Substrate Conformational Restriction and CD45-Catalyzed Dephosphorylation of Tail Tyrosine-Phosphorylated Src Protein*

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Abstract: Hydrolysis of the tail phosphotyrosine in Src family members is catalyzed by the protein tyrosine phosphatase CD45, activating Src family-related signaling pathways. Using purified recombinant phospho-Src (P-Src) (amino acid residues 83-533) and purified recombinant CD45 catalytic (cytoplasmic) domain (amino acid residues 565-1268), we have analyzed the kinetic behavior of dephosphorylation. A time course of phosphatase activity showed the presence of a burst phase. By varying the concentration of P-Src, it was shown that the amplitude of this burst phase increased linearly with respect to P-Src concentration. Approximately 2% of P-Src was shown to be rapidly dephosphorylated followed by a slower linear phase. A P-Src protein substrate containing a functional point mutation in the SH2 domain led to more rapid dephosphorylation catalyzed by CD45, and this reaction showed only a single linear kinetic phase. These results were interpreted in terms of a model in which P-Src exists in a relatively slow dynamic equilibrium between “closed” and “open” conformational forms. Combined mutations in the SH2 and SH3 domain or the addition of an SH3 domain ligand peptide enhanced the accessibility of P-Src to CD45 by biasing P-Src to a more open form. Consistent with this model, a phosphotyrosine peptide which behaved as an SH2 domain binding ligand showed approximately 100-fold greater affinity for unphosphorylated Src versus P-Src.

Surprisingly, P-Src possessing combined SH3 and SH2 functional inactivating point mutations was dephosphorylated by CD45 more slowly compared to P-Src completely lacking SH3 and SH2 domains. Additional data suggest that the SH3 and SH2 domains can inhibit accessibility of the P-Src tail to CD45 by interactions other than direct phosphotyrosine binding by the SH2 domain. Taken together, these results suggest how activation of Src family member signaling pathways by CD45 may be influenced by the
presence or absence of ligand interactions remote from the tail.
Introduction

The interplay between protein tyrosine kinases and protein tyrosine phosphatases regulate critical cellular processes (1-3). One of the few well-established examples of a protein tyrosine phosphatase-phosphoprotein enzyme-substrate relationship in cell signalling is that of CD45 and Src. The CD45 tyrosine phosphatase participates in the catalytic removal of the tail phosphotyrosine from the Src protein tyrosine kinases (4-6). Src kinases are maintained in a catalytically quiescent state by the presence of a tail phosphorylated tyrosine which is introduced by the action of the protein tyrosine kinase Csk (7-9). The CD45-catalyzed tail-dephosphorylation reaction involving phosphorylated Lck, Fyn, and perhaps other Src kinase family members is responsible for the stimulation of their tyrosine kinase activities. In the case of Lck, this catalytic stimulation results in T cell differentiation and activation (4-6).

CD45 is a receptor tyrosine phosphatase protein with an extracellular domain of unclear function and two intracellular domains, a PTPase catalytic (D1) and pseudocatalytic (D2) domain that appear to collaboratively effect dephosphorylation (4-6, 10, 11). The D1/D2 tandem is necessary and sufficient in vivo for tail-dephosphorylation of Src family members. The nine Src family members are composed of a weakly conserved N-terminal membrane docking domain, and three highly conserved modules: an SH3 domain, an SH2 domain, a catalytic domain, and a phosphorylatable tyrosine containing tail. Upon tail phosphorylation, Src adopts an intricate three-dimensional fold in which the SH3 domain interacts intramolecularly with the SH2-catalytic domain linker and the SH2 domain binds to the tail phosphotyrosine (7-9). Because the phosphorylable moiety appears to be buried
in a pocket in the SH2 domain, it is difficult to understand how CD45 might gain access to
the tail to remove its phosphate.

While the recombinant CD45 intracellular PTPase domains have been prepared and
studied as catalysts with phosphotyrosine peptide substrates (12, 13), there has not yet been
reported a detailed analysis of CD45-catalyzed dephosphorylation of Src phosphoproteins in
a purified system. To gain greater understanding of the molecular basis of CD45
recognition of a phosphoprotein substrate, we undertook a kinetic analysis of the
dephosphorylation of tail-phosphorylated Src (P-Src) and report the results here.

EXPERIMENTAL PROCEDURES

General – Hapes, Tris, DTT, ATP, BSA, sodium vanadate, Triton X-100 and activated
charcoal were obtained from Sigma; imidazole, MnCl₂ and EDTA were purchased from
Fisher. The [γ-³²P]-ATP (6000 Ci/mmol) was purchased from New England Nuclear. All
Fmoc amino acid derivatives and resins were obtained from Novabiochem. The 5-(and-6)-
carboxy-X-rhodamine succinimidyl ester and EnzChek phosphate assay kit were purchased
from Molecular Probes. Cytoplasmic domain CD45 and Csk were prepared exactly as
reported previously (14, 15).

Preparation of Src, Src-cat, Src-3, Src-2, Src-23 (see Figure 1 for structures) – The pET
expression plasmid encoding kinase inactive Src (aa 83-533, K295M) (16) was used to
generate the plasmids encoding point mutations in the SH3 domain (W118V, src-3), SH2
domain (R174A, src-2) and the double mutant (W118V/R174A, src-23) using the QuikChange method (Stratagene) and the constructs were confirmed by DNA sequencing of the entire open reading frames. The expression plasmid encoding the catalytic domain of kinase inactive Src (aa 260-533, K295M, src-cat) was prepared previously (16). Src proteins were prepared from these vectors by expression in *E. coli* along with the chaperones GroES and GroEL as previously described (16). Each of these proteins which contained N-terminal His tags was purified by chromatography over a Zn-chelating column as reported previously (16). Proteins were concentrated by ultrafiltration to 2-4 mg/mL (determined by Bradford protein assay used bovine serum albumin as standard (17)) and stored in buffer at 80°C. Protein purities estimated by SDS-PAGE stained with Coomassie Blue were >80%.

**Preparation of SH3-SH2-2** The coding region for amino acid residues 83-259 (containing an R174A mutation) of the chicken *c-src* gene was subcloned into the pGEX-6P-1 expression vector (Amersham Pharmacia Biotech) with *Bam*HI and *Xho*I sites on the 5’- and 3’- ends, respectively. The GST fusion protein was expressed in *E. coli* and immobilized on glutathione-agarose resin as described previously (18). Removal of the GST tag from the fusion protein was achieved on-resin by treatment with PreScission protease (Amersham Pharmacia Biotech) as described by the manufacturer. In brief, the cell lysate (obtained from 1 L of *E. coli* culture) containing GST-fusion protein was immobilized on 0.2 g glutathione-agarose resin suspended in 2 mL of cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT) and treated with 30 unit PreScission protease overnight at 4°C. The cleavage reaction mixture was further purified by chromatography over a MonoS (HR5/5) ion exchange column. The resultant
protein (ca. 70% pure by SDSPAGE stained with Coomassie Blue) was concentrated to 1.6 mg/mL (determined by Bradford protein assay), and stored at 80°C. The molecular weight of the recombinant protein fragment was confirmed by MALDI mass spectrometric analysis.

**Peptide Synthesis**  The phosphotyrosine-containing peptide (pY542) NH₂-CEpYTNIKYSLADQTSGD-CO₂H was prepared as described previously (19). The SH3 domain binding peptide (SH3BP) AcNH₂-VSLARRPLPPLP-CNH₂ (20) and the rhodamine-tagged, phosphotyrosine-containing peptide (Rhod-SH2BP) Ac-PQpYEEIPGGGK(Rhod)-NH₂ were prepared by solid phase peptide synthesis using the Fmoc strategy on a 0.1 mmol scale. For Rhod-SH2BP synthesis, phosphotyrosine was introduced in the phosphate unprotected form. Orthogonal protection of the \( \varepsilon \)-NH₂ group of the C-terminal Lys residue with Dde (dimethylidioxocyclohexyldene) allowed direct attachment of rhodamine (activated as a succinimide ester) before the final cleavage step (21). Peptides were cleaved and deblocked using reagent K (10 mL trifluoroacetic acid, 0.75 g phenol, 0.5 mL thioanisole, 0.25 mL ethanedithiol, 0.5 mL water) and purified to greater than 95% homogeneity by reversed phase HPLC using a water:acetonitrile:0.05% trifluoroacetic acid gradient. Correct peptide structures were confirmed by electrospray ionization mass spectrometry.

**Phosphorylation of Src, Src-cat, Src-3, Src-2 and Src-23** – Purified recombinant proteins were phosphorylated at Tyr-527 by Csk. General reactions were performed in a
volume of 0.5 mL at 30°C and pH 7.4 with 15-30 µM of Src protein or Src mutants (Figure 1), 30 nM Csk, 2 mM MnCl₂, 60 µM ATP (0.4 µCi of [γ-32P]-ATP), 60 mM Tris-HCl, 4 mM Na-Hepes, 10 mM DTT, 60 µg/mL bovine serum albumin for 30 min in a 1.5 mL plastic (Eppendorf) tube. Phosphorylated proteins were purified by chromatography on a Zn-chelating column as described previously (16) and were then dialyzed against phosphatase assay buffer (25 mM Na-Hepes, pH 7.5, 5 mM EDTA and 10 mM DTT). The proteins were concentrated by Centricon ultrafiltration (Millipore) and the concentration was determined by Bradford protein analysis. Stoichiometry of Src protein phosphorylation was determined to be >90% by radioactive counting. Less than 5% phosphorylation of the Src Y527F protein in the presence of Csk was observed indicating a specific labeling on the tail tyrosine residue Tyr-527 of above proteins.

**Phosphatase Assays with Phosphorylated P-Src or mutants** – Dephosphorylation activity was measured based on the release of inorganic phosphate. Reactions were performed in a volume of 0.03 mL at 25°C and pH 7.5 with 0.5-100 µM phosphorylated Src or mutants, 0.5-60 nM CD45, 25 mM Na-Hepes, pH 7.5, 5 mM EDTA and 10 mM DTT in a 0.6 mL plastic (Eppendorff) tube. Reactions were initiated with CD45 and aliquots (3-8 µL) of reaction mixture at fixed time points (up to 15 min) and were quenched with 400 µl of acidic 5% activated charcoal suspension, followed by immediate vortexing. The quenched mixtures were centrifuged for 20 min at 2000g and 200 µl of the supernatant was then removed and transferred to 9 ml scintillation fluid and radioactivity was then measured by scintillation counting. Dephosphorylation of P-Src and mutants was shown to occur
linearly with respect to CD45 concentration in the ranges used and linearly with respect to

time up to $\geq 20$ min (except for those reactions showing a burst phase, see below). The

assay was further validated by demonstrating that excess CD45 could completely (>95%)
dephosphorylate P-Src. The effectiveness of the quench was established by showing that no

further phosphate release occurred after vortexing with activated charcoal. 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%. Time course data were fitted either to a single-

phase, linear model or a two-phase kinetic model containing a first-order “burst-phase”

followed by a first-order steady-state phase (22):

$$P = A \left(1-e^{-kt}\right) + vt$$  
(Eq. 1)

P - product formation, A - burst amplitude, k - burst rate constant, v - steady-state rate, t -
time

The steady-state kinetic parameters were obtained from fitting data to the Michaelis-

Menten equation using a non-linear curve-fitting approach as described previously (16).

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*Preparation of partially dephosphorylated P-Src* Partial dephosphorylation of P-Src was
catalyzed by CD45 in a reaction with 10 $\mu$M P-Src, 30 nM CD45 at 25°C for 4 min. The
reaction was then quenched with 0.7 mM sodium vanadate. Under these conditions, it was
estimated that approximately 3% of the P-Src was hydrolyzed. The reaction mixture was
loaded on to a 0.5 ml Zn-chelating column and purified as reported previously (16). This
purified partially dephosphorylated P-Src (2 μM) was subsequently tested as a CD45 substrate as described above.

**Peptide Phosphate Assay** To assess the interaction of CD45 and SH3-SH2 domain of Src, EnzChek® phosphate assays with a short phosphopeptide substrate were performed as described by the manufacturer (Molecular Probes) based on the method of Webb (23). In brief, reactions were performed with 1 nM CD45, 1-8 μM SH3-SH2-2, 25 μM pY542 peptide, 12 μL MESG (2-amino-6-mercapto-7-methyl-purine riboside), 0.6 μL purine nucleoside phosphorylase, 3 μL 20X reaction buffer (400 mM Tris-HCl, pH7.5, 20 mM MgCl₂, 100 mM DTT, 1 M NaCl) in 60 μL reaction volume in a cuvette at 25 °C and quantitatively monitored by UV absorbance change at 360 nm. The reaction velocities were calculated based on the release of inorganic phosphate versus time. Control experiments showed no apparent change in CD45 activity with further increases of purine nucleoside phosphorylase, and that the phosphatase activity was linear with respect to time and enzyme concentration in this range. Furthermore, the concentration of peptide substrate used was well below its Kₘ in this system.

**Fluorescence Binding Measurements** – To measure the dissociation constants (Kₐ) for binding of a SH2 domain binding peptide to the phosphorylated P-Src and nonphosphorylated Src-Y527F proteins, titration reactions were conducted by titrating fixed concentrations (1 μM) of Rhod-SH2BP peptide with increasing amounts of the protein (0
90 µM) on a SPEX Fluoromax spectrofluorometer using a 3 mm square cuvette (24). The emission spectra were collected over the wavelength range 595 - 700 nm with an excitation wavelength of 574 nm. All measurements were performed in 20 mM Na-Hepes, pH 7.5, 5 mM DTT, 50 mM NaCl at 25 °C. The fluorescence intensity (F) at 605 nm was plotted against protein concentration to obtain the $K_D$ from Equations 1 and 2 after the background fluorescence of protein was subtracted from each spectrum ($F_0$ and $F_f$ are the initial and final fluorescence intensities, respectively),

$$F = F_0 - \{(F_0 - F_f)[\text{Peptide}]_{\text{tot}}/2\} \{b - (b^2 - 4[\text{Protein}]_{\text{tot}}[\text{Peptide}]_{\text{tot}})^{1/2}\}$$

(Eq. 2)

$$b = K_D + [\text{Protein}]_{\text{tot}} + [\text{Peptide}]_{\text{tot}}$$

(Eq. 3)

**Results**

*CD45-catalyzed dephosphorylation of P-Src shows a burst phase*

Recombinant human Src (83-533) was overproduced and purified from *E. coli* as described previously (16) and phosphorylated with $\gamma$-32P-ATP to near completion with recombinant Csk to generate P-Src (Figure 1). A kinase defective mutant of Src was employed to avoid autophosphorylation affecting the analysis (16). The presence of additional phosphotyrosine modifications in P-Src would complicate the interpretation of dephosphorylation results since CD45 would likely act on these other sites. As demonstrated previously (16, 18, 25), Csk is extremely selective for the C-terminal tyrosine
of Src family members which in part was confirmed here by demonstrating the lack of phosphorylation of the Y527F mutant. Specific radioactivity of labelled P-Src obtained in this manner suggested 95-100% labelling. The protein was purified away from residual ATP and Csk by affinity chromatography and subjected to CD45 dephosphorylation and the inorganic phosphate generation was monitored by partitioning with activated charcoal.

In a time course of dephosphorylation of P-Src, a burst phase of inorganic phosphate release was observed reproducibly followed by a slower phase of phosphate generation (Figure 2). This data was nicely fit by a burst phase kinetic model with two first-order rate constants $k_{\text{burst}} = 0.8 \text{ min}^{-1}$, $k_{\text{steady-state}} = 0.1 \text{ min}^{-1}$, and burst amplitude = 59 nM. In initial experiments, it was considered that this burst phase might be due to the initial single turnover by the enzyme (22), in part because the burst amplitude (40-60 nM) was somewhat similar to the concentration of CD45 present (30 nM determined by Bradford assay).

However, a plot of the time course as a function of different CD45 concentrations failed to show a significant change in the amplitude of the burst phase (data not shown). In contrast, the amplitude of the burst phase increased linearly with increasing P-Src concentration over a fairly wide range (Figure 3). The slope of a plot of burst amplitude versus P-Src concentration (Figure 3B) was approximately 0.02, suggesting that 2% of the P-Src was reacting with a relatively rapid rate constant and 98% with a somewhat slower value.

**P-Src behaves as a reversible equilibrium of isoforms**

Two possibilities were considered for the above behavior. In the first, two stable and presumably covalently different forms of P-Src were present, perhaps due to oxidation or
proteolysis of a minor amount of protein. A second possibility was that there are two forms of interconverting P-Src in an established equilibrium. In either case, the burst phase would be due to the minor component which is a more efficiently processed substrate. In order to distinguish between these models, the P-Src protein was treated with CD45 until approximately 3% of the P-Src was hydrolyzed and then quenched with vanadate. Theoretically, this should be sufficient to remove the minor component, estimated to be 2% as stated above. The quenched reaction mixture was eluted over a Zn chelate column to remove the CD45. After dialysis to remove vanadate, the pre-hydrolyzed P-Src was again exposed to CD45 and the time course of product formation was recorded. The time course with this pre-hydrolyzed P-Src (Figure 2 B) was essentially identical to untreated P-Src (Figure 2 A) arguing in favor of the model of interconverting forms of P-Src and against the concept that irreversible covalent changes are responsible for the burst phase behavior.

Effects of SH3 and SH2 mutations on P-Src dephosphorylation

In considering the structural basis for the minor component showing enhanced efficiency as a CD45 substrate, we considered the possibility that this form of the protein could contain a disruption in its known intramolecular SH2-pTyr or SH3-PPII linker interactions. To evaluate these possibilities, we prepared the following mutant proteins: P-Src-2, P-Src-3, P-Src-23, and P-Src-cat (Figure 1). The CD45-catalyzed dephosphorylation time courses for these proteins were obtained and shown in Figure 4 and the rates versus substrate concentrations plotted in Figure 5. It is apparent from Figure 4 that the burst phase associated with “wild type” P-Src protein substrate has essentially disappeared and the data for the mutants could be reasonably fit to linear time courses. Each
of the mutant proteins exhibited faster steady-state rates compared to “wild-type” P-Src. These data suggest that the initial burst phase in P-Src dephosphorylation is the result of a more open form of P-Src which is lacking the crystallographically observed intramolecular SH2-phosphotyrosine interaction (8, 9). Examination of Figure 5 indicates that P-Src and mutant P-Src proteins display reasonable fits to Michaelis-Menten kinetics although the $K_m$ for all but P-Src-cat was hard to ascertain because for these substrates it is much greater than 10 µM. Finally, the effects of the point mutations show cooperativity as suggested by the progression of steady state dephosphorylation rates ($V/E$) for P-Src (0.1 min$^{-1}$, calculated from Figure 2), P-Src-3 (0.28 min$^{-1}$), P-Src-2 (1.8 min$^{-1}$), and P-Src-23 (6.9 min$^{-1}$) (Figure 4).

Effect of SH3 peptide ligand (SH3BP) on dephosphorylation

That the substrate P-Src-3 showed a steady-state kinetic rate of dephosphorylation that was 3-fold faster than that of “wild-type” P-Src, and P-Src-23 showed a 3-fold faster steady-state rate compared to P-Src-2 suggested that the SH3-PPII linker interaction is modestly inhibitory to CD45 recognition and/or dephosphorylation. In order to further assess this possibility, the proline-rich high affinity ligand SH3BP was added to the P-Src and P-Src-2 dephosphorylation reactions as an independent way to disrupt the intramolecular SH3-linker interaction in P-Src. As can be observed in both cases (Figure 6A,B), the steady-state rate of dephosphorylation was enhanced by the presence of SH3BP by about 3-fold. While a burst phase for CD45-catalyzed dephosphorylation of P-Src still appears to be present, the burst phase amplitude is approximately 2-fold larger in the
presence of SH3BP. Taken together with the SH3 mutation data, these results suggest that
the SH2-phosphotyrosine and SH3-linker interactions cooperatively inhibit the
dephosphorylation reaction catalyzed by CD45.

**Affinity of an SH2 ligand for P-Src and unphosphorylated Src**

Since the burst amplitude corresponds to about 2% of the total P-Src protein
concentration, it was proposed that the ratio of concentrations at equilibrium between closed
and open P-Src might be approximately 50:1. To measure this equilibrium using an
independent approach, a fluorescently labelled phosphotyrosine containing peptide was
synthesized and its affinity with P-Src and unphosphorylated full length Src was
investigated (Figure 6C,D). The $K_d$ values for P-Src and unphosphorylated Src are 75 and
0.7 µM (suggesting an open/closed equilibrium of 100:1), respectively, in approximate
concordance with the apparent equilibrium constant deduced from the dephosphorylation
studies.

**Evidence for non-phosphate-mediated intramolecular domain interactions**

Interestingly, P-Src-cat is quite a bit more (ca. 20-fold) efficiently
dephosphorylated as a CD45 substrate compared to P-Src-23. Since two major
intramolecular interactions are thought to be interrupted in this protein, it was unclear how
the SH3 and SH2 domains were inhibiting CD45-catalyzed dephosphorylation. To gain
greater insight into this observation, the effect of the presence intermolecularly of the Src
SH2-SH3-2 fragment (bearing Arg→Ala SH2 domain mutation; Figure 1) on CD45
dephosphorylation of P-Src-cat was investigated. A dosage-dependent inhibition of CD45-catalyzed dephosphorylation of P-Src-cat by SH2-SH3-2 was observed (Figure 7), consistent with the finding that CD45 dephosphorylates intact P-Src less efficiently than P-Src-cat protein.

Two potential models could explain how SH2-SH3-2 could inhibit CD45-catalyzed dephosphorylation of P-Src-cat. In one model, SH2-SH3-2 could directly bind and inhibit CD45 as a competitive inhibitor or allosteric regulator. In a second model, SH2-SH3-2 could interact with P-Src-cat directly, blocking its accessibility to CD45. To distinguish between these models, the effect of SH2-SH3-2 on CD45-catalyzed dephosphorylation of a short, unrelated peptide (pTyr542) was investigated. In these studies, it was found that CD45 activity on pTyr542 was not inhibited by SH2-SH3-2. These results favor the model in which SH2-SH3-2 limits access to P-Src-cat by a direct interaction between these two protein fragments, presumably mimicking an intramolecular interaction in P-Src.

Discussion

There has been increased attention with regard to how protein phosphatases recognize and act on physiologic phospho-protein substrates (26, 27). These studies provide the first detailed analysis of purified CD45 catalytic domain with a tail-phosphorylated Src protein family member as substrate. It has been observed here that the behavior of the CD45-catalyzed dephosphorylation reaction of P-Src protein is more complex than that with P-Src-cat or phosphopeptide. It is envisioned that the intramolecular interaction between the phosphotyrosine and the SH2 domain limit accessibility of the phosphotyrosine to CD45 (Figure 8A). Perhaps more unexpected is that
the SH3 domain-PPII linker intramolecular interaction in P-Src also contributes significantly to inhibition of the CD45 dephosphorylation reaction. These intramolecular interactions appear to show cooperativity not only in preventing enzyme-catalyzed dephosphorylation as seen here but also in limiting the activity of the protein tyrosine kinase activity of Src family members (28-30).

One of the more interesting findings from these studies is the observation of a burst phase in CD45-catalyzed dephosphorylation of P-Src. This leads to at least two important conclusions regarding P-Src and its interaction with CD45. First, the equilibrium between the “closed” and “open” forms of P-Src (Figure 8A) can clearly influence the rate of dephosphorylation by CD45. Second, the rate-limiting step for dephosphorylation of P-Src by CD45 at steady-state appears to involve the opening of P-Src with the loss of the intramolecular SH2-phosphotyrosine interaction (Figure 8B). That is, the energetic barrier in going from the open to closed conformation is presumably higher than the energetic barrier to CD45-catalyzed dephosphorylation. If the barrier to going from open to closed state were lower than the CD45-catalyzed dephosphorylation barrier, no burst phase should be observed since a rapid equilibrium between the open and closed forms should exist. Thus, the CD45-dephosphorylation reaction offers a new approach to interrogating the rate of conformational change motions of P-Src. This should allow for the analysis of the effects of various ligands and mutations on the conformational state of the P-Src protein in a fashion complementary to measuring Src kinase activity or surface plasmon resonance (28-31).

In principle, the “opening” rate in Figures 8 should be equal to the $k_{cat}$ of P-Src.
dephosphorylation by CD45 at steady-state, which can be estimated to be 5 min\(^{-1}\) (Figure 5A). However, because the P-Src-23 rate of dephosphorylation catalyzed by CD45 is about 20-fold lower compared to P-Src-cat, it is not clear precisely what the “open” form really corresponds to structurally and what interactions are still present in this form of P-Src. Further studies with other phosphatases and complementary transient kinetic methods will likely be needed to provide increased insight into the physical basis for these rate constants.

The three-dimensional interactions in P-Src-23 that prevent it from being as efficiently processed as P-Src-cat present an intriguing structural biology problem. The obvious implication is that significant long range, interdomain interactions in unphosphorylated Src may contribute to its catalytic activity, substrate interactions, and regulation. Recent structural and enzymatic studies on the related protein tyrosine kinase Csk suggest that such long range interactions may have a significant impact on the structure and function of the kinase domain (18, 32, 33).

The implications of the kinetic behavior of CD45-catalyzed dephosphorylation of P-Src on the scope and mechanisms of Src in cell signalling pathways are worth considering here. That there is a burst phase in dephosphorylation of the tail by CD45 could allow a Src-related pathway to undergo rapid initiation by a subpopulation of Src molecules. This initiation may then be followed by a slower but more sustained activation as the bulk of the tail-phosphorylated Src is dephosphorylated. These results also point to a new role for ligands, such as phosphotyrosine proteins and proline-rich ligands that can bind to the SH2 domain and SH3 domain of Src and their mode of activation of the tail-phosphorylated Src protein. Obviously, it is possible that they can directly activate the P-Src protein by
restructuring the Src catalytic domain. But it is also now clear that they can enhance the tail
deposphorylation of Src which again could lead to sustained Src pathway activation.

A paradox concerning Src family member-CD45 interactions is that CD45 appears
to be able to inactivate these tyrosine kinases by dephosphorylation of the activation loop
phosphotyrosine site (4-6). It is not yet understood how activation loop dephosphorylation
catalyzed by CD45 might be affected by the presence of tail phosphotyrosine or its
interaction with SH2 domain. Moreover, it is not yet known how the lipid membranes that
serve as the physiologic environment for CD45 and P-Src might influence catalysis. Future
studies may allow a greater understanding of the effects of these complex variables on
CD45-Src interactions.

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members of the Cole lab for helpful discussions.
Abbreviations used: PTPase, protein tyrosine phosphatase; D1, catalytic domain of CD45; D2, pseudocatalytic domain of CD45; SH2, Src homology domain 2; SH3, Src homology domain 3; P-Src ("wild-type"), tail tyrosine phosphorylated Src (aa 83-533, K295M); P-Src-2, P-Src with a mutation (R174A) in SH2 domain; P-Src-3, P-Src with a mutation (W118V) in SH3 domain; P-Src-23, P-Src with two mutations (R174A/W118V) in the SH2 and SH3 domains; P-Src-cat, tail tyrosine phosphorylated kinase domain of Src (aa 261-533); SH3-SH2-2, Src SH3-SH2 fragment (aa 83-260) with a mutation (R174A) in the SH2 domain; pY542, a phosphotyrosine-containing peptide; Rhod-SH2BP, rhodamine-tagged, phosphotyrosine-containing peptide; SH3BP, SH3 domain binding peptide. Csk, C-terminal Src kinase; CD45, cytoplasmic domains of a receptor tyrosine phosphatase; ATP, adenosine triphosphate; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Hepes, N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid.
References

1) Hunter, T. (2000) *Cell* **100**, 113-127

2) Blume-Jensen P, and Hunter T. (2001) *Nature* **411**, 355-365

3) Tonks, N. K., and Neel, B. G. (2001) *Curr. Op. Cell Biol.* **13**, 182-195

4) Hermiston, M. L., Xu, Z., Majeti, R., and Weiss, A. (2002) *J. Clin. Invest.* **109**, 9-14

5) Qian, D., and Weiss, A. (1997) *Curr. Opin. Cell Biol.* **9**, 205-212

6) Thomas, M. L. (1994) *Curr. Opin. Cell Biol.* **6**, 247-252

7) Brown, M. T., and Cooper, J. A. (1996) *Biochem. Biophys. Acta* **1287**, 121-149

8) Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature* **385**, 595 – 602

9) Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385**, 602 – 609

10) Johnson, P., Ostergaard, H. L., Wasden, C., and Trowbridge, I. S. (1992) *J. Biol. Chem.* **267**, 8035-8041

11) Streuli, M., Krueger, N. X., Thai, T., Tang, M., and Saito, H. (1990) *EMBO J.* **9**, 2399-2407

12) Cho, H., Krishnaraj, R., Itoh, M., Kitas, E., Bannwarth, W., Saito, H., and Walsh, C. T. (1993) *Protein Sci.* **2**, 977-984.

13) Cho, H., Ramer S. E., Itoh, M., Kitas, E., Bannwarth, W., Burn, P., Saito, H., and Walsh, C. T. (1992) *Biochemistry* **31**, 133-138

14) Wang, Y., Guo, W., Liang, L., and Esselman, W. J. (1999) *J Biol Chem* **274**, 7454-7461

15) Grace, M.R., Walsh, C.T., and Cole, P.A. (1997) *Biochemistry* **36**, 1874-1881

16) Wang, D., Huang, X.-Y., and Cole, P. A. (2001) *Biochemistry* **40**, 200-2010

17) Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254.

18) Sondhi, D., and Cole, P. A. (1999) *Biochemistry* **38**, 11147-11155
19) Lu, W., Gong, D., Bar-Sagi, D., and Cole, P. A. (2001) Mol. Cell 8, 759-769

20) Feng, S., Kasahara, C., Rickles, R. J., and Schreiber S. L. (1995) Proc. Natl. Acad. Sci. USA 92, 12408-12415

21) Muir, T. W., Sondhi, D., and Cole, P. A. (1998) Proc. Natl. Acad. Sci. USA 95, 6705-6710

22) Ghose, C., and Raushel, F. M. (1985) Biochemistry 24, 5894-5898

23) Webb, M. R. (1992) Proc. Natl. Acad. Sci. U S A. 89, 4884-4887

24) Jiang Y.L., Kwon, K., and Stivers, J. T. (2001) J. Biol. Chem. 276, 42347-42354

25) Wang, D. and Cole, P. A. (2001) J. Am. Chem. Soc. 123, 8883-8887.

26) Wang, Z. X., Zhou, B., Wang, Q. M., and Zhang, Z. Y. (2002) Biochemistry 18, 7849-7857.

27) Zhao, Y., and Zhang, Z. Y. (2001) J. Biol. Chem. 276, 32382-32391

28) Porter, M., Schindler, T., Kuriyan, J., and Miller, W. T. (2000) J. Biol. Chem. 275, 2721-2726

29) LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J., and Miller, W. T. (1998) J. Biol. Chem. 273, 32129-32134

30) Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C. H., Kuriyan, J., and Miller, W. T. (1997) Nature 385, 650-653

31) Boerner, R. J., Kassel, D. B., Barker, S. C., Ellis, B., DeLacy, P., and Knight, W. B. (1996) Biochemistry 35, 9519-9525

32) Shekhtman, A., Ghose, R., Wang, D., Cole, P. A., and Cowburn, D. (2001) J. Mol. Biol. 314, 129-138

33) Ogawa, A., Takayama, Y., Sakai, H., Chong, K. T., Takeuchi, S., Nakagawa, A., Nada,
S., Okada, M., and Tsukihara, T. (2002) *J. Biol. Chem.* **277**, 14351-14354
Figure Legends

Fig. 1. Constructs of CD45, Src and mutants used in these experiments. The numbers with lettering above the construct indicate mutations and the phosphorylatable tail tyrosine is indicated by Y527. The numbers below the construct specify amino acid sequence numbering.

Fig. 2. CD45 catalyzed dephosphorylation of P-Src. (A) Time course of the hydrolysis of P-Src, and (B) P-Src, partially dephosphorylated by pretreatment with CD45. Conditions: concentration of P-Src or pretreated P-Src = 2 \( \mu \)M, CD45 = 30 nM, 25ºC. Data were fitted to a burst equation. For untreated P-Src, burst amplitude = 59±3 nM, burst rate = 0.8±0.1 min\(^{-1}\), steady-state rate = 0.1±0.01 min\(^{-1}\). For pretreated P-Src, burst amplitude = 53±4 nM, burst rate = 0.8±0.1 min\(^{-1}\), steady-state rate = 0.1±0.01 min\(^{-1}\). Each of these experiments was performed at least 4 times (duplicates on two separate occasions) and showed good reproducibility (±20%). See “Experimental Procedures” for experimental conditions.

Fig. 3. CD45 Dephosphorylation of P-Src. (A) Multiple time courses of the hydrolysis of P-Src at various P-Src concentrations. \( \times \), 1 \( \mu \)M; \( , \) 2 \( \mu \)M; \( s \), 4 \( \mu \)M; \( c \), 10 \( \mu \)M; \( g \), 15 \( \mu \)M; \( o \), 20 \( \mu \)M; \( l \), 30 \( \mu \)M. [CD45] = 30 nM, 25ºC. Each plot was fit to the burst phase equation described in the Experimental Procedures. (B) Plot of burst amplitude versus [P-Src]. Data was obtained from the burst equation fit from the data in Figure 3A. The data was fit to a
linear equation and gave a slope of 0.02.

Fig. 4. CD45 catalyzed hydrolysis of phosphorylated Src mutants. (A) Time course of P-Src-3, V/E=0.28±0.01 min⁻¹; (B) P-Src-2, V/E=1.8±0.03 min⁻¹; (C) P-Src-23, V/E=6.9±0.1 min⁻¹; (D) P-Src-cat, V/E=129±2 min⁻¹. P/E is the ratio of the concentration of the product formed divided by the concentration of the enzyme used. Data of the P-Src-3 dephosphorylation (A) were fitted to a linear equation because, although a possible burst phase was detected, it was too small to fit reliably to the burst phase equation. Data for B-D were also fitted to linear equations. Experimental conditions: [P-Src mutant] = 2 µM, [CD45] = 0.3 60 nM, 25ºC.

Fig. 5. Kinetic analysis of the CD45 dephosphorylation of P-Src and mutants. (A) Plot of velocity versus [P-Src], (B) [P-Src-2], (C) [P-Src-23], and (D) [P-Src-cat]. The steady-state velocities of P-Src were obtained from multiple time courses fit to the burst equation. Data were fit to the standard Michaelis-Menten equation. [CD45] = 1 - 60 nM, 25ºC. The steady-state catalytic efficiency (k_cat/K_m) for these proteins is as follows: P-Src, 1.1±0.1×10³ M⁻¹s⁻¹; P-Src-2, 1.5±0.2×10⁴ M⁻¹s⁻¹; P-Src-23, 4.7±0.5×10⁴ M⁻¹s⁻¹; and P-Src-cat, 1.6±0.1×10⁶ M⁻¹s⁻¹. For P-Src-cat, k_cat = 1.5±0.1×10⁳ min⁻¹, K_m = 15±3 µM.

Fig. 6. Effect of SH3BP, a SH3 domain binding peptide, on the CD45 dephosphorylation of P-Src and mutant, and binding of Rhod-SH2BP, a SH2 domain binding peptide, to
phosphorylated and unphosphorylated Src proteins. (A) Time course of the dephosphorylation of P-Src, and (B) P-Src-2 in the presence (i) and absence (o) of 100 µM SH3BP. Conditions: Concentration of P-Src or mutant = 2 µM, CD45 concentration was 30 or 5 nM for P-Src or P-Src-2, respectively. For P-Src, the burst amplitude = 48±5 nM in the absence of the peptide SH3BP and 99±8 nM in the presence of SH3BP. The steady-state rate (V/E) = 0.13±0.02 and 0.4±0.03 min⁻¹ without and with SH3BP, respectively. For P-Src-2, SH3BP increased V/E from 1.8±0.03 min⁻¹ to 5.1±0.1 min⁻¹. (C) Plot of fluorescence intensity at 605 nM versus the total concentration of added protein P-Src, and (D) Src-Y527F. The K_D values of 0.7 ± 0.4 µM and 75 ± 9 µM were obtained from a fit to Equations 2 and 3 for P-Src and Src-Y527F, respectively.

Fig. 7. Effect of SH2-SH3-2 on the CD45 dephosphorylation of P-Src-cat and pY542 peptide. (A) Plot of velocity versus [SH2-SH3-2] in the presence of a fixed P-Src-cat concentration (1 µM); [CD45], 1 nM. (B) Plot of velocity versus [SH2-SH3-2] in the presence of a fixed pY542 concentration (25 µM), [CD45], 1 nM. See Experimental Procedures for conditions.

Fig. 8. (A) Proposed model for CD45 dephosphorylation of P-Src. P-Src exists in equilibrium of open and closed forms. Burst rate is caused by the faster dephosphorylation of open P-Src whereas slower dephosphorylation of closed P-Src represents the steady-state rate. (B) Reaction coordinate diagram of CD45 dephosphorylation of P-Src. The energetic barrier from open P-Src to closed P-Src (AT.S.) is higher than that from open P-
Src to dephosphorylated Src (B.T.S.).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Dephosphorylation

CD45 slow steady-state phase

Dephosphorylation

CD45 fast burst phase

B. Reaction Coordinate

2.4 kcal/mol

AT.S.

B.T.S.

Closed P-Src

Open P-Src

Dephosphorylated Src

Figure 8
Substrate conformational restriction and CD45-catalyzed dephosphorylation of tail tyrosine-phosphorylated Src protein
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