

[a] Values for $Csk_{full\ length}$ taken from previous reports (*23*, *25*). [b] Values for GST−Csk_{cat} were determined as described in Materials and Methods. Kinetic constants are shown ± standard error.

were analyzed by running an SDS−12% or 15% polyacrylamide gel depending on the size of the protein being eluted. The Coomassie stained gels were scanned and the intensity of the bands was measured by using the Imagequant software package (Molecular Dynamics), allowing the ratio of bound versus unbound protein to be determined. Experiments with Csk domains were performed at least two times and duplicate runs gave results in good agreement (within 10%).

Affinity Purification of $Csk_{full\ length}$. Human recombinant Csk was overproduced in *E. coli* in combination with the chaperones GroES and GroEL as previously reported (*21*, *26*). After cell lysis, a portion of the supernatant (1 mL) was applied to the PEP peptide affinity column. A procedure analogous to the one described above was followed with the exception that the elution of the protein was carried out solely by competition with wash buffer containing the nonbiotinylated PEP peptide (1 mL of 2 mg/mL). All fractions were analyzed on a Coomassie-stained SDS−12% polyacrylamide gel.

Kinetic Assays of GST−Csk_{cat}: General. All experiments described below were performed at least two times. Duplicate runs generally gave results that agreed within 20%.

(A) Poly(Glu,Tyr) as Substrate. These were performed as described (*25*) where the phosporyl transfer of ^{32}P from [γ-^{32}P]ATP to poly(Glu, Tyr) was monitored. The standard assay conditions used were 60 mM Tris-HCl at 30 °C, pH 7.4, 10 mM DTT, 200 μg/mL BSA, 4 mM $MnCl_2$, 3 μCi of [γ-^{32}P]ATP, 60 μM ATP, 400 μg/mL poly(Glu,Tyr), and 500 nM GST−Csk_{cat} for 4 min and a 15 μL reaction volume. Activity was shown to be linear with time for 4 min and with enzyme concentration in the 0−1 μM range. Removal of the GST domain from GST−Csk_{cat} by factor Xa proteolysis or generation of Csk_{cat} by subtilisin proteolysis of $Csk_{full\ length}$ led to catalytic constructs that showed essentially identical kinase rates. For determination of K_m for ATP, a range of 0−100 μM ATP was employed, and for determination of K_m for poly(Glu, Tyr), a range of 0−400 μg/mL of poly(Glu, Tyr) was employed. Kinase assays for intermolecular activation of Csk_{cat} by SH3 domains of Csk, Abl, and Lck were also carried out with poly(Glu,Tyr) as the substrate under the conditions above. The range of concentrations tested for the various domains added in trans were as follows: Csk_{SH3} (0−20 μM), Csk_{SH3SH2} (0−20 μM), and $Csk_{SH3W47V}$ (0−20 μM). The other domains (Csk_{SH2}, Lck_{SH3}, and Abl_{SH3}) were employed at a concentration of 5 μM. The analysis of enzymatic reactions by SDS−PAGE, calculation of reaction velocities, and fit of the data to the Michaelis−Menten equation were carried out as described earlier (*25*). Viscosity experiments were performed with sucrose [0−35% w/v to ensure relative viscosity ratios of 1−3.1, respectively], in a manner analogous to previously described methods with saturating substrate concentrations (*25*). K_m values for ATP and poly(Glu,Tyr) were not significantly altered at the extremes of viscosity, ensuring that rates in the presence of viscogen represented k_{cat}.

(B) Lck as Substrate. GST−Csk_{cat} catalyzed phosphorylation of Lck was carried out according to previously described methods for $Csk_{full\ length}$ phosphorylation of Lck (*23*). In brief, reactions were performed at 30 °C, pH 7.4, with 100−500 nM GST−Csk_{cat}, 2 μM Lck (the Lck construct contains an N-terminal deletion of the first 63 amino acids and replacement of Lys-273 with Arg), 67 mM Tris-HCl, 8 mM Na-Hepes, 8 mM NaCl, 20 μM ATP, 67 μg/mL BSA, 5 mM $MnCl_2$, 10 mM DTT, and 3 μCi of [γ-^{32}P]ATP, in 15 μL for 4 min. Reactions were analyzed by SDS−PAGE and reaction velocities were determined as previously described (*23*).

(C) Peptides as Substrates. GST−Csk_{cat} − peptide phosphorylation was studied by a coupled spectrophotometric assay based on methods described previously (*26*). In summary, the reactions were carried out at 30 °C in 60 mM Tris-HCl (pH 7.4), 0.5 mM ATP, 190 μM NADH, 1 mM phosphoenol pyruvate, GST−Csk_{cat} (1−2 μM), excess lactate dehydrogenase−pyruvate kinase, 5 mM $MnCl_2$, and 10 mM DTT, and the peptide concentrations were 8 mM for EDNEYTA and 1 mM for KKKKEEIYFFF. In both cases, the kinase activity was below the level of detection (30-fold below the rate for $Csk_{full\ length}$).

RESULTS

Preparation and Catalytic Evaluation of the Catalytic Domain of Csk. To evaluate the possible influences of the SH2 and SH3 domains on Csk catalytic activity, the isolated Csk catalytic domain (Csk_{cat}) was subcloned into a GST fusion system (Figure 2). The N-terminal edge of the catalytic domain was chosen on the basis of sequence alignments to other protein kinases, and ∼10 additional residues were included because of the uncertainty about the precise requirements for a stable fold. The production of Csk_{cat} as a GST fusion protein allows soluble protein to be expressed without the need for GroES/GroEL chaperonin coexpression. After purification on a glutathione column (leading to ∼90% purity by SDS−PAGE), GST−Csk_{cat} was tested for catalytic activity. This domain was shown to be a very poor catalyst compared to $Csk_{full\ length}$. With the substrate poly(Glu,Tyr) and the physiologic substrate Lck, the overall specific activity was ∼100-fold lower compared to that of $Csk_{full\ length}$ (Table 1). The kinase activity with peptide substrates KKKKEEIYFFF and EDNEYTA was also reduced (below the limits of detection, at least 30-fold). Our first concern was that this reduced activity was related to potential perturbations that might be caused by the presence of the GST domain. Cleavage of the GST domain with factor Xa led to the formation of the GST-free Csk_{cat}, which was found to have very similar specific activity compared to GST−Csk_{cat}.

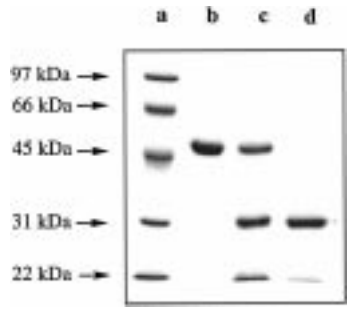

FIGURE 3: SDS−PAGE of Csk proteolysis reaction stained with Coomassie. Lane a, molecular mass markers; lane b, $Csk_{full\ length}$; lane c, $Csk_{full\ length}$ treated with subtilisin (see Materials and Methods for details); lane d, nonbinding fraction of the Csk proteolysis mixture passed over a phosphotyrosine column (see Materials and Methods for details).

We next considered the possibility that the reduced activity of the isolated Csk catalytic domain was an artifact due to protein instability or an improper fold during the production of the recombinant protein. We had observed previously that there is a subtilisin-sensitive cleavage site at the SH2 domain−catalytic domain border of Csk (*29*). Using subtilisin on a preparative scale, we were able to obtain ~1 mg Csk_{cat} by limited proteolysis (Figure 3). The SH3−SH2 moiety was removed by use of a phosphotyrosine affinity column, leaving the catalytic domain in greater than 95% purity by SDS−PAGE (Figure 3), and N-terminal sequencing confirmed that the cleavage occurred within a 7 amino acid region in the SH2 domain−catalytic domain border (three major N-termini were Ala-179, Asp-181, and Gly-187). The kinase activity of the Csk catalytic domain produced in this way, with substrates poly(Glu,Tyr) and Lck, was essentially identical (within 2-fold) compared to the analogous material produced in recombinant form (Csk_{cat}), suggesting that the reduced kinase activity was not an artifact of recombinant expression of the domain.

More detailed analysis of GST−Csk_{cat} by steady-state kinetics with poly(Glu,Tyr) as the tyrosine substrate showed that the effect was largely a k_{cat} effect, with the K_ms being within 2-fold of those of the full-length enzyme (Table 1). These data would suggest that the overall catalytic domain fold and the structure of the substrate binding sites is likely preserved and the reduced activity is probably a result of a subtle active-site structural alteration. Two principal explanations for k_{cat} reduction were considered: (i) Csk_{cat} could be a mixed (equilibrium) population of predominantly (~99%) kinase-dead protein and a small proportion (~1%) of fully active enzyme (with rate similar to the kinase activity of $Csk_{full\ length}$) or (ii) Csk_{cat} could be a homogeneous population of impaired catalyst. Assuming the catalyst was homogeneous, k_{cat} reduction could result from a very slow product release step [ADP or phospho-poly(Glu,Tyr)] or an impaired chemical step (phosphoryl transfer). To distinguish among these possibilities, the effects of viscosity on catalysis were examined (Figure 4). It has previously been shown that the $Csk_{full\ length}$ reaction shows a rate decrease in the presence of the microviscogen sucrose (*25*, *26*). The slope, +0.42, in a plot of k_{cat}(control)/k_{cat}(viscogen) vs relative viscosity ratio (Figure 4) is consistent with the product release being partially rate-determining (*25*). However, kinetic analysis of the active site Csk mutant D314E protein and viscosity experiments with $Csk_{full\ length}$ employing the alternative

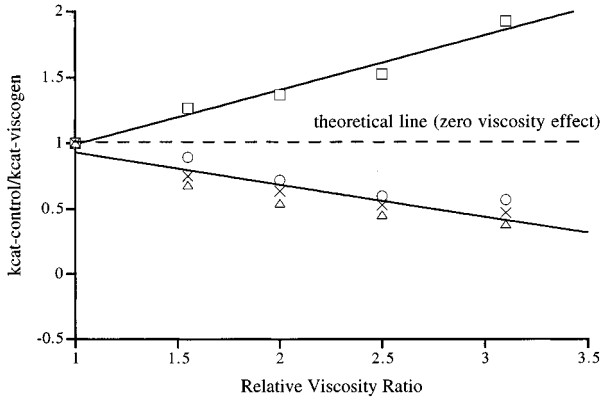

FIGURE 4: Sucrose microviscosity effect on k_{cat}. k_{cat}(control)/k_{cat}(viscogen) vs relative viscosity ratio for $Csk_{full\ length}$ with ATP (□, slope = +0.42 ± 0.04) and ATPγS (○, slope = −0.22 ± 0.03) (*25*); D314E $Csk_{full\ length}$ with ATP (△, slope = −0.28 ± 0.06) (*26*); GST−Csk_{cat} with ATP (×, slope = −0.24 ± 0.04). The best fit slopes (± standard error) were calculated by linear regression. Lines are shown for the fits from $Csk_{full\ length}$ with ATP (□) and GST−Csk_{cat} with ATP (×). See Materials and Methods for further details.

substrate ATPγS demonstrated a slight rate enhancement with increasing sucrose and modestly negative slopes (*25*, *26*). This nonspecific viscogen effect is general for Csk, related to sucrose and not macroviscosity in general, and has been discussed previously (*23*, *25*).[2] The results overall have been interpreted as indicating that the chemical step was rate-determining with "poor substrate" and "poor enzyme". Experiments with GST−Csk_{cat} revealed a similar modest negative slope (−0.24) essentially identical compared with D314E Csk (−0.28) and with ATPγS (−0.22) experiments. Thus it is likely that the chemical step is rate-determining for Csk_{cat} and furthermore that the loss of kinase activity was unlikely to be a consequence of a small proportion of fully active protein (which would have been expected to have a positive slope like that of $Csk_{full\ length}$) in a pool of completely dead enzyme.

Intermolecular Effects of SH3 and SH2 Domains on Csk_{cat} Catalysis. An obvious question that follows the observation of the decreased kinase activity associated with Csk_{cat} vs $Csk_{full\ length}$ pertains to the structural contributions of the SH2 and/or the SH3 domains in enhancing catalysis. Two different types of effects that might enhance the kinase domain are (i) indirect and/or weak interactions that would require an intact protein backbone and possibly sequences directly contiguous to the catalytic domain and (ii) direct and (most likely) higher affinity through space contacts between the catalytic domain and the SH2 and/or SH3 domain. To discriminate between these possibilities, the SH2, SH3, and linked SH3−SH2 protein domains of Csk were prepared by overexpression in *E. coli* and purified to near homogeneity (Figure 2). These domains were then tested in trans (in excess) at concentrations of 5 μM with GST−Csk_{cat} for their ability to affect kinase activity. Csk_{SH2} domain showed no effect (<20%) but the Csk_{SH3} and Csk_{SH3SH2} domains led to modest but reproducible enhancements of kinase activity (Figure 5). Examination of the concentration dependence of

[2] While in principle the nonspecific sucrose slope effect can be subtracted from the positive slope effect with wild-type enzyme and normal substrate to afford more precise rate constants, the relative correction is relatively minor and does not significantly affect the conclusions (*25*).

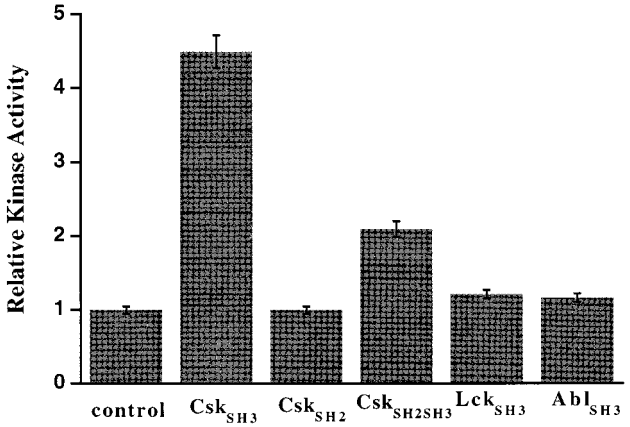

FIGURE 5: Activation of GST−Csk$_{cat}$ kinase activity by addition of various SH3 and SH2 protein domains (see Materials and Methods for details).

these enhancements showed saturation behavior, and the apparent K_ds of activation were found to be 2.2 ± 0.8 μM and 8.8 ± 0.9 μM, respectively for Csk$_{SH3}$ and Csk$_{SH3SH2}$ (Figure 6). The extrapolated maximal stimulation was similar for both (4−5-fold). It should be noted that the maximal activation rate is still 20-fold below the Csk$_{full\ length}$ k_{cat}, however, suggesting that the intermolecular Csk$_{SH3}$−Csk$_{cat}$ complex is still somewhat structurally defective compared to Csk$_{full\ length}$. These effects were not related to the GST domain on the GST−Csk$_{cat}$ construct, as the kinase activity of the GST-free catalytic domain was affected by Csk$_{SH3}$ in an identical fashion. Furthermore, Csk$_{SH3}$ domain had no impact on the kinase activity of Csk$_{full\ length}$, nor was it significantly phosphorylated by either Csk$_{full\ length}$ or Csk$_{cat}$ in the concentration ranges investigated.

Stimulation of the Csk$_{cat}$ kinase activity by Csk$_{SH3}$ and not by Csk$_{SH2}$ suggests that there is an interaction site between the catalytic domain and the SH3 domain. To determine the specificity of this interaction, two other SH3 domains, Abl$_{SH3}$ and Lck$_{SH3}$, were probed for their effect on the Csk$_{cat}$. Abl$_{SH3}$ and Lck$_{SH3}$ showed no effect (<20%, with 5 μM of either SH3 domain) on the catalytic domain kinase activity. Since it was possible that these SH3 domains could bind to the catalytic domain without activation, these domains were tested as competitive inhibitors of Csk$_{cat}$ activation. In this experiment, no inhibition (<20%) of the activation was detected. These results suggest that the Csk$_{SH3}$−Csk$_{cat}$ interaction is specific to the Csk$_{SH3}$ sequence and not conferred by the general SH3 protein fold.

PEP Peptide Effects on Binding and Catalysis. The observation of a specific interaction between the SH3 domain and catalytic domain would reasonably be expected to involve the SH3 surface that binds type II helix polyproline sequences. Indeed, Csk$_{cat}$ has several stretches that contain multiple prolines. To further probe the Csk$_{SH3}$−Csk$_{cat}$ interaction, a proline-rich peptide (PEP peptide) was synthesized on the basis of its reported ability to bind the Csk SH3 domain (*28*). A portion of this peptide was chemically biotinylated on its N-terminus and used to generate an affinity column with streptavidin. As expected, this column was shown to specifically pull down the Csk$_{SH3}$ domain (98% bound) compared to the control column that lacked PEP peptide (15% bound), suggesting a high-affinity interaction between the Csk SH3 domain and this proline-rich peptide.

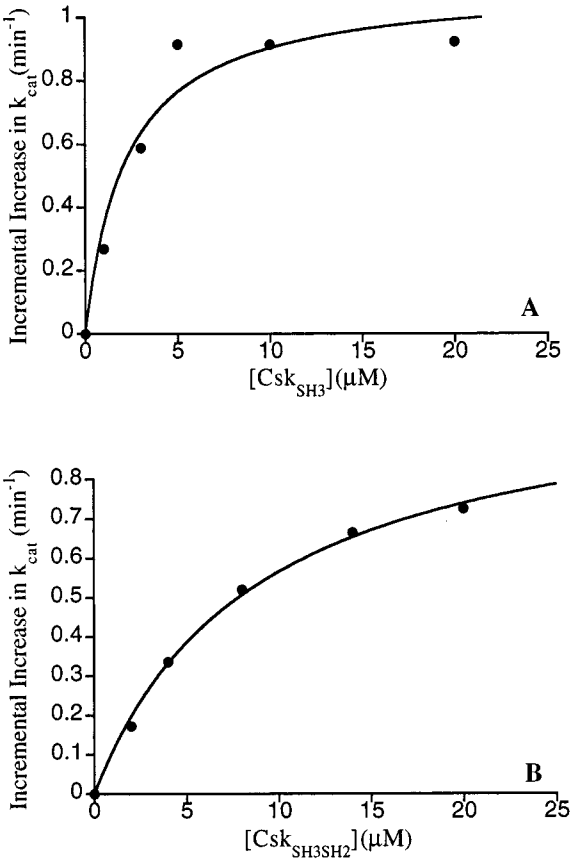

FIGURE 6: Activation of kinase activity GST−Csk$_{cat}$ vs SH3 protein domain concentration. (A) Incremental increase in k_{cat} as a function of Csk$_{SH3}$ (K_d of activation = 2.2 ± 0.8 μM, activation maximum = 5.4 ± 0.6-fold); (B) Incremental increase in k_{cat} as a function of Csk$_{SH3SH2}$ (K_d of activation = 8.8 ± 0.9 μM, activation maximum = 4.4- ± 0.2-fold). Data were fit in both cases to a standard rectangular hyperbola ligand−receptor interaction equation and the kinetic constants (±standard error) were calculated by a nonlinear curve fit with the KaleidaGraph computer program (Macintosh). Kinase reactions were carried out with saturating ATP (100 μM) and poly(Glu,Tyr) (400 μg/mL) concentrations, and all concentrations of substrates and activating domains were at least 10-fold greater than that of GST−Csk$_{cat}$. See Materials and Methods for further details.

Elution of the Csk$_{SH3}$ domain from the column required either denaturant (1% SDS) or the nonbiotinylated proline-rich peptide (2 mg/mL). The PEP peptide column was unable to specifically pull down Abl$_{SH3}$ (17% bound) and Lck$_{SH3}$ (<20% bound).

Having confirmed a specific interaction between the isolated Csk$_{SH3}$ domain and PEP peptide, PEP peptide was examined for its ability to bind Csk$_{full\ length}$. If the SH3 domain was interacting with the catalytic domain with a surface that the SH3 domain used to interact with PEP peptide, it might be expected that the SH3 domain of full-length Csk would be unavailable for interaction with the PEP peptide affinity column. In fact, Csk$_{full\ length}$ binds to the PEP peptide affinity column quite well (77% bound), albeit possibly slightly more weakly than the isolated SH3 domain (98% bound). As a control, it was shown that nonspecific binding of Csk$_{full\ length}$ to the column that lacked PEP peptide was low (23% bound). To further demonstrate the specificity of this interaction, this PEP peptide affinity column was used to purify recombinant Csk$_{full\ length}$ to near homogeneity from *E. coli* cell extracts (Figure 7).

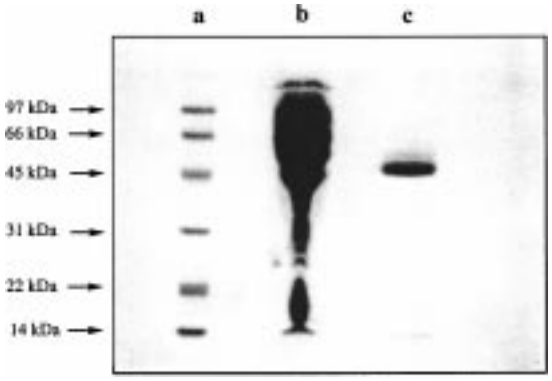

FIGURE 7: Coomassie-stained SDS−PAGE showing affinity purification of $Csk_{full\ length}$ using PEP peptide resin. Lane a, molecular weight markers; lane b, *E. coli* cell extracts containing recombinant $Csk_{full\ length}$; lane c, fraction (major band is $Csk_{full\ length}$) that was specifically eluted from the column with PEP peptide. See Materials and Methods for details.

Given the inability of the Csk catalytic domain to antagonize the Csk SH3 domain from binding to PEP peptide, it was next of interest to determine the impact of PEP peptide on kinase activity of the alternative constructs of Csk. Addition of PEP peptide (500 μM) to $Csk_{full\ length}$ had no effect (<20%) on its phosphorylation of poly(Glu,-Tyr) or the full-length physiologic substrate Lck. Addition of PEP peptide to a Csk_{SH3}/Csk_{cat} mixture mildly attenuated, but did not abolish, the ability of Csk_{SH3} to activate Csk_{cat}. In the presence of PEP peptide, the K_d of activation of Csk_{SH3} rose to 6.3 ± 0.4 μM (3-fold increase) while the maximal activation was unchanged. These results suggest that engagement of Csk_{SH3} by PEP peptide can have some impact on its intermolecular binding to or activation of Csk_{cat}, but it is likely to be a minor, indirect effect and not a direct, competitive relationship.

Effect of Mutant SH3 Domain on Binding and Catalytic Activation. To further clarify the nature of the Csk_{SH3}−Csk_{cat} interaction, a point mutant in the isolated SH3 domain was generated, $Csk_{SH3W47V}$. This highly conserved residue, tryptophan 47, is expected to be crucial for binding type II helix polyproline peptides. As predicted, $Csk_{SH3W47V}$ showed greatly diminished binding to PEP peptide containing resin (26% bound), which was near background levels (10−20%). However, $Csk_{SH3W47V}$ demonstrated wild-type ability to activate Csk_{cat} (K_d of activation for $Csk_{SH3W47V}$ was 1.6 ± 0.3 μM compared to 2.2 ± 0.8 μM for Csk_{SH3}; similar 4−5-fold maximal activation rates). These results further support the proposal that distinct surfaces of Csk_{SH3} are involved in PEP peptide as opposed to Csk_{cat} interaction.

DISCUSSION

Delineating the structural and functional roles of noncatalytic domains in protein tyrosine kinases and phosphatases is an intensive area of research. The general view is that the catalytic domains of these enzymes are fully capable of catalyzing phosphoryl transfer and that noncatalytic domains are likely to play roles in substrate selection and cellular localization or as modulatory switches in interconverting active versus inactive states. Consistent with these ideas, many recombinant catalytic domains of protein tyrosine kinases and protein tyrosine phosphatases appear to be essentially fully catalytically active, including those isolated from the insulin receptor kinase, Src, Fps, and the tyrosine phosphatases SHP-1 and SHP-2 (*34*−*42*). Perhaps the two best studied cases in revealing structure−function relationships among these are Src and SHP-2. In both examples, the catalytic domains are at least as active as the full-length proteins, depending on their phosphorylation states or the presence of allosteric regulatory peptides/proteins. The SH2 domains in both of these proteins undergo intramolecular interactions that lead to inhibition of catalytic activity, which can be relieved by intermolecular phosphotyrosine peptide competitive displacement. In the case of Src, this interaction was shown to be a canonical SH2 domain−tail phosphotyrosine interaction that enforced an additional intramolecular SH3-type II helix interaction (Figure 1). For SHP-2, the SH2−catalytic domain interaction was shown to involve alternative contacts, preventing access of the substrate to the phosphatase active site.

Consequences of Domain Deletions in Csk. In light of the above paradigms, and because of the obvious importance of Csk in signal transduction, we undertook a detailed functional analysis of the Csk catalytic domain. The catalytic domain of Csk is clearly deficient as a catalyst when removed from the noncatalytic SH2 and SH3 domains. The consistency of catalytic behavior of the domain, whether produced in recombinant form or by proteolysis of the full-length protein, strengthens the assertion that the catalytic domain is a stable structure but defective in protein kinase function. Furthermore, the consistent rate reduction for unstructured peptides [EDNEYTA, KKKKEEIYFFF, poly(Glu,Tyr)] and a physiologic protein substrate Lck, as well as viscosity experiments, suggests that the problem is a mechanical deficiency in catalyzing phosphoryl transfer rather than simply a problem in tyrosine substrate recognition. Indeed, the normal K_ms for ATP and poly(Glu,Tyr) substrates and the basically normal protein kinase fold revealed by X-ray crystal structure of this domain (*43*) support the idea that the loss of activity is likely due to a rather subtle structural defect.

The ability of Csk_{SH3} to rescue a portion of the catalytic reduction in an intermolecular fashion was unexpected and points to a likely important intramolecular native interaction between these domains within full-length Csk. That this interaction was specific—it could not be substituted by other SH3 domains—and had relatively high apparent affinity (2 and 9 μM for SH3 and SH2) further points to its relevance. The observation of only partial recovery (5-fold out of 100-fold loss) suggests that the intermolecular Csk_{SH3}−Csk_{cat} interaction does not afford a completely native catalytic domain structure and that the chemical step is fully rate-limiting in the kinase reaction catalyzed by this complex.

The general and well-understood mode for SH3 domain protein−protein interactions involve the SH3 domain type II helix binding surface. Although many SH3 domain−type II helix interactions have been studied in vitro and in vivo, a relatively small number have been convincingly demonstrated to be physiologically relevant in signal transduction. Such biologically important interactions include Grb2−Sos (*7*), Src−intramolecular (*12, 13*), and Src−arrestin (*44*).

Extensive thermodynamic, structural, and biochemical studies have been carried out on SH3−type II helix interactions. SH3 domains have a characteristic fold, two triple-stranded antiparallel pleated β-sheets. Two loop regions known as the Src loop and the RT loop participate in forming

a binding groove on the SH3 domain for type II helix interactions. A highly conserved tryptophan residue within SH3 domains has been demonstrated to be essential for such interactions. SH3 domains often bind the peptide sequence PPXPP, a consensus recognition sequence with the secondary structure characteristic of a type II polyproline helix (*7*). The location of a positive charge of an Arg or Lys side chain contiguous with the polyproline sequence is believed to mediate orientation of the polyproline helix in the SH3 binding groove.

To examine in more detail the $Csk_{SH3}-Csk_{cat}$ interaction, a proline-rich peptide (PEP peptide) was employed to probe the nature of the SH3 binding surface involved. The PEP peptide sequence was originally identified in a two-hybrid screen by Veillette and co-workers, and the specific interaction sequence within the protein tyrosine phosphatase has been mapped previously (*27*, *28*). On the basis of this sequence, we synthesized a 25-amino acid peptide (PEP peptide), and a portion was used for preparation of an affinity column via N-terminal biotinylation. As expected, this sequence was able to specifically pull down Csk_{SH3} as well as $Csk_{full\ length}$ and could be specifically eluted by PEP peptide. Indeed, we envisage that this interaction will be useful as an alternative to phosphotyrosine chromatography for the rapid and efficient purification of Csk from cell extracts (Figure 7).

That the immobilized PEP peptide could bind nearly as well to $Csk_{full\ length}$ as it could to Csk_{SH3} was the first of several pieces of evidence that the $Csk_{SH3}-Csk_{cat}$ interaction did not involve the same Csk_{SH3} surface as the $Csk_{SH3}-$PEP peptide interaction. Other evidence pertaining to this point was that addition of PEP peptide (500 μM) failed to significantly affect $Csk_{full\ length}$ kinase activity and only weakly attenuated the intermolecular activation of Csk_{cat} by Csk_{SH3}. Perhaps the most persuasive data demonstrating that Csk_{SH3} interacts with Csk_{cat} and PEP peptide in different ways came from site-directed mutagenesis studies. $Csk_{SH3W47V}$ was unable to bind PEP peptide but showed potency akin to wild-type Csk_{SH3} in Csk_{cat} activation. It should be noted that these studies help explain the potentially discordant findings obtained in the reports by Koegl et al. (*33*) and by Howell and Cooper (*31*). In the previous studies, point mutations in the Csk SH3 and SH2 domains in full-length Csk failed to affect Csk phosphorylation of the physiologic Src substrate whereas SH3−SH2 domain deletions resulted in complete loss of Csk catalytic activity.

To our knowledge, the novel way in which the Csk SH3 domain supports catalysis by Csk via a presumed intramolecular interaction with the Csk catalytic domain (Figure 8) represents the first clear biological assignment of an SH3 domain functioning in protein−protein interactions without using its type II helix binding surface. While the Nef−Hck SH3 interaction involves regions outside of the standard SH3 binding groove (*7*), it also involves the canonical SH3 type II helix binding site. Interestingly, studies on the SH3 domain by Kim and co-workers (*45*) identified a D-amino acid-containing peptide by phage display that appeared to bind an SH3 domain in an atypical fashion. However, the biological relevance of this interaction has not been established. In the case of Csk, the relatively high apparent affinity of the SH3−catalytic domain interaction deduced from enzyme activation (K_d = 2.2 μM and 8.8 μM for the free

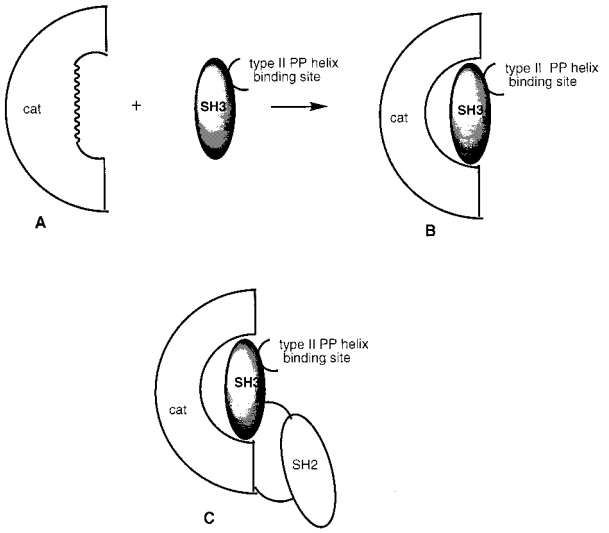

FIGURE 8: Schematic illustrating the $Csk_{cat}-Csk_{SH3}$ interaction. (A) Free Csk_{cat} and free Csk_{SH3}; (B_ Csk_{cat} bound to Csk_{SH3}; (C) $Csk_{full\ length}$. Type II PP helix indicates a type II polyproline helix.

SH3 and the SH3−SH2 domains, respectively) is of a level typical of strong SH3-peptide interactions and further underscores its physiological significance.

The detailed molecular basis of the Csk SH3−catalytic domain interaction is still unsettled and must await higher resolution studies. Sequence alignment of the Csk SH3 domain versus other SH3 domains does not reveal a single obviously distinctive site. The X-ray structure of the Csk SH3 domain was shown to contain a standard SH3 protein fold (*46*). It was noted that Csk SH3 residues Tyr-18 and Tyr-64 appeared to mediate intermolecular interactions in the crystal, and the authors speculated that these groups could be involved in interdomain interactions in the Csk full-length structure (*46*). However, these residues are reasonably well-conserved in several SH3 domains (*7*) including Abl_{SH3}, which lacked ability to interact with Csk_{cat}.

Protein Tyrosine Kinase Csk Substrate Recognition, Regulation, and Function. The studies reported herein give further insight into the authentic roles of noncatalytic domains in Csk cellular function. It seems clear that engagement of the Csk SH3 domain by a type II helix proline-rich peptide neither enhances nor detracts from Csk's ability to catalyze phosphoryl transfer. These studies complement previously reported experiments that a phosphotyrosine peptide that can bind to the Csk SH2 domain does not affect Csk's catalytic activity (*29*). Moreover, Csk's noncatalytic domains do not appear to be important in enhancing substrate selectivity as the rate decline of Csk_{cat} with the random copolymer poly(Glu,Tyr) substrate was similar to that with the physiologic substrate Lck.

A recent high-resolution X-ray structure of the Csk catalytic domain (residues 177−450) demonstrates that it shares a fold nearly identical with those of other catalytic domains of protein kinases (*43*). It is noteworthy that this structure showed a disordered regulatory loop, which is a common feature of inactive states of protein kinases (*12*, *13*). While few details were provided in the report about the catalytic activity of the construct used for crystallography, on the basis of the results herein it can be safely assumed that the crystallized protein showed diminished kinase

activity compared to $Csk_{full\ length}$. Based on these findings, a reasonable speculation would have the Csk SH3 domain interacting with the Csk catalytic domain in $Csk_{full\ length}$ in such a way that the regulatory loop is rigidified. This intramolecular interaction could compensate for the lack of an activating phosphorylation event in the case of Csk. A direct interaction between the Csk SH3 domain and the Csk regulatory loop solely involving the loop region seems unlikely, however, since a synthetic peptide based on the regulatory loop was unable to disrupt intermolecular Csk_{SH3}-mediated Csk_{cat} kinase activation (unpublished data from this lab).

The concept that domains outside of the catalytic core of protein kinases can provide constitutive catalytic enhancement has not been well-established in protein tyrosine kinase function. Csk may have evolved in such a way as to replace a reversible regulatory loop activating phosphorylation by accessory intramolecular interactions from its noncatalytic domains. Src has the same linear modular layout as Csk but appears to have very different intramolecular domain interactions (*12*, *13*, *29*). While the activation of tyrosine kinase domains by phosphorylation or the binding of partner proteins via intermolecular events is now well-accepted, the ability of intramolecular noncatalytic domains to enhance the rate of the chemical step in protein kinases as in the Csk case may provide a paradigm for constitutively active members of the protein kinase superfamily.

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