Src Tail Phosphorylation Is Limited by Structural Changes in the Regulatory Tyrosine Kinase Csk

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Src family tyrosine kinases are down-regulated through phosphorylation of a single C-terminal tyrosine by the nonreceptor tyrosine kinase Csk. Despite the fundamental role of Csk in controlling cell growth and differentiation, it is unclear what limits this key signaling reaction and controls the production of catalytically repressed Src. To investigate this issue, stopped-flow fluorescence experiments were performed to determine which steps modulate catalysis. Both Src binding and phosphorylation can be monitored by changes in intrinsic tryptophan fluorescence. Association kinetics are biphasic with the initial phase corresponding to the bimolecular interaction of both proteins and the second phase representing a slow conformational change that coincides with the rate of maximum turnover. The kinetic transients for the phosphorylation reaction are also biphasic with the initial phase corresponding to rapid phosphorylation and the release of phospho-Src. These data, along with equilibrium sedimentation and product inhibition experiments, suggest that steps involving Src association, phosphorylation, and product release are fast and that a structural change in Csk participates in limiting the catalytic cycle.

The Src family tyrosine kinases (SFKs) play important roles in cellular growth and differentiation. Studies of transgenic mice indicate that SFKs serve key functions in the development of the immune response (1–3), whereas unregulated SFK activities have been detected in human malignancies including tumors of the breast and colon (4, 5). Such findings underscore the need for a thorough understanding of the regulation of these kinases in human disease. All of the members of this kinase family contain three important structural domains: a C-terminal tyrosine kinase domain flanked on the N terminus by noncatalytic SH2 and SH3 domains. The activity of the kinase domain of Src, a prototype for the SFKs, is controlled through autophosphorylation in the kinase domain on the activation loop and through phosphorylation in the C-terminal tail segment (6). The latter phosphorylation event catalyzed by the Csk (C-terminal Src kinase) represses activity through a substantial conformational change involving all three domains. In the active form of Src, only the SH3 domain interacts with the kinase domain (7). However, phosphorylation of the C terminus by Csk induces large segmental movements of the SH2-SH3 domains across the small lobe of the kinase domain, resulting in a high affinity interaction between the phosphorylated tail sequence and the SH2 domain (8–10). In the repressed form, the SH2 domain now makes strong contacts with the large lobe of the kinase domain. This motion induces a closed conformation of Src that is apparently not capable of binding and phosphorylating protein substrates. This modification and subsequent conformational change has profound biological significance for the regulation of all SFKs. For example, mice with targeted disruptions in the Csk gene die in utero at week 9, and cells harvested from these embryos display unusually high SFK activity (11).

Csk is a nonreceptor tyrosine kinase that shares much homology with the SFKs that it regulates. Csk is also composed of essential kinase, SH2, and SH3 domains, but these domains are organized uniquely with both SH2 and SH3 domains interacting with the small lobe of the kinase domain (12). Csk is not regulated by autophosphorylation of its activation loop in the kinase domain and does not contain a phosphorylatable C terminus (13). Thus, unlike the SFKs, Csk is considered a constitutively active kinase that does not require any further chemical modifications for activity. In the cell, Csk is cytosolic but can phosphorylate SFKs at the membrane when recruited by an adaptor protein, Cbp (Csk-binding protein) (14, 15). Cbp is a transmembrane protein that may be phosphorylated at a specific tyrosine side chain, a modification that provides a binding site for Csk and colocalizes Csk near membrane-associated SFKs. The binding of Csk and Cbp is driven by the high affinity interaction between phospho-Cbp and the SH2 domain of Csk. This interaction has also been shown to increase the catalytic activity of the tyrosine kinase domain of Csk, further enhancing the repression of SFK activity (16).

Mutagenesis studies have shown that Csk recognizes not only residues directly in the C terminus but also residues in the large lobe of the kinase domain of Src (17–20). Several side chains located in helix D outside the active site of Csk in a docking groove are important in maintaining efficient Src phosphorylation kinetics (19). In a previous study we showed that Csk does not bind with high affinity to Src based on equilibrium sedimentation and single turnover experiments (21). Rather, the low $K_m$ for Src stems from a fast phosphoryl transfer step that clamps the substrate into the active site, a mechanism also shown to be operative in another kinase-protein substrate system (22). Given these findings, it is likely that residues out-
side the active site of Csk play a large role in enhancing the phosphoryl transfer step, thereby promoting efficient recognition of poorly interacting substrates. Although Csk has clearly evolved to support a highly efficient phosphoryl transfer step, it is still unclear what factors modulate Src turnover ($k_{cat}$), the maximum rate constant at which phosphoryl-Src is generated at high Src concentrations. To investigate what controls this parameter, we developed a stopped-flow fluorescence assay to monitor Src association and phosphorylation. Using these techniques, we found that phospho-Src is generated and released rapidly and does not limit turnover. Instead, a slow conformational change associated with the catalyst limits the rate of multiple catalytic turnovers. This represents the first demonstration that a slow structural change in a protein kinase may control the phosphorylation of a physiological substrate target.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, ADP, Mops, MgCl$_2$, KCl, acetic acid, DE52 resin, and liquid scintillant were obtained from Fisher. AMPPNP was purchased from Sigma. [$\gamma$-$^32$P]ATP was obtained from PerkinElmer Life Sciences.

**Protein Purification**—A full-length form of human Csk with a C-terminal polyhistidine tag was expressed in Escherichia coli strain BL21(DE3) and purified with a Ni$^{2+}$-agarose column according to a previously published procedure (23). A GST form of Csk (GST-Csk) was also expressed in E. coli strain BL21(DE3) and purified as previously described (24). A mutant form of chicken Src lacking the first 82 residues and possessing a lysine-to-methionine substitution at position 295 was coexpressed with GroEL/ES chaperonin proteins in E. coli strain BL21(DE3) and purified using a Ni$^{2+}$-agarose spin column.

**Synthesis of Phospho-Src**—Phospho-Src was synthesized using Src (20 µM), GST-Csk (50 nM), and excess ATP (1 mM) in 100 mM Mops (pH 7), 2 mM DTT, 10 mM MgCl$_2$. The reaction was run for 1 h and dialyzed into 100 mM Mops, 100 mM KCl (pH 7), 2 mM DTT. Phospho-Src was then separated from GST-Csk using a Ni$^{2+}$-agarose spin column.

**Rapid Quench Flow Kinetic Assays**—The phosphorylation of Src was monitored using a KinTek Corporation Quench Flow apparatus model RGF-3 following a previously published procedure (26). The experiments were typically executed by loading equal volumes of Csk, [$\gamma$-$^32$P]ATP (300–700 cpm pmol$^{-1}$) and MgCl$_2$ into one sample loop and Src and MgCl$_2$ into the other loop in 100 mM Mops (pH 7), 100 mM KCl, 2 mM DTT. The reactions were quenched using 30% acetic acid, and phosphoprotein was separated from unreacted [$^32$P]ATP using the column separation assay. Control experiments were performed to determine the background phosphorylation (i.e. phosphorylation of substrate in the presence of quench) using previously published protocols (22). The time-dependent concentration of phosphoprotein was then determined by considering the total cpm of the flow-through, the specific activity of the reaction mixture, and the background phosphorylation.

**Steady-state Kinetic Assays**—Steady-state kinetic assays for phospho-Src were performed with a Beckman Optima XL-I instrument using an An60Ti rotor. A mixture of Csk (5 µM) and Src (5 µM) in 100 mM Mops (pH 7), 100 mM KCl, 2 mM DTT, and 10 mM MgCl$_2$ was run in the absence and presence of 50 µM ATP or AMPPNP. Absorbance changes as a function of radial position were measured at a rotor speed of 20,000 at 25 °C. Equilibrium was reached after 14 h at this rotor speed. The data were then analyzed using the Origin software package provided by Beckman. The program Sednterp (28) was used to calculate the partial specific volumes and solvent densities of both proteins based on their amino acid sequences.

**Kinetic Data Analysis**—The initial velocity versus substrate concentration data were fitted to the Michaelis-Menten equation to obtain $k_{cat}$ and $K_m$ values for Src. The production of
Regulation of Src by Csk

phospho-Src in the rapid quench flow experiments was fitted to Equation 1,

\[ [P] = \alpha \left[ 1 - \exp \left( - k_b t \right) \right] + Lt \]  

(Eq. 1)

where \([P]\) is the concentration of phospho-Src, \(\alpha\) is the amplitude of the “burst” phase, \(k_b\) is the burst phase rate constant, \(L\) is the linear rate, and \(t\) is time. The Src dependence on the burst phase amplitude was analyzed using Equation 2,

\[ \alpha = \alpha_{\text{max}} \left( \frac{[S]}{[S] + K_m} \right)^2 \]  \hspace{1cm} (Eq. 2)

where \(\alpha_{\text{max}}\) is the maximum burst amplitude at infinite Src concentration, and \([S]\) is the total concentration of Src. Equations 1 and 2 were taken from a previous report (29). The stopped-flow fluorescence data were fitted to either single or double exponential functions.

RESULTS

Phosphoryl Transfer Step Is Fast—The phosphorylation kinetics of Src were monitored using rapid quench flow mixing and a radioactive-based assay. In these experiments, Csk, pre-equilibrated with \(^{32}\)P-ATP, is mixed with Src, the reaction is mechanically stopped at discrete times with acetic acid, and \(^{32}\)P-Src is measured using scintillation counting. Using 2 \(\mu\)M Csk and 10 \(\mu\)M Src, the production of \(^{32}\)P-Src follows a biphasic time course over the first 5 s (Fig. 1A). The exponential burst phase corresponds to the fast incorporation of \(^{32}\)P into Src in the first enzyme turnover (~300 ms), whereas the linear phase corresponds to the slower phosphorylation of Src under multturnover, steady-state conditions. The data were fitted to Equation 1 to obtain the burst phase rate constant \((k_b)\) of 8 s\(^{-1}\) and the linear, steady-state phase rate \((L)\) of 0.2 \(\mu\)M/s. The amplitude of the burst phase under these conditions represents 50% of the total enzyme concentration \((\alpha = 1 \mu\text{M})\), implying that Src binds only a portion of the available active sites in the first turnover. These data indicate that the phosphoryl transfer step is fast and does not limit Src turnover.

Active Site Titration of Csk—The burst phase amplitude for Src phosphorylation does not reflect all of the available active sites in solution under our assay conditions (Fig. 1A). To determine whether this reflects some unproductive enzyme or an inability to saturate the active site, we measured the burst amplitude as a function of Src concentration (5–40 \(\mu\)M) at a fixed concentration of Csk (0.5 \(\mu\)M) over a time frame of 0.5–5 s in the rapid quench flow apparatus (Fig. 1B). To achieve accurate measures of the burst phase, we focused on obtaining data on in the linear phase. Both the linear rates and amplitudes normalized to the total Csk concentration were then plotted as a function of total Src concentration. The linear rates were fitted to a hyperbolic function to obtain \(k_{\text{cat}}\) and \(K_m\) values of 0.15 s\(^{-1}\) and 4 \(\mu\)M. To verify these measurements, Csk (100 nM) and varying Src (2–20 \(\mu\)M) were allowed to react for 60 s in manual mixing experiments to obtain \(v\) versus \([S]\) plots with \(k_{\text{cat}}\) and \(K_m\) values of 0.12 s\(^{-1}\) and 5 \(\mu\)M, respectively (data not shown). The burst amplitudes were fitted to Equation 2 to obtain a maximum, normalized amplitude \((\alpha/[E])\) of 0.95 ± 0.05. The fitting of the data also provides an estimate of \(K_m\) for Src (4 \(\mu\)M), which is similar to the value of this parameter measured by both manual and rapid quench flow mixing. The \(K_m\) can be measured in the pre-steady-state phase using Equation 2, because we showed previously that Src exchanges rapidly with the active site of Csk (26). Overall, these data imply that a minimum of 90% of the Csk active sites are viable and can bind and phosphorylate Src.

Phosphorylation Dependence on Complex Formation—In prior studies we showed that mixing stoichiometric amounts of Src and Csk resulted in an equilibrium sedimentation curve reflecting the average monomer molecular mass of the two individual proteins (~56 kDa), suggesting that a stable complex of Csk and Src does not form appreciably near the \(K_m\) (26). To determine whether phospho-Src might bind with higher affinity to Csk and thus provide a means of limiting Src turnover, we incubated an equimolar Csk and Src (3 \(\mu\)M) in the presence of ATP or AMPPNP (50 \(\mu\)M) and then subjected both samples to sedimentation in an analytical ultracentrifuge (Fig. 2A). In the presence of AMPPNP, the fitted molecular mass for the mixture was consistent with the average mass of the two proteins.
Inhibition with Phospho-Src—To provide further evidence for weak protein-protein interactions, we next investigated the binding affinity of phospho-Src under catalytic conditions. We synthesized and purified phospho-Src and then determined whether this product could inhibit the steady-state phosphorylation reaction (Fig. 2B). Increasing the concentration of phospho-Src up to 8 μM at a constant amount of Src (5 μM) had no impact on the phosphorylation reaction (Fig. 2B). In this assay, less than 10% of the total Src is phosphorylated after a 1-min reaction time so that the band intensities can be used to determine initial velocities. In control experiments where Src was removed from the reaction, no radiolabeled phospho-Src was generated, confirming that none of the labeled phospho-Src in the autoradiograms resulted from impurities in the phospho-Src preparation. If 8 μM phospho-Src does not lower the initial velocity by more than 5%, the error limits for this assay, a $K_I$ of ~90 μM for the product can be estimated. These findings corroborate the sedimentation data and indicate that phospho-Src binds weakly to Csk with a lower limit of ~90 μM for the $K_I$.

Association Kinetics of Csk and Src—Because phospho-Src binds poorly according to the above data, and ADP is not rate-limiting based on previous work (26), we next wondered whether a conformational change in Csk could limit turnover. To address this possibility, the association kinetics of Csk and Src were monitored using a stopped-flow fluorimeter. We found that mixing Csk with varying Src led to a biphasic increase in tryptophan fluorescence (Fig. 3A). The rate constant for the initial fast phase ($k_1$) increases as a function of Src concentration consistent with a simple bimolecular association event. The apparent association ($k_{	ext{on}}$) and dissociation rate con-
Regulation of Src by Csk

stants \(k_{\text{off}}\) can be estimated from a plot of observed rate versus Src concentration (30) (Fig. 3B). The slope and intercept values of this plot provide \(k_{\text{on}}\) and \(k_{\text{off}}\) values of 0.9 \(\mu M^{-1} s^{-1}\) and 7 s\(^{-1}\), respectively. The ratio of \(k_{\text{off}}\) and \(k_{\text{on}}\), termed the “kinetic” \(K_d\) \(\left(\frac{k_{\text{off}}}{k_{\text{on}}}\right)\), is 7.8 \(\pm\) 2.0 \(\mu M\), a value much lower than the estimates of the overall \(K_d\) (~100 \(\mu M\)) from equilibrium binding data (Fig. 2). The amplitude for the fast phase varies linearly up to 20 \(\mu M\) Src, the maximum concentration attained in this study. In comparison, the slower phase of this reaction \(k_c\) did not vary significantly over this concentration range. The average rate constant for the slow phase is 0.8 s\(^{-1}\). The overall association kinetics suggest that Src and Csk initially interact rapidly, but a slow conformational change may limit the complete formation of the complex.

Changes in Tryptophan Fluorescence Accompany Src Phosphorylation—Because Src is a fluorescent protein that undergoes a large conformational change upon phosphorylation, we wondered whether the Csk reaction could be monitored if the spectral properties of the substrate and phosphoprotein are sufficiently different. To accomplish this, Csk was used to phosphorylate Src in the stopped-flow fluorimeter, and changes in tryptophan emission were collected. In Fig. 4A, Src and Csk in one syringe were mixed with ATP from the second syringe. Using 2 \(\mu M\) Csk, 10 \(\mu M\) Src, and 50 \(\mu M\) ATP, a single exponential fluorescence decrease was observed. Removing ATP or replacing it with ADP resulted in no significant changes in fluorescence beyond a small linear decrease of only 0.3 mV/min most likely because of photobleaching (Fig. 4A). It has been shown previously that the \(K_d\) for ADP is 3 \(\mu M\), indicating that the absence of a fluorescence change in the presence of ADP is not the result of poor binding affinity (31). Omitting either Csk or Src led to no significant changes in fluorescence emission upon mixing with ATP (data not shown), indicating that nucleotide binding to either protein does not cause fluorescence changes on the time frame observed with both proteins. Together, these data suggest that the observed changes in tryptophan fluorescence are associated with an ATP-dependent process requiring both Csk and Src.

Fluorescence Changes in Src upon Phosphorylation—To determine the source of the fluorescence change in the phosphorylation reaction, progress curves were repeated using varying concentrations of both proteins. Halving the Csk concentration in Fig. 4A (2 to 1 \(\mu M\)) at constant Src (10 \(\mu M\)) had no effect on the overall amplitude of the reaction (~20 mV), although the rate constant for the approach to equilibrium decreased. To determine whether Src fluorescence quenching could be the source of the observed changes, the enzyme reaction was performed at varying amounts of Src at fixed Csk. Using low Csk concentrations (0.5 \(\mu M\)), changes in reaction amplitude varied in proportion to the total amount of Src (Fig. 4B). At 2, 5, and 10 \(\mu M\) Src, fluorescence amplitudes of 5, 11 and 20 mV, respectively, were obtained. Thus, unlike Csk, a direct proportional relationship between total Src and fluorescence change was obtained (~2 mV/\(\mu M\) Src). These data suggest that the phosphorylation of Src by Csk results in quenching of tryptophan fluorescence in Src.

Fluorescence Burst in Phospho-Src Production—Because steady-state production of phospho-Src could be readily monitored using catalytic amounts of Csk (Fig. 4), we wondered whether we could also detect product formation in the first enzyme turnover in the stopped-flow instrument. To accomplish this, high Csk concentrations were used (2.5–7.5 \(\mu M\)) in the second loop of the rapid quench flow apparatus. Final concentrations are 10 \(\mu M\) Src, 1 \(\mu M\) Csk (blue), or 2 \(\mu M\) Csk (red), and 50 \(\mu M\) ATP or ADP. The rate constants and amplitudes are 19 \pm 1 mV and 0.026 \pm 0.002 s\(^{-1}\) at 2 \(\mu M\) Csk and 20 \pm 1 mV and 0.014 \pm 0.002 s\(^{-1}\) at 1 \(\mu M\) Csk. In the presence of ADP and no nucleotide, the fluorescence changes are fitted to linear functions with slopes of ~0.3 mV/min. B, effects of Src concentration on the reaction curves. Csk and Src in one reaction loop is mixed with ATP in the second loop of the rapid quench flow apparatus. Final concentrations are 0.5 \(\mu M\) Csk, 2 \(\mu M\) Src (black) or 5 \(\mu M\) Src (blue), or 10 \(\mu M\) Src (red), and 50 \(\mu M\) ATP. The rate constants and amplitudes are 5.4 \pm 0.2 mV and 0.010 \pm 0.002 s\(^{-1}\) at 2 \(\mu M\) Csk, 11 \pm 0.5 mV and 0.013 \pm 0.001 s\(^{-1}\) at 5 \(\mu M\) Csk, and 20 \pm 1 mV and 0.014 \pm 0.002 s\(^{-1}\) at 10 \(\mu M\) Src. All of the transients were collected at an identical photomultiplier tube voltage (800 V) and slit widths (0.4 nm). All of the data are set arbitrarily to an initial start fluorescence of 20 mV at time 0.
Regulation of Src by Csk

Repression of Src activity involves large conformational changes induced by the regulator, Csk (7). Given the preeminent role of Csk in catalyzing this phosphorylation-driven reaction, it is important to understand what factors limit this signaling event. In rapid quench flow experiments, Csk rapidly phosphorylates Src within ~300 ms in the first enzyme turnover (Fig. 1A). Indeed, the rate of Src phosphorylation by these methods is about 100-fold faster than net Src turnover ($k_{cat}$), a comparatively sluggish process that occurs at a rate of ~0.2 $s^{-1}$. These findings now raise the question: what controls the production of phospho-Src? We showed previously that the net release rate of ADP ($k_{off}$) is much faster than $k_{cat}$, indicating that other events control this parameter (26). Here, we investigated whether the release of phospho-Src or some structural event in Csk limits turnover. In prior equilibrium sedimentation studies, we showed that Src does not form a stable complex with Csk, indicating that the overall $K_{d}$ for Src is higher than $K_{m}$ (26). Weak affinity has been recapitulated in single turnover experiments (i.e. [Csk] > [Src]) where the observed phosphoryl transfer rate increases linearly up to 15 $\mu$M Csk, suggesting that the $K_{d}$ is likely to greatly exceed this value (26). Consistent with our observations, Cole and co-workers (18) showed that a mutant containing a Tyr-to-Phe substitution in the C terminus of Src (Y527F) does not inhibit phosphorylation from the Csk active site is fast.

DISCUSSION

Phosphorylation Does Not Improve Src Affinity to Csk—Although Src binds poorly to Csk, phosphorylation could stabilize this interaction and provide a means for limiting turnover. To address this possibility, we performed equilibrium sedimentation and product inhibition experiments. Both studies were unable to detect formation of a stable Csk-Src complex (Fig. 2). This poor binding affinity for both substrate and phospho-product is not without precedence. Recently, we showed that the protein kinase Sky1p has very poor affinity for its natural substrate, Npl3, and releases the phosphorylated product, phospho-Npl3, at a rate constant in excess of maximum turnover (22). Furthermore, it is likely that the active sites of protein kinases do not generally provide interactions that preferentially stabilize phosphorylated versus unphosphorylated ligands. Studies performed on cAMP-dependent protein kinase indicate that the binding affinity of a short peptide substrate is not enhanced by phosphorylation (32).

Phospho-Src Dissociation is Fast—Although the above binding data monitor only equilibrium processes, we can use these studies to understand the dynamics of the Csk-Src complex. Most relevant for these mechanistic studies, we can estimate the overall release rate for phospho-Src ($k_{off}$) to determine whether it might play a role in limiting $k_{cat}$. To obtain a burst rate constant of 8 $s^{-1}$ at 10 $\mu$M Src in Fig. 1A, $k_{off}$ for Src must be at least 1 $\mu$M$^{-1}$$s^{-1}$, an estimate that is in line with the stopped-flow experiments in this present report (0.9 $\mu$M$^{-1}$$s^{-1}$; Fig. 3B).
Regulation of Src by Csk

Using the estimated $K_d$ of 100 μM, we can then calculate a lower limit of 100 s$^{-1}$ for the Src $k_{off}$ from the relationship: $k_{off} = k_{on} \times K_d$. Because phosphorylation does not enhance binding affinity (Fig. 2), a similar $k_{off}$ can be calculated for phospho-Src. These analyses suggest that phospho-Src dissociates at a rate constant that exceeds $k_{cat}$ by about 3 orders of magnitude and thus does not limit turnover.

**Slow Structural Changes Accompany Src Binding**—Because phospho-Src dissociates rapidly, we investigated whether a structural change in Csk might control turnover. The results of the stopped-flow studies suggest that in addition to a bimolecular phase, a slow conformational change accompanies binding (Fig. 3). The data presented here are most consistent with the minimal kinetic mechanism in Scheme 1, where a conformational step (pre-equilibrium) precedes Src binding based on two observations. First, the kinetic $K_d$ (i.e. $k_{off}/k_{on} = 8 \mu$M) for the bimolecular phase is lower than the overall $K_d$ for the Csk-Src interaction based on equilibrium sedimentation and product inhibition studies ($K_d \approx 100 \mu$M). These findings suggest that the interaction of the two proteins cannot be described by a one-step mechanism. Second, the amplitude for a binding transient is expected to vary in a hyperbolic manner with ligand concentration according to the overall $K_d$ (Eq. 3). Finally, although the data requires an unfavorable pre-equilibrium constant that exceeds $k_{cat}$ by about 3 orders of magnitude and thus does not limit turnover.

**Both Forms of Csk Can Bind Src**—The transient state kinetics for Src binding indicate that an unfavorable pre-equilibrium precedes association. Although we detect no fluorescence signal in support of binding to the dominant species in Scheme 1 (Csk), the pre-steady-state kinetic data indicate that this species must be accessible to Src. In rapid quench flow experiments we observe a large burst amplitude that extrapolates to a minimum of 90% of the active sites (Fig. 1B). The kinetic mechanism in Scheme 1 would not provide for such a large burst phase but would instead generate an amplitude of only ~10% based on our estimates of the pre-equilibrium constant ($k_{on}/k_{off} \geq 12$). Thus, it is likely that Src can bind to Csk in Scheme 1, although the affinity for this form must be low to satisfy the overall $K_d$ limits ($K_d = \sim 100 \mu$M). This modified binding mechanism, displayed in Scheme 2, includes two enzymes or Csk with different affinities. The inability to detect the second binding event in the stopped-flow instrument may be due to small changes in fluorescence and/or to a large $k_{off}$ for this step. These limitations along with the inability to achieve a wider concentration range for Src makes it difficult to assign all of the individual rate constants in Scheme 2. Nonetheless, the observed rate constant for the conformational change is close in value to $k_{cat}$ (Fig. 3B), suggesting that this structural change could play some role in limiting turnover (see below).

**Conformational Changes Occur after Phospho-Src Release**—The data presented herein indicate that the phosphoryl transfer and phospho-Src release steps are fast, whereas a conformational change in Csk is slow. Where does this latter step lie within the catalytic cycle? Because the placement of this conformational change prior to the phosphoryl transfer step would prohibit a burst in product formation in the first enzyme turnover, an observation contrary to our data (Fig. 1), we investigated whether a conformational change in Csk after the phosphoryl transfer step but prior to substrate binding may control turnover. To investigate this possibility, we developed a fluorescence-based assay that allows us to potentially detect conformational changes along the phosphorylation pathway. In this assay, the Csk reaction is monitored through changes in tryptophan fluorescence after the C terminus of Src is modified (i.e. *Csk) and redistribute the population of Csk and *Csk in the bound form (i.e. *Csk/k_{cat} \gg k_{off}/k_{on}).
it can be employed as a conformational reporter that complements the $^{32}$P assay, which is insensitive to conformational dynamics after phosphorylation. If a slow structural change in Csk must precede phospho-Src release, a lag in the fluorescence-based assay would be detected that reflects the rate-limiting conformational change in $k_{cat}$. On the other hand, if no slow conformational change occurs prior to phospho-Src release, then a burst in fluorescence, similar to that for the $^{32}$P-based assay will be observed. As shown in Fig. 4, a large fluorescence burst phase is detected, consistent with the absence of any conformational changes prior to release. Thus, any conformational changes in Csk that limit the Src phosphorylation reaction must occur after the product release step.

Factors Controlling Single and Multi-turnover—The current data indicate that all the steps leading to generation of the first phospho-Src molecule (e.g. the association and phosphorylation of Src and the release of phospho-Src and ADP) are fast, yet the steady-state generation of phospho-Src is comparatively slow. Although we provide evidence that a slow conformational change in Csk limits Src binding, this event cannot limit the first enzyme turnover. However, this step could play a role after the first turnover. Such a possibility is described in Fig. 6, where two slowly equilibrating forms of the catalyst (Csk and $^*\text{Csk}$) participate in either the first or all subsequent turnovers. Based on our binding data, these two forms display unique specificities for Src with the higher affinity species ($^*\text{Csk}$) possibly representing a more open, accessible form ($K_a \sim 8 \mu M$) and the lower affinity species (Csk) representing a more closed, less accessible form ($K_a \sim 100 \mu M$). As the dominant species at equilibrium, Csk rapidly binds and phosphorylates the substrate and releases the products in the burst phase. Here, no slow conformational changes limit the synthesis and release of the first phospho-Src molecule. We propose that ATP-dependent phosphorylation of the Src tail induces the open enzyme form ($^*\text{Csk}$). Although $^*\text{Csk}$ can then bind Src efficiently ($K_{a} \sim 10 \mu M$) and lock the enzyme into the catalytic cycle, the resulting binary species ($^*\text{Csk-Src}$) must proceed through a slow, unfavorable conformational change to regenerate the active enzyme form. In the stopped-flow experiments, the conformational change step is ~5-fold higher than $k_{cat}$ (0.8 versus 0.15 s$^{-1}$) (Fig. 3A). Because the transient state kinetic data provide a sum of the forward and reverse rate constants ($^*k_c + ^*k_{-c}$) for the conformational change, $^*k_c$ will be much lower than $^*k_{-c}$ because this equilibrium step is unfavorable (i.e. $^*k_{-c} > k_c$, Scheme 2). Thus, it is likely that $^*k_c$ is much closer to $k_{cat}$ and consequently plays a larger role in controlling turnover.

Structural Underpinning of Csk Forms—Currently, it is unknown what structural changes are associated with the interconversion of the enzyme forms in Fig. 6. The x-ray structure of Csk reveals two forms that differ by movements of the SH2 domain (12). These two crystalline states suggest that cantilever motions of the SH2 domain occur in solution and possibly control catalysis. We have shown using theoretical calculations that strain develops at pivot points around the SH2-kinase linker region, suggesting that cantilever motions of the SH2 domain may occur in solution and provide some rate limitation for the enzyme reaction (27). Mutations in this linker region between the two domains substantially lower turnover and alter amide exchange properties within the SH2 domain and active site (27).

Overcoming Weak Affinity Interactions—Although the overall affinity of Src for Csk is poor, the $K_m$ for this interaction is paradoxically favorable ($K_{i} > K_a$). We showed in previous analyses of the burst phase that Csk may accomplish this feat by facilitating a very fast, thermodynamically favorable phosphoryl transfer step that kinetically clamps an otherwise weakly bound substrate in the active site (26). By this phenomenon, we showed that the $K_m$ for Src can be lowered from a high $K_a$ by a large internal equilibrium constant ($K_{int}$) for the transfer step ($K_m \sim K_a/K_{int}$). We now show that this "clamping" mechanism serves an essential role in the first enzyme turnover by recruiting Src to a weak binding form of the enzyme (Csk in Fig. 6) so that the first molecule of phospho-Src can be quickly generated despite high energy barriers for binding ($K_a/K_m \sim 30$). The new data indicate that once the enzyme is locked into the catalytic cycle, the phosphoryl transfer step takes on a new function. Src now interacts with the higher affinity form of the enzyme ($^*\text{Csk}$), and the phosphoryl transfer step is now recruited to overcome a preceding, unfavorable conformational transition (i.e. $k_{-c} > k_c$). For steady-state phosphorylation, the $K_m$ will reflect mostly the affinity of Src for $^*\text{Csk}$. Thus, Csk maintains efficient recognition and phosphorylation of Src despite a dominant equilibrium species (Csk) with very weak binding affinity for its natural substrate target. In this mechanism the fast phosphorylation reaction is a clamp not only for initial recognition but also for surmounting an unfavorable conformational equilibrium in subsequent catalytic turnovers. Finally, whereas these studies establish the role of a conformational change in controlling Src turnover ($k_{cat} = ^*k_c$), the mech-

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**FIGURE 6. Catalytic cycling of Csk during Src phosphorylation.** Src binds two forms of Csk (Csk and $^*\text{Csk}$) that differ in affinity (8 μM versus 100 μM). Csk is the dominant species at equilibrium, whereas $^*\text{Csk}$ dominates under steady-state conditions. Phosphoryl transfer and product release steps are fast. The rate-limiting step is a conformational change preceding the phosphoryl transfer step. Src is displayed in green with a black C-terminal tail. The phosphorylated Src tail residue is shown in red.

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**Regulation of Src by Csk**
anism in Fig. 6 indicates that this step also plays a role in controlling phosphorylation rates at lower Src concentrations as well (\(k_{\text{cat}}/K_m = *k_{c}/K_d\)). Thus, the Csk conformational change is expected to participate in modulating phosphorylation rates irrespective of the physiological levels of Src.

REFERENCES

1. Abraham, N., Stojdl, D. F., Duncan, P. I., Methot, N., Ishii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., Koromilas, A. E., Brown, E. G., Sonenberg, N., and Bell, J. C. (1999) J. Biol. Chem. 274, 5953–5962
2. Ernst, M., Inglese, M., Scholz, G. M., Harder, K. W., Clay, F. J., Bozinovski, S., Waring, P., Darwiche, R., Kay, T., Syl, P., Collins, R., Turner, D., Hibbs, M. L., Anderson, G. P., and Dunn, A. R. (2002) J. Exp. Med. 196, 589–604
3. Hibbs, M. L., Harder, K. W., Armes, J., Kountouri, N., Quilici, C., Casagrande, F., Dunn, A. R., and Tarlinton, D. M. (2002) J. Exp. Med. 196, 1593–1604
4. Irby, R. B., and Yeatman, T. J. (2000) Oncogene 19, 5636–5642
5. Frame, M. C. (2002) Biochim. Biophys. Acta 1602, 114–130
6. Cole, P. A., Shen, K., Qiao, Y., and Wang, D. (2003) Curr. Opin Chem. Biol. 7, 580–585
7. Cowan-Jacob, S. W., Fendrich, G., Manley, P. W., Jahnke, W., Fabbro, D., Liebetanz, J., and Meyer, T. (2005) Structure (Camb.) 13, 861–871
8. 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
9. Chong, Y. P., Mulhern, T. D., and Cheng, H. C. (2005) Growth Factors 23, 233–244
10. Shaffer, J., and Adams, J. A. (1999) Biochemistry 38, 12072–12079