Peptide and Protein Phosphorylation by Protein Tyrosine Kinase Csk: Insights into Specificity and Mechanism†

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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 down-regulation of Src family members. The molecular basis of Csk's substrate specificity and catalytic mechanism with a protein substrate was investigated. Using a peptide library approach, preferential amino acids which are unrelated to the conserved Src C-terminal sequence were identified. The validity of these preferences was confirmed by synthesizing a short consensus peptide and demonstrating its high catalytic efficiency with Csk. These results underscore the difficulties of relying on amino acids neighboring tyrosine in protein sequences as predictors of protein kinase substrate specificity for in vivo analysis. In addition, a catalytically inactive version of the Src family member, Lck (lymphoid cell kinase), was expressed, purified, and evaluated as a Csk substrate. It was proven to be the most catalytically efficient substrate yet identified for Csk. The high efficiency of purified Csk phosphorylating a pure, unphosphorylated Src family member argues against the importance of an SH2−phosphotyrosine docking interaction or the involvement of extra recruitment proteins in facilitating Csk phosphorylation of Src family members. Kinetic studies revealed that the chemical step is at least partially rate-determining in Csk-mediated phosphoryl transfer to the Lck protein. Other properties including preferences for Mn over Mg, thio effects, and K_m's for ATP also correlate fairly well between protein and peptide phosphorylation. The lack of a significant impact of increased salt on the K_m for Lck phosphorylation differs from Csk-mediated poly(Glu,Tyr) phosphorylation, and argues against the importance of electrostatic effects in the Csk−Lck binding interaction. The failure of the Lck phosphorylation product (phosphotyrosine-505) to significantly inhibit Csk phosphorylation of Lck is consistent with a catalytic model involving multidomain structural interactions between substrate and enzyme.

Protein tyrosine kinases, enzymes critical in cell signal transduction, catalyze the phosphorylation of proteins on tyrosine residues (*1*). It is widely held that specificity for particular substrates and well-defined timing with respect to cellular events are crucial for the biological functions of protein tyrosine kinases. For the majority of protein tyrosine kinases, the biologically relevant in vivo substrates have not yet been identified. In fact, the molecular basis of protein tyrosine kinase substrate selectivity in vitro and in vivo is poorly understood. Sequence selection for tyrosine over serine or threonine may well be mediated in large measure by the defined spatial separation between the peptide backbone and the side chain hydroxyl (*2, 3*). Yet, how a particular protein is chosen, let alone a single tyrosine among many in a large protein, has not been adequately addressed for any protein tyrosine kinase (*4*−*7*). Furthermore, outside of autophosphorylation studies, there has not been previously reported any quantitative assessment of phosphorylation catalyzed by a protein tyrosine kinase on a physiologically relevant, structured protein substrate using pure proteins.

Protein tyrosine kinase Csk[1] (C-terminal Src kinase) phosphorylates Src family members on their conserved C-terminal tyrosines, thereby substantially reducing the Src family members own catalytic activity (*8*). That a major and biologically relevant group of Csk substrates is the Src family is strongly supported by a variety of in vitro and in

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[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Csk, C-terminal Src kinase; Lck, lymphoid cell kinase; Lck-ΔNH2, Lck with amino-terminal deletion of first 63 amino acids; Lck-ΔNH2-R, Lck with amino-terminal deletion of first 63 amino acids and replacement of lysine-273 with arginine; Lck-ΔNH2-R-505-P, Lck-ΔNH2-R which is phosphorylated on tyrosine-505; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; FMOC, fluorenylmethoxycarbonyl; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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vivo studies (*9*−*11*). Csk appears to be a highly selective protein tyrosine kinase. Overexpression of Csk in eukaryotic cells does not generally lead to an increase in phosphotyrosine content (*12*). Unlike many protein tyrosine kinases, Csk does not significantly undergo autophosphorylation (*13*).

Amino acid sequences surrounding the phosphorylated tyrosine from the C-terminus of Src, Lck, Fyn, and other Src family members are highly conserved (*14*). We (unpublished data) and others (*15*−*17*) have shown that short to medium length peptides (9−40 amino acids) based on this well-established sight of phosphorylation are rather poor Csk substrates. These substrate-based peptides have K_m's greater than 1 mM and a catalytic efficiency (k_{cat}/K_m) 1000-fold lower than poly(Glu,Tyr), the heretofore best characterized and most catalytically active substrate. The molecular basis of Csk's selectivity for peptides and the catalytic properties of Csk toward proteins have not been well-defined. We have pursued two approaches described herein to address these problems. The first is a peptide library analysis that was used to investigate whether Csk shows preferences for particular amino acids surrounding the phosphorylated tyrosine in short peptides (*4*). The second is a quantitative study of phosphorylation of the Src family member Lck by Csk.

MATERIALS AND METHODS

Chemicals. Tris, DTT, ATP, BSA, PEP, and NADH were from Sigma. $MnCl_2$, $MgCl_2$, and EDTA were from Fisher. ATPγS, pyruvate kinase, and lactate dehydrogenase were from Boehringer. [^{35}S]ATPγS (1500 Ci/mmol) and [γ-^{32}P]-ATP (6000 Ci/mmol) were from New England Nuclear.

Csk Production and Purification. Human recombinant Csk was expressed in *E. coli* in combination with the chaperones GroES and GroEL and purified using phosphotyrosine affinity chromatography as described previously (*18, 19*). D314E Csk was also expressed and purified as previously reported (*18, 19*). Specific activity was checked using a poly(Glu,Tyr) phosphorylation assay (*20*).

Peptide Library Work. The library was prepared according to previously described methods (*4*). Phosphorylation by Csk, separation of the phosphotyrosine-peptides on an Fe−iminodiacetic acid column, and amino acid sequencing were performed as previously reported (*4*). Note that the absence of quantitation of aspartate in the first two sequencing cycles (see Figure 1) is due to a technical limitation of the sequencing process that limits the accuracy of this measurement.

Peptide Synthesis. The peptide KKKKEEIYFFF was synthesized using FMOC solid-phase peptide synthesis. It was purified using reversed-phase HPLC on a C-18 column with an acetonitrile/water (0.05% trifluoroacetic acid) gradient, and the peptide was shown to have the correct molecular weight by mass spectrometry.

Csk−Peptide Phosphorylation. Steady-state kinetic parameters were obtained using a coupled assay based on methods as described previously (*20*). Briefly, reactions were carried out at 30 °C in 60 mM Tris-HCl (pH 7.4), 0.5 mM ATP, 190 μM NADH, 1 mM PEP, wild-type Csk, lactate dehydrogenase−pyruvate kinase, 5 mM $MnCl_2$, 10 mM DTT, and peptide concentrations varied 20-fold around the K_m. The absorption at 340 nm was monitored spectrophotometrically versus time in the linear range (less than 10% turnover of the limiting substrate), and reaction velocities were calculated based on an extinction coefficient of 6300 cm^{-1} M^{-1} for NADH at 340 nm. Reactions with $MgCl_2$ used 1 mM ATP and 15 mM $MgCl_2$.

Lck Preparation and Purification. Lck-ΔNH2-R was expressed in a baculovirus/SF9 cell system and purified essentially as previously described for wild-type Lck-ΔNH2 (*21*). Lck-ΔNH2-R was further purified, and defined phosphorylation states were isolated (Tyr-505-phosphorylated, nonphosphorylated) with a combination of phosphotyrosine affinity and Q-Sepharose chromatography. These proteins appeared homogeneous using 10% SDS−PAGE with Coomassie staining. Phosphorylation states were confirmed with mass spectrometry and native PAGE. Protein concentrations were quantitated spectrophotometrically in the denatured state (*22*) and using the Bradford assay.

Assays of Csk Phosphorylation of Lck-ΔNH2-R. General reactions were performed at 30 °C, pH 7.4 with 1−10 nM Csk, 1−10 μM Lck-ΔNH2-R, 67 mM Tris-HCl, 8 mM Na-HEPES, 1−30 μM ATP, 3 μCi of [γ-^{32}P]ATP, 8 mM NaCl, 10 mM DTT, 67 μg/mL BSA, and 5 mM $MnCl_2$, in 15 μL for 2 min in a 0.6 mL microcentrifuge tube. Reactions were initiated with Lck-ΔNH2-R with vigorous mixing with a pipetman and quenched with 7.5 μL of aqueous Na-EDTA (100 mM, pH 8). The quenched mixtures were treated with 5.7 μL of 5× SDS gel load [40% v/v glycerol, 10% w/v SDS, bromophenol blue (trace), Tris-HCl (250 mM), and 25% v/v β-mercaptoethanol], vortexed, and heated to 90 °C for 60 s. A 20 μL aliquot from the mixtures was then loaded onto a 1 mm 10% SDS−PAGE (Bio-Rad miniprotean gel apparatus) at constant voltage (200 V), being careful not to let the dye front proceed closer than 2 cm to the gel's bottom. The gels were stained with Coomassie stain for approximately 30 min and destained for 20 min. Lck-ΔNH2-R bands were easily visualized and removed by cutting with a blade either before or after drying the gel (there was less than 10% difference in quantitation using these alternative methods). Quantitation was performed either by phosphorimage analysis (Fuji phosphorimager, McBas software) or by direct scintillation counting of the bands (Beckman LS scintillation counting). Counting efficiency was taken as 100% with ^{32}P.

Lck-ΔNH2-R autophosphorylation (i.e., ^{32}P incorporation in the absence of Csk) was shown to be above background (<0.01% total cpm in the reaction) and was dependent on Lck-ΔNH2-R concentration. The radioactivity of background autophosphorylation (typically less than 20% of the total) was subtracted at all Lck-ΔNH2-R concentrations from the total number of cpm to afford the Csk-mediated Lck-ΔNH2-R phosphorylation. Exclusive phosphorylation at Tyr-505 of Lck-ΔNH2 by Csk was further verified by two-dimensional peptide mapping. Although Csk and Lck comigrated using 10% SDS−PAGE, there was shown to be insignificant Csk autophosphorylation under the conditions of these assays.

The optimal Mn concentration was shown to be 3−7 mM using 20 μM ATP and 2 μM Lck-ΔNH2-R. Activity was linear with respect to both time (up to at least 5 min) and Csk concentration (up to at least 10 nM) (see Figure 3 and data not shown). Measurements of K_m(apparent)'s for ATP and Lck-ΔNH2-R employed the concentration range shown

in Figure 4. Note that for the measurement of the Lck-ΔNH2-R K_m it was only possible to achieve a maximal substrate concentration 2 times K_m, to some extent limiting the accuracy of this measurement. Calculations were done by fitting the data to the Michaelis−Menten equation using a nonlinear least-squares approach, and there was good agreement with fits using double reciprocal analysis. The kinetic constants are shown ± the standard error in the text.

The optimal Mg concentration was found to be 4−10 mM. Kinase activity was also shown to be linear with respect to time and enzyme concentration with Mg. The K_m(apparent) for ATP was obtained at 2 μM Lck-ΔNH2-R with an ATP concentration range from 5 to 80 μM. The K_m for Lck-ΔNH2-R was above 10 μM at 40 μM ATP but was not determined precisely because of the high background Lck-ΔNH2-R autophosphorylation at higher concentrations of Lck-ΔNH2-R. However, using Lck-ΔNH2-R substrate concentrations from 1 to 10 μM, the K_m was estimated to be 20−30 μM from both double-reciprocal and rectangular hyperbola plots.

The product of Csk phosphorylation of Lck-ΔNH2-R, Lck-ΔNH2-R-505-P, was evaluated as a Csk substrate with 8 nM Csk and 9.5 μM Lck-ΔNH2-R-505-P with Mn as the divalent ion. Csk-mediated phosphorylation of Lck-ΔNH2-R-505-P was undetectable (at least 500-fold slower compared to its rate of phosphorylation of Lck-ΔNH2-R). Lck-ΔNH2-R-505-P was also tested as an inhibitor of Csk phosphorylation of Lck-ΔNH2-R using the concentration range 0−9.5 μM. Background autophosphorylation of Lck-ΔNH2-R and/or Lck-ΔNH2-R-505-P was subtracted at every concentration of Lck-ΔNH2-R-505-P by comparing the radioactivity when Csk was omitted from the assays, and was less than 20% of the Csk-mediated phosphorylation.

All assays were performed at least twice. In all cases, reaction of the limiting substrate did not exceed 10%.

ATPγS Experiments. Reactions with ATPγS were carried out analogous to previously described methods (*18*). Briefly, since the counting efficiency is much lower with ATPγS because of its lower energy emission, the polyacrylamide gel was broken down by oxidation under acidic conditions with gentle heating. Independent controls were performed showing that there were no significant losses of ^{35}S upon heating the sample in gel load for 90 s and staining and destaining the gel. Background autophosphorylation of Lck-ΔNH2-R was found to be insignificant in these assays, and the overall background cpm were less than 0.01% of the total cpm in a reaction. Enzyme activity was shown to be linear vs time and enzyme concentration with ATPγS. All assays were performed at least twice. In all cases, reaction of the limiting substrate did not exceed 10%.

RESULTS

Peptides. To address the potential local amino acid preferences surrounding a phosphorylated tyrosine of peptides as substrates for Csk, a library of peptides $MAX_1X_2X_3X_4YX_5X_6X_7X_8AKKK$ was synthesized where sites 4 amino acids upstream and downstream of a target tyrosine incorporated up to 15 different amino acids at each site (*4*). This peptide library thus contained up to 15^8 members. The library was phosphorylated with [γ-^{32}P]ATP to partial completion. The phosphorylated peptides were separated using a ferric−iminodiacetic acid column, and the resultant peptides were sequenced as described previously (*4*). No particular amino acid site showed greater than a 1.9-fold enhancement compared to a random amino acid (see Figure 1). The most highly preferred residues at the randomized sites were:

Position:	-4	-3	-2	-1	+1	+2	+3	+4
Residue:	E(1.6)	E(1.9)	D/E(1.3)	I(1.9)	F(1.4)	F(1.4)	F(1.8)	F(1.7)

The most highly preferred residue is shown under the amino acid position (referenced to tyrosine = 0), and the selectivity factor (referenced to a random amino acid) is shown in parentheses. E(1.9), for example, implies that glutamate showed the greatest preference and that it was 1.9-fold more likely to be present in a substrate at this position than a random amino acid. Although in theory there is no upper limit, practically speaking 15 is the largest selectivity factor that has been observed using this approach in other systems. Examination of these sequence data reveals that the preferred amino acids bore little resemblance to the in vivo target site EGQYQPQP. In fact, these amino acids showed the following preferences: E (1.9), G (0.9), Q (0.8), Q (0.7), P (0.6), Q (0.9), P (0.9). The overall average of these preferences (0.96) was not significantly different from a random sequence.

Based on the preferences shown above, several "consensus" peptides were designed and synthesized, most of which (including KKKKEEEIYFFFF) were poorly soluble. One somewhat simplified peptide that was designed based on this sequence (and truncated for synthetic ease) was KKKKEEIYFFF. This peptide proved to be the most potent small peptide substrate yet identified for Csk with k_{cat} = 200 ± 10 min^{-1} and K_m = 550 ± 100 μM with the divalent ion Mn, an overall catalytic efficiency at least 500-fold greater than that of EGQYQPQPG and within a factor of 4 of poly(Glu,Tyr). The presence of the extra lysines not only aids in solubility but also allows binding to phosphocellulose, providing the potential for a convenient direct assay. With the nonoptimal but physiologic divalent ion Mg, the k_{cat} and K_m were 190 ± 20 min^{-1} and 1.8 ± 0.4 mM, and k_{cat}/K_m were only 3-fold reduced compared to Mn. Thus, the high efficiency of this peptide is not dependent on the divalent ion involved.

Characterization of Lck-ΔNH2-R as a Csk Substrate. In order to address the catalytic efficiency of phosphorylation of a Src family member by Csk, we chose to study Lck (lymphoid cell kinase) which has a defined role in T-cell activation (*23, 24*). The technical issues associated with analysis of Lck as a substrate are not insignificant. Included are the difficulty of expression and purification of Lck, the presence of microheterogeneity in the sample particularly due to the multiple N-terminal phosphorylation sites, and the fact that Lck is itself a protein tyrosine kinase which undergoes autophosphorylation. Using a baculovirus/SF9 cell expression system, N-terminally truncated Lck (Lck-ΔNH2, see Figure 2) was expressed and purified to near-homogeneity, but it was quickly discovered that its rate of autophosphorylation was too rapid to allow for investigation of initial conditions (<10% turnover) for Csk-mediated Lck-

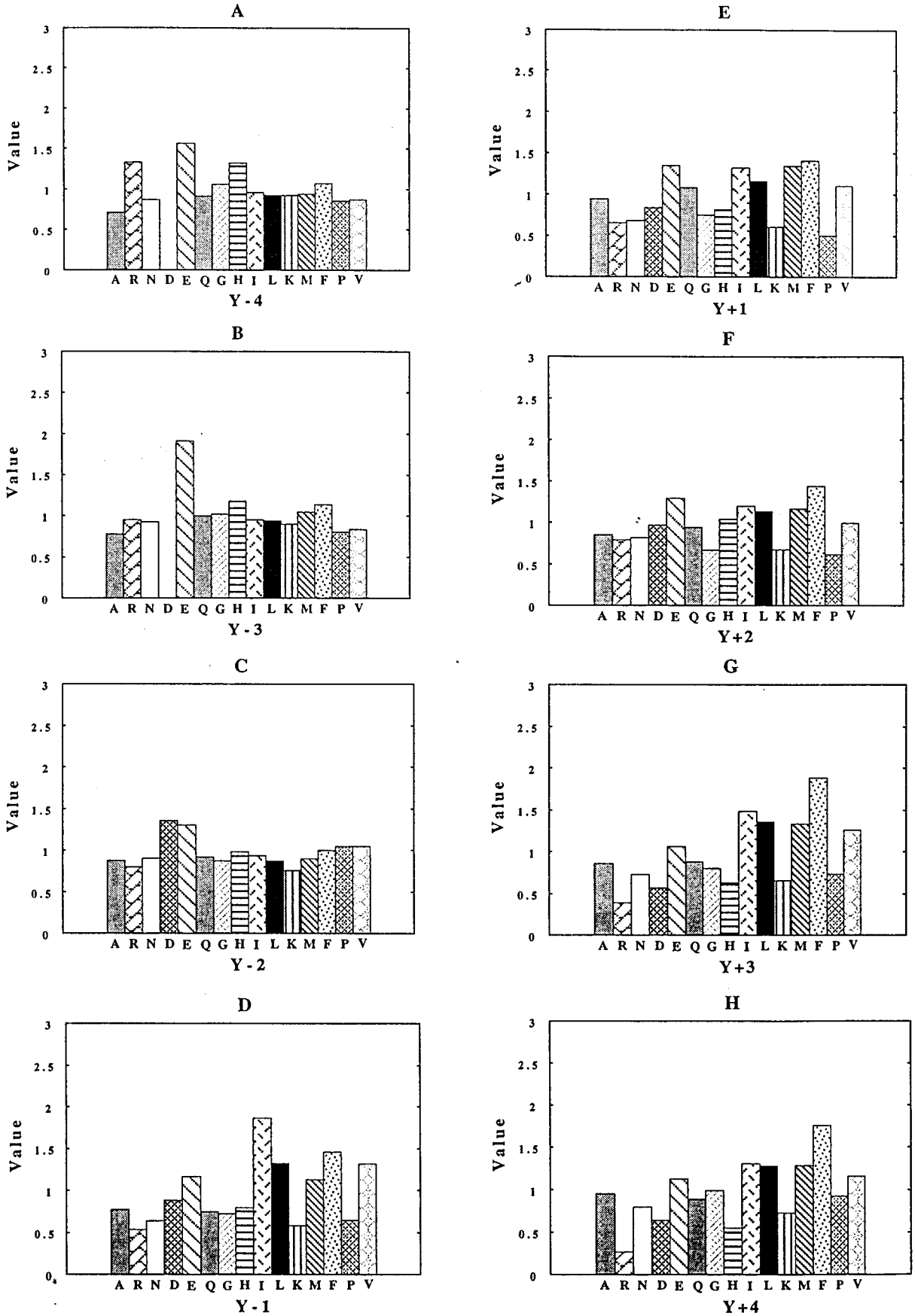

FIGURE 1: Csk kinase substrate selectivity using a peptide library. Panels A−H refer to the distribution of the sequenced amino acid residues at Tyr−4 to Tyr+4, respectively. The *y*-axis is the selectivity factor for a given amino acid standardized to the mean amino acid at that position. See Songyang *et al.* (*4*) and Materials and Methods for further experimental details.

phosphorylation. We decided therefore to evaluate the suitability of Lck-ΔNH2-R (containing a K273R mutation) as a Csk substrate. Lck-ΔNH2-R was anticipated to be catalytically impaired by virtue of a mutation in the highly

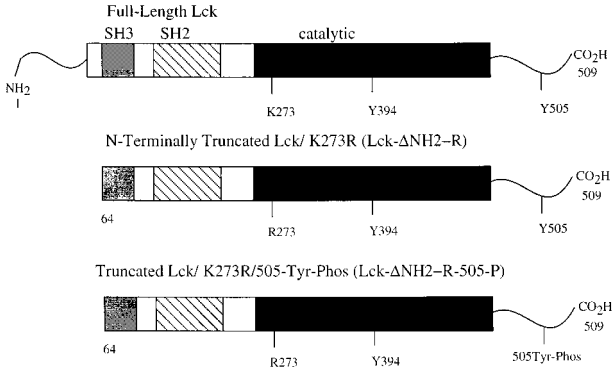

FIGURE 2: Lck constructs used in these experiments.

conserved, active site lysine which is expected to salt-bridge with the β-phosphate of ATP (*25*).

Lck-ΔNH2-R was expressed and purified using the identical expression system as for Lck-ΔNH2 (*21*). Indeed, the autophosphorylation rate of Lck-ΔNH2-R at a concentration of 1 μM was measured to be at least 100-fold lower than Lck-ΔNH2. Background Lck-ΔNH2-R phosphorylation was detectable, however, and its rate was second-order with respect to Lck-ΔNH2-R concentration. Whether this background phosphorylation was autophosphorylation or due to trace levels of a contaminating kinase is not known. Evidence supporting the latter possibility comes from the fact that autophosphorylation levels were somewhat Lck-ΔNH2-R batch dependent, varying up to 5-fold, while the level of Lck-ΔNH2-R's phosphorylation by Csk was quite consistent from batch to batch (less than 2-fold variation).

However, the background phosphorylation rate proved to be much less than Csk-catalyzed Lck-ΔNH2-R phosphorylation at most of the Lck-ΔNH2-R concentrations studied. At higher Lck-ΔNH2-R concentrations (10 μM and higher), the rate of background phosphorylation was significant (20−30% of the signal) compared to the rate of Csk-catalyzed Lck-ΔNH2-R phosphorylation. Nevertheless, with careful attention to background subtraction, we could use an Lck-ΔNH2-R concentration range giving reliable and reproducible Michaelis−Menten kinetic behavior (see below). That Csk was directly responsible for the phosphorylation (and not an allosteric activator) was most convincingly demonstrated by using D314E Csk. Previous studies demonstrated that D314E Csk lacks gross structural perturbations but shows a 10^4-fold k_{cat} reduction compared with wild-type Csk enzyme (*18*). In our current investigation, attempts at phosphorylation of Lck-ΔNH2-R by D314E Csk demonstrated that phosphorylation was reduced at least 1000-fold.

It was first established that the rate of Csk-catalyzed Lck-ΔNH2-R phosphorylation was linear with respect to time and Csk enzyme concentration (Figure 3). With the divalent ion Mn, the optimal Mn concentration was found to be in the range of 2−8 mM, similar to results with poly(Glu,Tyr) as the Csk substrate (*18*). The K_m(apparent) for ATP was 4.9 ± 0.2 μM, and the K_m for Lck-ΔNH2-R was 5.4 ± 0.5 μM with a k_{cat} of 106 ± 4 min^{-1} (see Figure 4). The K_m for ATP was about 2-fold lower than its value in the Csk reaction with poly(Glu,Tyr) (*13*). The effect of added NaCl, which caused a dramatic rise in the K_m for poly(Glu,Tyr) (*18*), led to no K_m effect on Lck-ΔNH2-R (4.3 ± 1.4 μM at 100 mM NaCl compared to 5.4 ± 0.5 μM at 8 mM NaCl) and small effects on the ATP K_m [ATP K_m(apparent)) = 8.0 ± 1.2

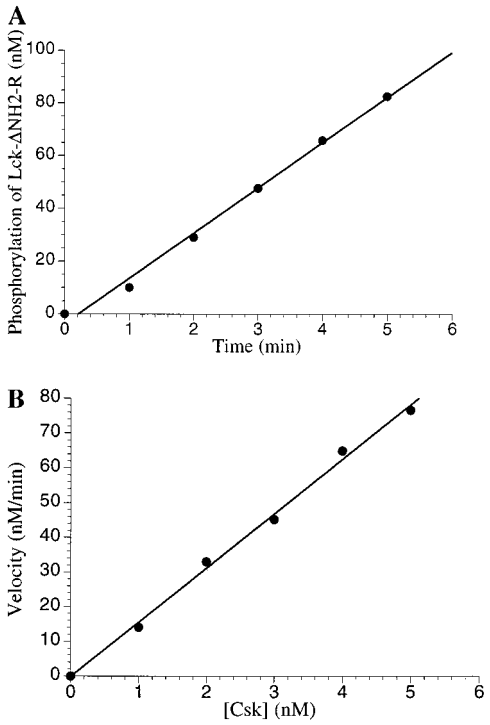

FIGURE 3: Csk-catalyzed phosphorylation of Lck-ΔNH2-R as a function of time and Csk concentration. (A) Csk-mediated phosphorylation of Lck-ΔNH2-R versus time. (B) Enzyme velocity versus Csk concentration. Conditions: [$MnCl_2$], 5 mM; [ATP], 20 μM; [Lck-ΔNH2-R], 2 μM. Assays were carried out as described under Materials and Methods.

μM at 100 mM NaCl compared to 4.9 ± 0.2 μM at 8 mM NaCl] and the k_{cat} (42 ± 7 min at 100 mM NaCl compared to 106 ± 4 min^{-1}). The K_m for Lck-ΔNH2-R proved to be nearly identical at high (30 μM) and low (2 μM) ATP concentrations (data not shown). This behavior is consistent with a rapid equilibrium random sequential Bi-Bi kinetic mechanism as observed in the phosphorylation of poly(Glu,-Tyr) by Csk (*18*). This suggests that binding of Lck-ΔNH2-R to Csk is not directly influenced by ATP, and the nucleotide and protein substrate binding sites are functionally independent.

Lck-ΔNH2-R Phosphorylation Kinetics with Mg. Recent studies demonstrated that Mg, the likely in vivo relevant divalent ion, is less efficient for poly(Glu,Tyr) phosphorylation by causing increased K_m's for ATP and poly(Glu,-Tyr) while leading to a modest increase in k_{cat} (*18*). Studies with Mg with Lck-ΔNH2-R substrate were therefore initiated. As with Mn, the activity was linear with time and enzyme concentration (data not shown). There appeared to be a broad Mg concentration optimum at 2 μM Lck and 20 μM ATP, and therefore 5 mM Mg was chosen as a working concentration. The K_m(apparent) for ATP (15 ± 3 μM) was found to be somewhat higher than with Mn (5 μM) although the relative increase (3-fold) was less than with poly(Glu,-Tyr) (13-fold). The K_m of Lck-ΔNH2-R could not be accurately measured because it was significantly above 10 μM, where background autophosphorylation becomes high, but it was estimated to be about 20−30 μM. This increase was also qualitatively similar to results with poly(Glu,Tyr) (*18*). The estimated k_{cat} (55 min^{-1}) was within 2-fold of the k_{cat} of the Mn reaction.

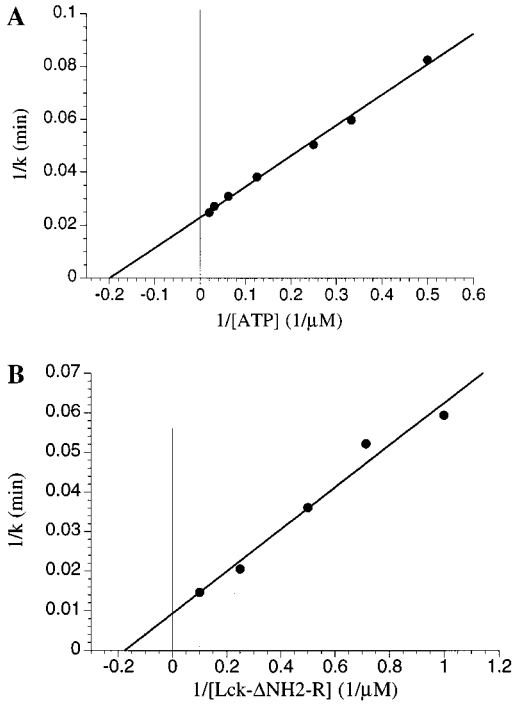

FIGURE 4: K_m measurements of Csk phosphorylation of Lck-ΔNH2-R with ATP. (A) Plot of 1/velocity (1/*k*) versus 1/[ATP] at a fixed Lck-ΔNH2-R concentration (2 μM). The kinetic constants were determined by nonlinear curve-fitting. K_m (apparent) = 4.9 ± 0.2 μM. (B) Plot of 1/velocity (1/*k*) versus 1/[Lck-ΔNH2-R] at a fixed ATP concentration (30 μM). The kinetic constants were determined by nonlinear curve-fitting. K_m = 5.4 ± 0.8 μM and k_{cat} = 106 ± 8 min^{-1}. Conditions: [$MnCl_2$], 5 mM; [Csk], 1 nM. Assays were carried out as described under Materials and Methods.

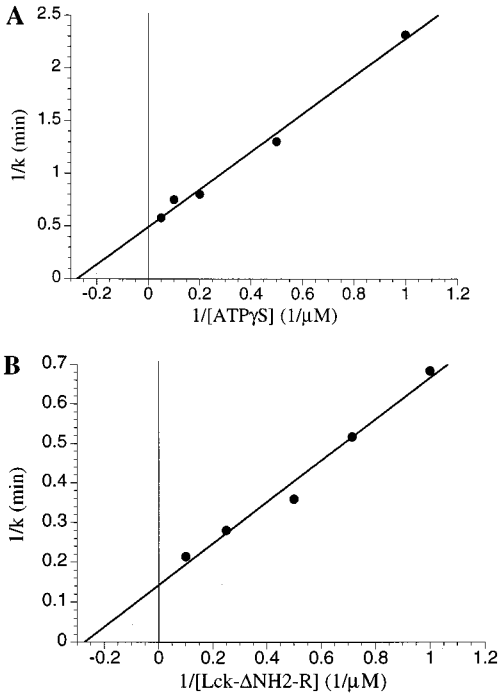

FIGURE 5: K_m measurements of Csk phosphorylation of Lck-ΔNH2-R with ATPγS. (A) Plot of 1/velocity (1/*k*) versus 1/[ATPγS] at a fixed Lck-ΔNH2-R concentration (2 μM). The kinetic constants were determined by nonlinear curve-fitting. K_m(apparent) = 3.2 ± 0.6 μM. (B) Plot of 1/velocity (1/*k*) versus 1/[Lck-ΔNH2-R] at a fixed ATPγS concentration (20 μM). The kinetic constants were determined by nonlinear curve-fitting. K_m = 2.7 ± 0.3 μM and k_{cat} = 6.0 ± 0.3 min^{-1}. Conditions: [$MnCl_2$], 5 mM; [Csk], 20 nM. Assays were carried out as described under Materials and Methods.

Substrate and Inhibition Studies with Lck-ΔNH2-R-505-P. As the singly-phosphorylated Lck-ΔNH2-R-505-P, the product of Csk phosphorylation of Lck-ΔNH2-R, was also available in pure form, it was also tested as a Csk substrate and inhibitor. In particular, it was of interest to measure a lower limit for the selectivity of Csk's preference for phosphorylation of Tyr-505 of Lck-ΔNH2-R compared to the 16 other tyrosines in the protein. Indeed, phosphorylation of Lck-ΔNH2-R-505-P by Csk was essentially undetectable and reduced by a factor of at least 500. Thus, Csk was highly specific for Tyr-505 of Lck-ΔNH2-R.

Since Lck-ΔNH2-R-505-P was not a substrate for Csk, we decided to test it as a possible competitive inhibitor of the enzyme. At 9.5 μM Lck-ΔNH2-R-505-P, there was only approximately 25% inhibition of Csk-mediated Lck-ΔNH2-R (2 μM) phosphorylation (data not shown). The K_i was estimated by Dixon analysis assuming a competitive inhibition model to be >20 μM for Lck-ΔNH2-R-505-P (the K_d of Lck-ΔNH2-R under these conditions was 3−5 μM). Lck-ΔNH2-R-505-P, predicted to have a very different three-dimensional fold compared with unphosphorylated Lck (*25*−*27*), was not a potent Csk binder.

ATPγS Kinetics with Lck-ΔNH2-R. The rate-limiting steps for Csk-mediated poly(Glu,Tyr) phosphorylation have been previously determined using a combination of viscosity effects and thio effects (*13*). It was shown that the product release step (most likely ADP release) was similar in rate (1 s^{-1}) to the chemical step in the presence of the divalent ion Mn, and both were partially rate-determining (*13*). The poly(Glu,Tyr) reaction showed an overall k_{cat} thio effect [k_{cat}(ATP)/k_{cat}(ATPγS)] of 19 with very similar K_m's. We wanted to test whether the physiologic and highly efficient substrate Lck-ΔNH2-R might show an even faster chemical step relative to product release, and therefore a predicted smaller thio effect. It seemed unlikely that diffusional release of Lck-ΔNH2-R-505-P would be slow because of its relatively low affinity as described above. After showing that phosphorylation kinetics with ATPγS were linear with respect to enzyme concentration and time, measurements of k_{cat} and substrate K_m's were undertaken with ATPγS using Mn as the divalent ion (see Figure 5). The K_m's for nucleotide and protein substrate were very similar to those of the standard ATP reactions: ATPγS K_m(apparent) = 3.2 ± 0.6 μM and Lck-ΔNH2-R K_m = 2.7 ± 0.3 μM. These similarities were also observed in the poly(Glu,Tyr) reactions (*13*). The k_{cat} with ATPγS (6 ± 0.3 min^{-1}) was about 18-fold lower than with ATP (i.e., thio effect = 18), nearly identical to the overall thio effect for the Csk-mediated poly-(Glu,Tyr) reaction in the presence of Mn (thio effect = 19) (*18*). The k_{cat}/K_m thio effect with Mg was about 30. Taken together, these results suggest that the chemical step is at least partly rate-determining in Csk-catalyzed phosphoryl transfer to Lck-ΔNH2-R.

DISCUSSION

The molecular basis of protein kinase substrate selectivity represents a major unsolved problem in the area of signal transduction. Clearly, with some kinases the local amino acid sequence surrounding the target site dictates, to a significant extent, the residue to be phosphorylated. A classic

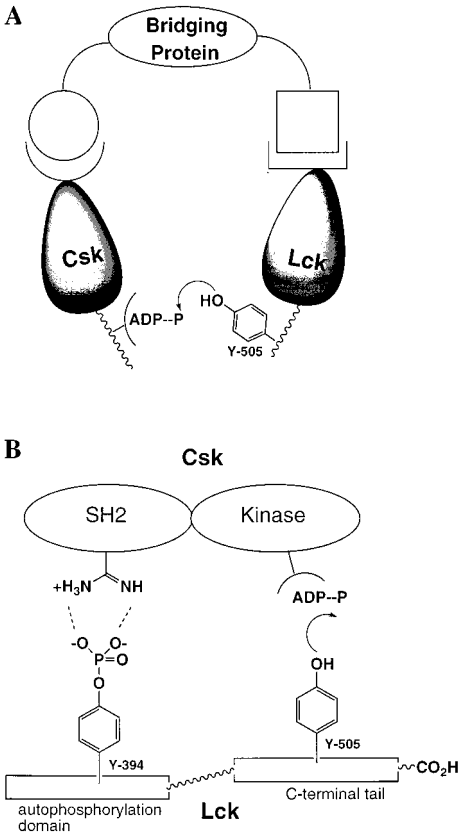

FIGURE 6: Alternate models proposed for Csk phosphorylation of Src family members. (A) Phosphorylation of Lck by Csk mediated by a protein complex with one or more recruitment proteins (*30*). (B) Phosphorylation of Lck by Csk by an SH_2−phosphotyrosine docking interaction between the Lck autophosphorylation site and the Csk SH_2 domain (*31*).

example is protein kinase A, a serine/threonine kinase which phosphorylates a serine residue in the context of a short, unstructured sequence with great catalytic efficiency (*28, 29*). In contrast, the protein tyrosine kinase Csk, whose in vivo target site is well-established and highly specific for 1 of approximately 20 tyrosine residues of Src family members, weakly phosphorylates the designed short peptides that correspond to this region. This represents the opposite pole in the spectrum of local versus global factors in protein kinase selectivity. Various hypotheses have been put forth to attempt to explain the molecular basis of Csk's selectivity. One theory suggests that phosphorylation of Src by Csk is achieved through a protein complex with several other proteins (the "third party model") (*30*) (Figure 6A). Another model proposes that docking between the autophosphorylation site of Src family members (phosphotyrosine-394 in Lck) and the Csk SH2 domain (Figure 6B) is crucial for achieving Csk specific phosphorylation of Src family members (*31*).

To attempt to probe more deeply into the molecular basis of Csk specificity for Src family members, two approaches were taken. The first was designed to examine whether in the context of small peptides there was a preference for certain amino acids surrounding the phosphorylated tyrosine and whether such preferences would correspond to those found in Src family members. In fact, what was observed was no strong preferences for local amino acids (all less than 3-fold) and that the conserved Src C-terminal tail amino acids were not generally preferred. Similar findings were recently reported with Csk purified from rat spleen (*32*). Interestingly, a peptide designed with even the modest preferences uncovered in the library approach, KKKKEEIYFFF, was a relatively good Csk substrate. The k_{cat}/K_m for Csk phosphorylation of KKKKEEIYFFF was within about 4-fold of the previously best-characterized substrate poly(Glu,Tyr) and at least 1000-fold greater than the C-terminal tail peptide EGQYQPQPG. KKKKEEIYFFF peptide, the most catalytically active short peptide substrate yet reported for Csk, should therefore be useful for mechanistic studies and inhibitor design. These studies also suggest that while Csk has no strong local amino acid preferences, it has modest selectivity. The lack of even these mildly preferred amino acids in the physiologic substrates is enigmatic. Clearly, understanding the selectivity of Csk cannot be accomplished solely by examining short peptide substrates.

We next chose to investigate a relevant protein substrate for Csk, Lck-ΔNH2-R. This study represents the first acquisition of accurate steady-state kinetic parameters of a protein tyrosine kinase's phosphorylation of a pure, physiologic substrate. Lck-ΔNH2-R was found to have high catalytic efficiency as a Csk substrate, approximately 20-fold greater than the next best characterized substrate poly(Glu,Tyr). Its behavior in terms of the K_m for ATP and the effects of Mg vs Mn on catalysis was similar with the poly(Glu,Tyr) results previously reported. Furthermore, the rate-limiting steps are also likely to be analogous based on a similar thio effect. Interestingly, the effects of NaCl on K_m were much less marked than previously observed with poly(Glu,Tyr), suggesting that electrostatic interactions may be unimportant for Csk-Lck recognition.

Thiophosphorylation of Lck-ΔNH2-R with ATPγS was also revealing. The presence of sizable thio effects in themselves suggests that the chemical step in the enzymatic transfer is at least partially rate-determining. As the magnitude of the thio effect is comparable to thio effects measured for peptides (*13*), it is probable that the chemical step has a similar rate and transition state for both protein and peptide substrates. Since in the ATPγS reaction, the chemical step is slow and fully rate-determining, it is likely that the binding and release of substrates are fast compared to the chemical step (a "rapid equilibrium" mechanism). Therefore, the K_m's likely correspond to K_d's (dissociation constants) for these reactions. This gives a true measure of affinity between Lck-ΔNH2-R and Csk in the presence of Mn and nucleotide. The K_d of 2.6 μM from our work is comparable to a dissociation constant between wild-type Lck and Csk recently determined using surface plasmon resonance (ca. 1 μM) (*31*).

Overall, these studies validate the use of unstructured peptide substrates for studies concerning the nature of the chemical mechanism of transfer for Csk. In contrast, these findings point to the inadequacy of peptides as models for substrate selectivity.

The results of these studies argue strongly that Csk-mediated phosphorylation of Src family members can be quite efficient in the absence of an autophosphorylated catalytic domain and without "third-party" proteins being present (as shown in Figure 6). Whether "third-party" proteins and/or Lck-ΔNH2-R autophosphorylation can further increase the specificity is unknown. However, the necessity for such enhancement in vivo is questionable since the phosphoryl transfer efficiency is already high, and the

k_{cat}/K_m is comparable to the most rapid values known for any protein kinase/substrate pair (*29*, *33*).

Lck-ΔNH2-R is phosphorylated at least 500 times faster than Lck-ΔNH2-R-505-P by Csk. As there are 16 tyrosines in Lck-ΔNH2-R, the selectivity for C-terminal tail tyrosine phosphorylation over other tyrosine residues is at least 8000-fold. Furthermore, the overall relative increase in the catalytic power of Csk for Lck-ΔNH2-R compared with short C-terminal tail peptides is at least 10^4. This corresponds to a large difference in energy ($\Delta G = 5.5$ kcal/mol), which must be accounted for in binding and catalysis. Precision docking using the modular domains outside the catalytic domains of the two groups of proteins could conceivably contribute to enhancing catalytic efficiency. The lack of Lck-ΔNH2-R-505-P to serve as a potent inhibitor of Csk phosphorylation of Lck is consistent with this idea as both the SH2 and SH3 domains of Lck are expected to be tied up in this structure (*26*, *27*). In Src family members, the SH2 domain binds in an intramolecular fashion to the phosphotyrosine tail, and the SH3 domain interacts intramolecularly with a type II polyproline helix located at the SH2 domain−catalytic domain junction. This conformationally constrained structure of tail-phosphorylated Lck may thus be unable to bind efficiently to Csk.

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