Molecular Determinants for Csk-Catalyzed Tyrosine Phosphorylation of the Src Tail†

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ABSTRACT: Phosphorylation of a critical tail tyrosine residue in Src modulates its three-dimensional structure and protein tyrosine kinase activity. The protein tyrosine kinase Csk is responsible for catalyzing the phosphorylation of this key Src tyrosine residue, but the detailed molecular basis for Src recognition and catalysis is poorly understood. In this study, we investigate this phosphorylation event using purified recombinant Csk and Src proteins and mutants. It was shown that the apparent k_{cat} and K_m values for Csk phosphorylation of catalytically impaired Src (dSrc) are similar to the parameters for Csk-catalyzed phosphorylation of the Src family member Lck. The SH3 (Src homology 3) and SH2 (Src homology 2) domains of dSrc were fully dispensable with respect to rapid phosphorylation, indicating that the catalytic domain and tail of dSrc are sufficient for the high efficiency of dSrc as a substrate. Of the eight Src tail residues examined, only the fully conserved Glu (Y-3 position) and Gln (Y-1 position) investigated by alanine scanning mutagenesis caused large reductions (10−40-fold) in dSrc substrate efficiency. The Y-3 Glu requirement was stringent as conservative replacements with Asp or Gln were no better than Ala whereas replacement of the Y-1 Gln with Ile was readily tolerated. Interestingly, en bloc replacement of the tail with a seven amino acid consensus sequence derived from a peptide library analysis was no better than the wild-type sequence. Surprisingly, the dSrc Y527F protein, although not a Csk substrate, enhanced Csk-catalyzed phosphorylation of dSrc. These results and other data suggest that Src dimerization (or higher order oligomerization) is important for high-efficiency Csk-catalyzed phosphorylation of the Src tail.

Protein kinase substrate selectivity is a critical feature in cell signal transduction (*1*, *2*). Despite its importance, the molecular details governing how protein kinases recognize their protein substrates are in many cases poorly understood. Synthetic peptide libraries have been used to enhance our understanding of protein kinase substrate recognition (*3*, *4*). In favorable instances, the consensus sequences show great predictive value for identification of in vivo targets. A salient example of successful prediction is in the case of protein kinase A, a serine/threonine kinase, where optimized peptides correlate well with several cellular protein targets of this enzyme (*5*, *6*).

In contrast, simple sequence algorithms appear to break down in the tyrosine phosphorylation of Src[1] catalyzed by Csk (C-terminal Src kinase) (*7*). Csk is a 50 kDa protein tyrosine kinase comprised of three modular domains, SH3, SH2, and the catalytic domains (*8*). Unlike many protein kinases, Csk does not undergo autophosphorylation and for this reason has been somewhat simpler to study enzymologically. Much has been learned recently about the details of its chemical mechanism of phosphorylation (*9*−*13*). Csk's SH3 and SH2 domains are important for enhancing the catalytic activity of the Csk kinase domain but appear to be dispensable for peptide and protein substrate recognition (*14*).

A major class of protein substrates for Csk are members of the Src family. Src and its family members are protein tyrosine kinases that have a similar architectural layout to Csk but also include an "SH4" variable domain (*15*, *16*). These enzymes are highly regulated by phosphorylation, including an autophosphorylation event in the activating loop. Csk is the enzyme responsible for catalyzing phosphorylation of a special tail tyrosine residue (Tyr-527 of chicken Src) of Src family members. This phosphorylation event has important structural and functional consequences for Src and its family members (*15*−*22*). Upon tail phosphorylation, Src family members undergo a conformational change initiated by an intramolecular interaction between the phosphotyrosine tail and the SH2 domain, and show reduced kinase activity (*15*−*22*).

Short peptides modeled on physiologic sequences in Src family member protein tails are poor Csk substrates (*23*, *24*).

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[1] Abbreviations: ATP, adenosine triphosphate; Csk, C-terminal Src kinase; Lck, lymphoid cell kinase; Src, protein tyrosine kinase of Rous sarcoma virus; dSrc, Src with amino-terminal deletion of the first 82 amino acids and replacement of arginine-295 with methionine and attachment of an N-terminal 6 histidine tag; SH2, Src homology 2; SH3, Src homology 3; SH4, Src homology 4; CE-dSrc, combinatorially engineered dSrc; IPTG, isopropyl thiogalactoside; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Moreover, an optimized peptide from a combinatorial library study showed little similarity to Src family tails (*23*). It has been shown with the Src family member Lck that purified, recombinant Lck protein (catalytically impaired) is an efficient substrate for Csk (*23*). Thus, the information for Csk's high selectivity toward substrate Lck is encoded somewhere within Lck's amino acid sequence and/or three-dimensional structure and does not rely on extra proteins or other posttranslational modifications. The precise details for Csk's recognition and phosphorylation of Src family members remain mysterious.

One of the technical factors which has slowed progress in this area has been the reliance on eukaryotic expression systems to generate purified recombinant Src and its family members in milligram quantities. Possibly as a result, a systematic analysis of the efficiency of Csk's phosphorylation of purified mutant Src proteins has not been undertaken. In this report, we describe an improved *E. coli* expression of catalytically impaired Src protein which has been exploited in the study of Csk's selectivity for Src substrates. The key findings include the dispensability of Src's SH2 and SH3 domains in phosphorylation by Csk, the strong requirement for two Src residues for efficient phosphorylation, and the unexpected cooperativity of Src polypeptides as substrates for Csk.

MATERIALS AND METHODS

General. Tris, DTT, ATP, BSA, poly(Glu,Tyr), and Triton X-100 were obtained from Sigma; imidazole, $MnCl_2$, and EDTA were obtained from Fisher. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from NEN. Csk was prepared as reported previously (*12*, *25*). Anti-Src antibody GD11was purchased from Upstate Biotechnology.

Overproduction and Purification of dSrc and Mutant Proteins (Figure 1). The coding region for amino acid residues 83−533 of chicken *c-src* gene was subcloned into pRSET vector (Invitrogen) with *Bam*HI and *Hin*dIII sites on the 5′- and 3′-ends, respectively. The site-directed mutant *c-src* K295M (*dsrc*) was made with Promega's Gene Editor system. The constructed plasmid pRSET-dsrc was cotransformed into BL21(DE3) strain of *E. coli* with the GroES and GroEL expression plasmid DNA in the presence of ampicillin (100 μg/mL) and kanamycin (50 μg/mL) (*25*). The cells were grown in 2XYT media in shaker flasks at 37 °C to reach OD_{600} = 0.5 on a 1 L scale and then induced with 0.5 mM IPTG at 25 °C for 14 h. After harvesting, the cell paste was resuspended in 60 mL of lysis buffer (20 mM Na-Hepes, pH 7.5, 200 mM NaCl, 1 mM DTT, 1% Triton X-100) and lysed by a single passage through a French pressure cell (13 000 psi). The resultant suspension was centrifuged for 30 min at 23000*g* at 4 °C, and the supernatant was collected.

dSrc and mutant proteins were purified by chromatography over a Zn-chelating column (Amersham Pharmacia Biotech). The lysate was loaded onto the preequilibrated column (20 mL) with 3 column volumes of washing buffer (lysis buffer plus 10 mM imidazole) at a flow rate of 1 mL/min. This was followed by washing with 2 column volumes of 10 and 50 mM imidazole containing washing buffer. The protein was eluted from the column with a stepwise gradient−100 mM, 200 mM, and 300 mM imidazole-containing buffers (20 mM Na-Hepes, pH 7.5, 200 mM NaCl, 1 mM DTT, 2 column volumes each), and 10 mL fractions were collected. Purified dSrc protein and mutants eluted at the 200 and 300 mM imidazole eluting steps. Fractions containing dSrc were dialyzed at 4 °C in a Slide-A-Lyzer (Pierce, 10 000 MWCO) first against intermediate buffer (20 mM Na-Hepes, pH 7.5, 200 mM NaCl, 5% glycerol, 5 mM DTT, and 100 mM imidazole) and then against storage buffer (20 mM Na-Hepes, pH 7.5, 200 mM NaCl, 5% glycerol, and 10 mM DTT). dSrc protein was concentrated by Centriprep ultrafiltration (Millipore) to a final concentration of ∼3 mg/mL and determined to be >90% pure by Coomassie-stained 10% SDS−PAGE. The protein yield was about 15 mg/L of *E. coli* cell culture as determined by Bradford analysis.

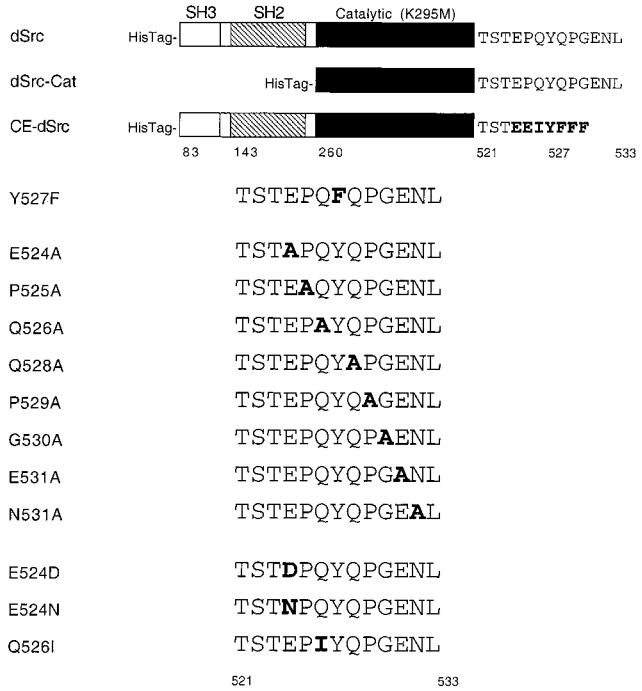

FIGURE 1: Constructs of dSrc and mutants used in these experiments. Residues mutated in these studies are highlighted as boldface letters. CE-dSrc is combinatorially engineered dSrc, and the optimal residues from peptide library studies replace those in the natural Src tail.

The plasmids for dSrc mutants containing single-residue mutations were prepared by the QuikChange method (Stratagene) using pRSET-dsrc as a template. The His-tagged *dsrc-cat* construct coding for the kinase domain and C-terminal tail (aa 260−533) was obtained by PCR amplification with primers containing a *Bam*HI site and a *Hin*dIII site. The amplified fragment was then subcloned in-frame into pRSET A vector. The CE-dsrc DNA plasmid was prepared by PCR amplification of the CE-dsrc segment, which was inserted in-frame into the pRSET A vector via *Bam*HI and *Hin*dIII restriction sites. Each construct was confirmed by DNA sequencing of the entire open reading frame. Mutant proteins were overexpressed and purified following the same procedure described above. The final purity of mutant proteins was typically about 70−80% as judged by visual inspection of the dried Coomassie-stained gels. The molecular weights of several of these proteins were confirmed by MALDI mass spectrometer analysis, indicating the absence of proteolytic digestion.

Csk Kinase Assays with dSrc and Mutant dSrc Protein Substrates. Steady-state kinetic assays with Csk phosphorylating dSrc and mutant proteins were carried out analogous to previously described methods (*23*). In brief, reactions were performed at 30 °C and pH 7.4 with 1−12 nM Csk, 0.4−15 μM Src or mutants, 2 mM $MnCl_2$, 50 μM ATP, 0.2 μCi of [γ-^{32}P]ATP, 60 mM Tris-HCl, 30 mM NaCl, 4 mM Na-Hepes, 10 mM DTT, and 200 μg/mL BSA in a 15 μL reaction volume for 2 min. Phosphorylation of dSrc was shown to occur linearly with respect to time (to 6 min) and Csk concentration in the ranges used. The optimal Mn concentration was shown to be 1−4 mM. For determination of the apparent K_m for ATP, a range of 0−60 μM ATP and a fixed concentration of 18 μM dSrc were employed. Reactions were initiated with Csk and quenched with 7.5 μL of aqueous Na-EDTA (100 mM, pH 8). A 20 μL aliquot of the quenched reaction mixtures was loaded onto a 10% SDS−PAGE. The gels were stained with Coomassie which revealed the protein band corresponding to phosphorylated dSrc or its mutants, which was subsequently excised with a blade. The radioactivity of gel slices was quantitated by liquid scintillation counting. All assays were performed at least twice, and duplicates typically agreed within 20%. In all cases, reaction of the limiting substrate did not exceed 10%. Calculations using the Michaelis−Menten equation were carried out using a nonlinear curve-fitting approach as described previously (*9*).

For dSrc and mutant assays, a control measurement was performed using the maximal concentration of the dSrc substrate in the absence of Csk. Under these circumstances, the radioactivity of background autophosphorylation from dSrc or its mutants was negligible (less than 10% of the signal produced in the presence of Csk and generally not distinguishable from minus dSrc background). When observed, this very low background activity was shown to be dSrc concentration dependent [as was observed for Lck previously (*23*)] and thus was negligible for the lower dSrc concentrations as well. Because of its very low level, this background rate did not affect significantly the rate measurements or subsequent calculations reported in this study.

Kinase Assays in the Presence of dSrc-Y527F Mutant. To measure the effect of nonphosphorylated dSrc-Y527F on the phosphorylation of protein and peptide substrates, the kinase reactions were carried out with fixed dSrc (0.4 μM) or poly-(Glu,Tyr) (1000 μg/mL) and varying concentrations of dSrc-Y527F as described below.

Kinase Assays in the Presence of Anti-Src Antibody. These reactions were carried out with a fixed concentration of dSrc (0.6 μM), Csk (3 nM), and antibody (+ or −, 0.3 μM) using the above-described conditions. This monoclonal GD11 antibody (IgG1) recognizes residues 82−169 of Src protein. Csk did not catalyze phosphorylation of this antibody or related impurities with the concentrations employed. The antibody did not detectably increase the background dSrc autophosphorylation rate.

RESULTS

Overproduction of dSrc and Mutants. Catalytically inactive Src (dSrc) containing the SH3−SH2−catalytic domains and containing a K295M mutation (Figure 1) was expressed

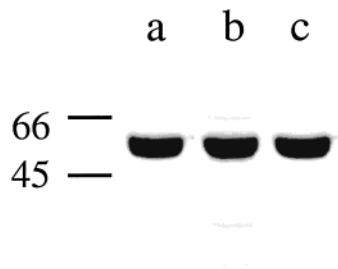

FIGURE 2: Coomassie-stained 10% SDS/PAGE of purified dSrc and two other dSrc recombinant protein mutants. Lane a, dSrc; lane b, dSrc-E524A; lane c, dSrc-Y527F. See Materials and Methods for details.

c-Src	STEPQ**Y**QPGENL	533
Lck	ATEGQ**Y**QPQP	509
Hck	ATESQ**Y**QQQP	505
c-Yes	ATEPQ**Y**QPGENL	543
c-Fgr	SAEPQ**Y**QPGDQT	519
Fyn	ATEPQ**Y**QPGENL	537
Yrk	ATEPQ**Y**QPGDNQ	536
Lyn	ATEGQ**Y**QQQP	512
Blk	ATEGQ**Y**ELQP	498

FIGURE 3: Amino acid sequences of the C-terminal tails of the Src family members. Phosphorylated Tyr is highlighted as a boldface single-letter abbreviation in each sequence.

containing an N-terminal 6 histidine tag under the control of a T7 promoter. Soluble expression of the dSrc protein was enhanced by coexpression with the chaperonins GroES and GroEL which were encoded on a separate plasmid. In this way, ~15 mg of dSrc protein could be purified from each liter of *E. coli* cell culture. Desired mutants (missense, deletions) of this original construct (Figure 1) prepared by standard PCR or the QuikChange method were also produced in this fashion with yields of these proteins being 4−20 mg/L of *E. coli* culture. Purification was accomplished on a Zn affinity column and led to removal of GroES and GroEL, and typical purity of the proteins was >70% as assessed by SDS−PAGE (Figure 2). In all cases, proteins could be obtained at concentrations of 20 μM or greater which was sufficient for the work described below.

Steady-State Kinetics with dSrc. Previous work (*23*) showed that the catalytically impaired SH3−SH2−catalytic construct of Lck (analogous to dSrc) is an efficient Csk substrate (apparent $k_{cat} = 106$ min^{-1}, apparent $K_m = 5.4$ μM). Given the sequence divergence of Src and Lck, especially in the tail region (*26*) (Figure 3), it was not clear how these two proteins would compare as Csk substrates. The full-length dSrc protein (Figure 1) was evaluated as a Csk substrate and shown to be quite efficient (apparent $k_{cat} = 78$ min^{-1}, apparent $K_m = 3.5$ μM), with similar kinetic parameters to those for catalytically impaired Lck (Table 1). Likewise, the K_m for ATP [K_m(apparent) = 6.2 ± 0.7 μM] and the dependence on Mn concentration were also similar for the two protein substrates (data not shown). To validate phosphorylation site-specificity of Tyr-527 in dSrc, the Y527F dSrc protein variant was prepared, and no phosphorylation by Csk was observed (detection limit <1% the rate

Table 1: Steady-State Kinetic Parameters for dSrc and Mutants Catalyzed by Csk[a]

substrate	k_{cat} (min^{-1})	K_m(app) (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
dSrc	78.2 ± 7.3	3.5 ± 0.7	3.7×10^5
dSrc-cat	114.2 ± 8.3	2.0 ± 0.4	9.5×10^5
CE-dSrc	84.3 ± 7.7	6.5 ± 1.2	2.2×10^5
E524A	—[b]	—[c]	1.0×10^4
E524D	—[b]	—[c]	8.0×10^3
E524Q	—[b]	—[c]	1.8×10^4
P525A	56.7 ± 4.9	5.2 ± 1.0	1.8×10^5
Q526A	—[b]	—[c]	3.5×10^4
Q526I	173.7 ± 20.1	7.8 ± 1.9	3.8×10^5
Q528A	100.7 ± 5.3	2.3 ± 0.3	7.3×10^5
P529A	178.0 ± 19.8	6.9 ± 1.3	4.3×10^5
G530A	56.7 ± 3.6	4.9 ± 0.6	1.9×10^5
E531A	70.6 ± 8.3	5.7 ± 1.3	2.1×10^5
N532A	68.4 ± 4.5	5.1 ± 0.8	2.2×10^5
Lck[d]	106	5.4	2.0×10^5

[a] Steady-state kinetic parameters determined as described under Materials and Methods. For situations where K_m could not be accurately determined, the Michaelis−Menten equation can still be used to provide the second-order rate constant (k_{cat}/K_m) with precision using the same nonlinear curve fit. [b] k_{cat} could not be accurately determined. [c] K_m(app) > 20 μM, beyond the detection limit. [d] For catalytically impaired Lck taken from ref *23*.

for dSrc). Despite their divergence in length and sequence, these results suggest that the tail sequences of Lck and Src proteins are handled similarly by Csk. Furthermore, the N-terminal 6 histidine tag present in dSrc, not present in the Lck substrate, does not appear to be interfering with Csk recognition.

Effects of Deletion of Src SH2 and SH3 Domains on Substrate Efficiency. The contributions of the Src SH2 and SH3 domains toward Csk recognition were next explored. The dSrc-cat protein lacking the SH3 and SH2 domains (Figure 1) was expressed and purified using the same *E. coli* expression system. This dSrc-cat protein was found to be an effective Csk substrate with a 3-fold increase in catalytic efficiency for dSrc-cat (apparent $k_{cat}/K_m = 9.5 \times 10^5\ M^{-1}\ s^{-1}$) versus full-length dSrc (apparent $k_{cat}/K_m = 3.7 \times 10^5\ M^{-1}\ s^{-1}$). This slightly enhanced efficiency rules out the involvement of important interactions between the dSrc SH2 and/or SH3 domains with Csk, as determinants of Csk-catalyzed phosphorylation of the dSrc tail. Therefore, the key information necessary for tail phosphorylation of dSrc by Csk is contained within the Src catalytic domain and/or tail sequence.

dSrc Tail Mutants and Csk Phosphorylation. To further understand the contributions of Src tail residues in Csk recognition, a systematic replacement of the residues from the Y − 3 to the Y + 5 position was undertaken. Initially, an "alanine-scanning" mutagenesis approach was used, and the proteins were expressed and purified. Most of the residues had minimal impact on tyrosine phosphorylation by Csk. For residues at Y − 2, Y + 1, Y + 2, Y + 3, Y + 4, and Y + 5, the apparent k_{cat}/K_m values were within 2-fold of the parent dSrc construct (Table 1). In contrast, replacement of Y − 3 (Glu) and Y − 1 (Gln) with Ala led to more marked declines in Csk-mediated phosphorylation of the dSrc construct. A ~40-fold drop in the apparent k_{cat}/K_m for E524A and a ~10-fold decrease for Q526A were observed. A significant component of the changes in the apparent k_{cat}/K_m for these mutants resulted from elevations in apparent K_ms which were greater than 20 μM in both cases. It was not possible to fully analyze the values of k_{cat} and K_m for these mutants because the apparent K_ms exceeded the maximal dSrc mutant protein concentrations that could be employed.

The specific role of the Glu-524 residue was further explored by mutating that amino acid to Asp and Gln. Interestingly, these relatively conservative mutations led to comparable reductions in dSrc processing efficiency (20−50-fold) with respect to E524A, intimating that precise geometric and electrostatic properties of the glutamate side chain are required for efficient dSrc processing.

To directly compare protein and peptide selectivities, it was of interest to determine the consequences of a complete replacement of the dSrc tail with the consensus residues obtained from the combinatorial approach (*23*): Y − 3 (E), Y − 2 (E), Y − 1 (I), Y + 1 (F), Y + 2 (F), Y + 3 (F) (Figure 1). This combinatorially engineered protein (CE-dSrc) was produced in *E. coli*, and although its expression level was approximately 4-fold lower than for dSrc, it was obtained in comparable purity to the other Src mutants. Strikingly, CE-dSrc (apparent $k_{cat}/K_m = 2.2 \times 10^5\ M^{-1}\ s^{-1}$) showed similar catalytic efficiency compared to the parent dSrc itself (apparent $k_{cat}/K_m = 3.7 \times 10^5\ M^{-1}\ s^{-1}$). Therefore, the natural sequence, which is at least 500-fold less efficient in the context of short synthetic peptides (*23*), is processed as efficiently as the consensus sequence when contained in the dSrc protein.

Given the fact that isoleucine was tolerated in CE-dSrc at the Y − 1 position, the dSrc mutant Q526I was generated. As expected from CE-dSrc results, Q526I was as good a substrate as parent dSrc, suggesting that the Gln side chain in the Y − 1 position of the parent dSrc is needed for steric bulk or hydrophobicity rather than hydrogen-bonding capabilities.

Activation of Csk-Catalyzed Phosphorylation of dSrc by dSrc Y527F. In an effort to obtain an apparent K_i for dSrc Y527F, an experiment was designed in which a range of concentrations of dSrc Y527F was added to block Csk phosphorylation of dSrc, following a typical Dixon protocol. Rather than inhibition, modest but reproducible activation of dSrc phosphorylation was observed with increasing dSrc Y527F concentration up to 8 μM dSrc Y527F (Figure 4A). This enhanced Csk kinase rate was shown not to be an artifact related to dSrc autophosphorylation or Csk phosphorylation of dSrc Y527F by performing appropriate controls. A plausible explanation for this behavior was that cooperativity between Src molecules is occurring. By careful inspection of some of the previous experiments with Csk phosphorylation of dSrc or dSrc mutants at low dSrc concentrations, it was reproducibly observed that a mild "S" shape was present in the Michaelis−Menten plots. Accordingly, a more extensive set of data points was collected for dSrc, and analysis of the data using a modified Michaelis−Menten equation incorporating a Hill coefficient for the dSrc substrate concentration gave a Hill coefficient of 1.73 ± 0.17, which indicates significant cooperativity (Figure 5A). The incorporation of the Hill coefficient gave an improvement in the fit versus the standard Michaelis−Menten equation (Figure 5A). A similar pattern was observed with dSrc-cat as substrate for which a Hill coefficient of 1.81 ± 0.11 was obtained (Figure 5B).

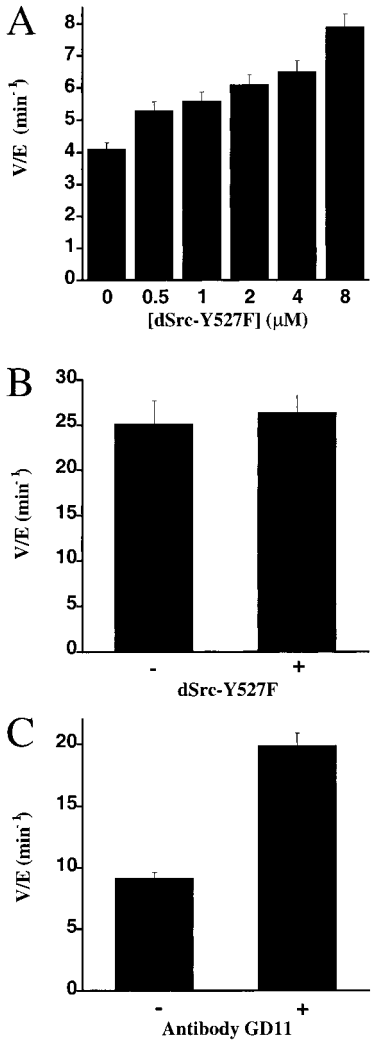

FIGURE 4: Effect of dSrc-Y527F and antibody on the Csk phosphorylation of dSrc and poly(Glu, Tyr). (A) Plot of velocity versus [dSrc-Y527F] in the presence of a fixed dSrc concentration (0.4 μM); [Csk], 1.5 nM. (B) Csk phosphorylation of poly(Glu, Tyr) (1000 μg/mL) in the absence and presence of 8 μM dSrc-Y527F; [Csk], 20 nM. (C) Csk phosphorylation of dSrc (0.6 μM) in the absence and presence of 0.3 μM anti-Src antibody; [Csk], 3 nM. Each of these experiments was performed at least 4 times (duplicates on two separate occasions) and showed good reproducibility.

At least two models could explain the apparent dSrc cooperativity. In model **A**, one dSrc molecule binds to a portion of Csk and directly stimulates Csk's catalytic activity toward a second substrate molecule. In model **B**, two (or more) dSrc protein molecules directly interact with one another, thereby enhancing binding and/or catalytic processing by Csk. Model **A** makes the prediction that dSrc mutant Y527F stimulation of Csk kinase activity should extend to non-dSrc substrates, whereas model **B** suggests that Src Y527F mutant stimulation of Csk should be confined to dSrc substrates. Consequently, we investigated dSrc Y527F's ability to stimulate Csk's phosphorylation of poly(Glu,Tyr), a peptide substrate for Csk. The protein dSrc Y527F (up to 8 μM) had no significant effect on Csk-catalyzed poly(Glu,-Tyr) phosphorylation (Figure 4B). This finding is consistent with the Y527F dSrc activation model in which dSrc dimerization is important (Figure 6). It is established that Src can self-associate since these enzymes can catalyze intermolecular autophosphorylation quite efficiently (*27*).

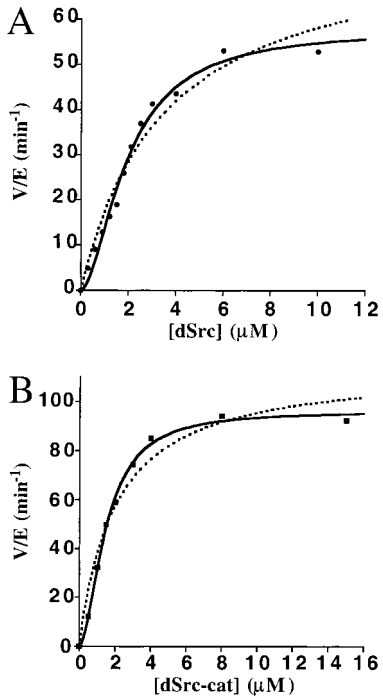

FIGURE 5: Hill analysis of the Csk phosphorylation of dSrc and dSrc-cat. (A) Plot of velocity versus [dSrc], and (B) [dSrc-cat]. Data were fitted to a modified (Hill) Michaelis−Menten equation: $[S]^n/(K' + [S]^n)$ (—) (*37*) and standard Michaelis−Menten equation (- - -). Conditions: [Csk], 1.5 nM; [ATP], 50 μM. For dSrc, parameters from the standard equation fit were: K_m(apparent) = 3.5 ± 0.7 μM, k_{cat} = 78.2 ± 7.3 min^{-1}, χ^2 = 144; parameters from the modified equation fit were: K' = 3.2 ± 0.3 μM, k_{cat} = 58.0 ± 3.0 min^{-1}, Hill coefficient n = 1.73 ± 0.18, χ^2 = 48. For dSrc-cat, standard fit: K_m(apparent) = 2.0 ± 0.4 μM, k_{cat} = 114.2 ± 8.3 min^{-1}, χ^2 = 340; modified fit: K' = 2.0 ± 0.1 μM, k_{cat} = 96.3 ± 1.8 min^{-1}, Hill coefficient n = 1.81 ± 0.11, χ^2 = 23.

To further test this model, Csk phosphorylation of dSrc was examined in the presence of a monoclonal antibody (mAb) which recognizes the Src SH3 domain. At a 1:2 mAb/dSrc stoichiometry, there was an approximate 2-fold increase in the rate of Csk-catalyzed phosphorylation of dSrc (Figure 4C). Thus, antibody which should enhance dSrc dimerization appears also to facilitate phosphorylation, consistent with the proposals outlined in Figure 6.

DISCUSSION

The interplay between local amino acid interactions, secondary and tertiary protein structure, and distal domain effects governs the molecular recognition of protein substrates by protein kinases. Certain serine-threonine kinases appear to show high specificity for a defined series of amino acids in short linear peptide sequences that correlate well with sequences of residues that are naturally phosphorylated in proteins (*3*−*6*). In contrast, there is a significant disparity between peptide site phosphorylation selectivity and protein site phosphorylation specificity for the protein tyrosine kinase Csk (*7*, *23*). Src family members are physiologically relevant substrates for Csk, and a single tyrosine out of approximately 20 in these family members is phosphorylated by Csk with high selectivity. How this selectivity is achieved is unknown. While a protein (Csk binding protein) has recently been reported to recruit Csk to the plasma membrane and thereby possibly enhance its rate of Src family member phosphorylation (*28*), it had been shown earlier with purified Csk

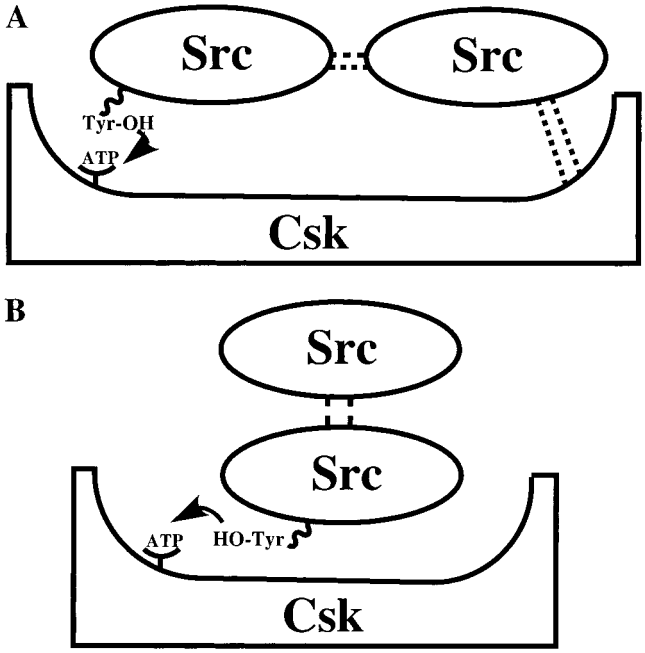

FIGURE 6: Two potential Src dimerization models proposed for Csk phosphorylation of Src protein. (A) Two Src polypeptides bind to each other and both directly to Csk, enhancing by recruitment phosphorylation of one of the Src molecules by Csk. (B) Only one Src polypeptide interacts directly with Csk, but a second Src molecule somehow conformationally enhances the first Src polypeptide's interaction with Csk.

and Lck proteins that much of the specificity underlying the interaction is dictated by the structures of the kinase and substrate themselves (*23*).

In this study, an improved bacterial expression system is described which has made it possible to conveniently express and purify quantities of catalytically inactive Src (dSrc) and dSrc mutants. This has allowed for a more detailed and direct analysis of Csk-catalyzed Src phosphorylation than has been performed to date. As noted for a variety of other proteins previously (*25*), coexpression with GroES and GroEL proved to be effective in enhancing soluble dSrc protein expression. The His6-tagged dSrc protein was purified to near-homogeneity readily with a Zn affinity column. This technology should find additional uses for experiments requiring purified Src protein.

It was interesting that dSrc showed similar efficiency as a Csk substrate compared to the previously reported results with catalytically inactive Lck. Deletion of dSrc's SH2 and SH3 domains did not diminish the efficiency with which dSrc was phosphorylated by Csk. Previous qualitative data available from cellular experiments were also suggestive that the Src SH2 and SH3 domains were dispensable for Csk-mediated phosphorylation (*29*), and the biochemical data here confirm this hypothesis and provide a quantitative assessment of the interactions. Taken together with previous Csk deletion experiments, these results strongly support the concept that the key specificity information in the Src−Csk interaction is located within the catalytic domains of the two proteins as well as the Src tail. This simplifies significantly future biophysical dissection of the Csk−Src interaction.

It was of considerable interest to quantitate the contributions of individual Src tail residues to the substrate efficiency of Src phosphorylation. Alanine scanning mutagenesis showed that two residues were highly important in recognition and catalysis, Y − 3 and Y − 1, and the others were replaceable with little impact. The 40-fold greater rate observed with Y − 3 (Glu) and 10-fold greater rate with Y − 1 (Gln) compared to Ala at these sites were considerably larger than the selectivities observed in peptide library studies (2−3-fold selectivities for Y − 3 Glu versus Ala and Y − 1 Ile versus Ala) within short sequences. It was also quite striking that the optimal short peptide sequence, which was >500-fold more efficient than the natural sequence within a short peptide, was no better than the naturally occurring sequence in the context of the dSrc protein. These results suggest that Csk recognition of Src is unlikely to be two independent interactions that Csk shows toward the non-tail Src protein and the Src tail. In this case, the binding energies for the two interactions should be strictly additive, and the catalytic efficiency for the tail-optimized Src should have been much greater than the natural material. Rather, a simple interpretation of the data involves a synergistic presentation of the Src tail by the Src catalytic domain. In this presentation, the Y − 3 and Y − 1 residues are highly influential in governing Csk recognition. At least two models, or some hybrid, are plausible to account for such behavior. In one model, Y − 1 and Y − 3 Src residues interact with some upstream region in the Src catalytic domain (either inter- or intramolecularly) and trigger a distal Src−Csk interaction. In the second model, Y − 1 and Y − 3 residues interact directly with Csk, but these interactions are facilitated indirectly by remote interactions between Src and Csk. The previously reported peptide library experiments suggest that model 2 has merit since in the absence of the Src catalytic domain there is some preference for the Y − 3 Glu residue (*23*). Moreover, in the absence of Csk, the Src family member Lck tail is thought to be relatively unstructured (and not interacting with the catalytic domain) based on the absence of electron density associated with the tail by X-ray crystallographic analysis (*22*). Further structural work will be necessary to settle this question more definitively.

It was somewhat surprising at first that the Ile residue was tolerated at the Y − 1 position in the CE-dSrc protein given the 10-fold preference for Gln versus Ala at this position. However, the Q526I dSrc mutant was equally efficiently processed compared to the parent dSrc protein, indicating that the hydrophobic/ steric properties of the Y − 1 side chain rather than hydrogen bonding are essential for Csk phosphorylation.

Csk phosphorylation of the Src tail is associated with down-regulation of Src's own tyrosine kinase activity. Consequently, there has been a substantial body of work aimed at identifying Src tail mutants that show transforming potential in cellular growth studies (*15*, *16*, *29*−*34*). Indeed, a subset of human colon cancers was shown to have a related mutation in which Src was truncated in the tail and resistant to negative regulation (*30*). It is noteworthy that only two residues other than the key tyrosine are strictly conserved among Src family member tails in the Y − 3 to Y + 5 region (Figure 3). Comparison of the preferences of the requirements for efficient Csk phosphorylation of dSrc can be used to account for the behavior of some of the mutants which showed transforming phenotype. For example, MacAuley and Cooper showed that mutations in Src Glu-524 to Gly, Val, or Lys led to cellular transformation (*32*, *33*). Likewise,

Gln-526 conversion to Lys or Pro also led to a transforming phenotype. These results are clearly consistent with the expectations from the results of Ala scanning experiments described here. More difficult to account for is the fact that mutants containing E524D and Q526G showed a nontransforming phenotype. As described above, Src E524D was similarly poor as a Csk substrate compared to E524A and would have been predicted to induce transformation. While no obvious explanation accounts for this behavior, it can be hypothesized that the E524D Src transforming potential is disabled in some other way in these cells. We believe this underscores the limitations of deriving definitive conclusions about specific protein−protein interactions from experiments done only in living systems.

Perhaps the most unexpected finding in the current study was the apparent cooperativity observed between Src molecules in the Csk phosphorylation event. It is worth commenting on the fact that the detailed kinetic studies described here using purified proteins allowed this rather subtle but potentially important feature to be revealed, which would probably have been impossible to observe using traditional kinase measurements made in cell biology. Somewhat related activating protein schemes have been proposed in MAP kinase cascades (*35*, *36*) although in these cases the activating protein is different from substrate.

How cooperativity is generated at the atomic level in the Csk−Src pathway is unclear but likely only involves the catalytic domains of the Src proteins given its occurrence with dSrc-cat. Two potential models for cooperativity which cannot currently be distinguished are shown in Figure 6. In one model, both Src molecules interact directly with the Csk protein (Figure 6A). In the second model, one Src molecule converts a second Src substrate molecule into a more efficient substrate by a conformational change (Figure 6B). It is presumed that such cooperativity directly contributes to the apparent differences in selectivity between peptide and protein substrates for Csk. It is also noteworthy that such cooperativity could well be fostered by recruitment of Src and Csk to cellular membranes where diffusion is limited to two dimensions.

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