Conformational Basis for SH2-Tyr(P)527 Binding in Src Inactivation*

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Src protein-tyrosine kinase contains a myristoylation motif, a unique region, an Src homology (SH) 3 domain, an SH2 domain, a catalytic domain, and a C-terminal tail. The C-terminal tail contains a Tyr residue, Tyr527. Phosphorylation of Tyr527 triggers Src inactivation, caused by Tyr(P)527 binding to the SH2 domain. In this study, we demonstrated that a conformational contribution, not affinity, is the predominant force for the intramolecular SH2-Tyr(P)527 binding, and we characterized the structural basis for this conformational contribution. First, a phosphopeptide mimicking the C-terminal tail is an 80-fold weaker ligand than the optimal phosphopeptide, pYEEI, and similar to a phosphopeptide containing three Ala residues following Tyr(P) in binding to the Src SH2 domain. Second, the SH2-Tyr(P)527 binding is largely independent of the amino acid sequence surrounding Tyr(P)527, and only slightly decreased by an inactivating mutation in the SH2 domain. Furthermore, even the unphosphorylated C-terminal tail with the sequence of YEEI suppresses Src activity by binding to the SH2 domain. These experiments demonstrate that very weak affinity is sufficient for the SH2-Tyr(P)527 binding in Src inactivation. Third, the effective intramolecular SH2-Tyr(P)527 binding is attributed to a conformational contribution that requires residues Trp260 and Leu255. Although the SH3 domain is essential for Src inactivation by Tyr(P)527, it does not contribute to the SH2-Tyr(P)527 binding. These findings suggest a conformation-based Src inactivation model, which provides a unifying framework for understanding Src activation by a variety of mechanisms.

Src protein-tyrosine kinases (PTK) are important enzymes in several signal transduction pathways initiated by a variety of cell surface receptors, such as receptor tyrosine kinases (1), cytokine receptors and antigen receptors (2), and G-protein-coupled receptors (3). In the cell, Src normally exists in the inactivated form and can be activated by numerous mechanisms in response to upstream signals (4). Activated Src phosphorylates a large number of proteins to activate the mitogen-activated protein kinase pathways, regulate the cell cycle, stimulate DNA synthesis, and trigger cytoskeletal rearrangement and cell migration (4). Constitutive activation of Src results in cell transformation (5) and is associated with numerous human cancers (6), making Src an important proto-oncogene and target for drug discovery (7). Elucidating the regulatory structure-function relationships of Src is important for understanding physiological and oncogenic signal transduction.

The foundation for Src regulation is its complex multidomain organization and domain-domain interaction (4). It has an N-terminal myristoylation motif responsible for association with the plasma membrane, followed by a unique region, an SH3 domain that binds to Pro-rich peptide sequences, an SH2 domain, and a C-terminal regulatory tail. Phosphorylation of a Tyr residue (Tyr527) on the C-terminal tail by a pair of cytosolic PTKs, C-terminal Src kinase (Csk) or Csk homologous kinase, inactivates Src (8). Phospho-Tyr527 on the C-terminal tail (Tyr(P)527) binds to the SH2 domain, stabilizing a compact and inactive conformation (9). Dephosphorylation of Tyr(P)527 results in Src activation because of SH2-tail dissociation. The crystal structures of the inactivated Src (10, 11) and an active form of Src (12) have been determined.

In the inactivated structure (10, 11), the SH3 and the SH2 domains are located in the backside of the catalytic domain (Fig. 1). The SH2 domain binds to Tyr(P)527 of the C-terminal tail. The SH2-catalytic linker (2-C linker) is sandwiched between the SH3 domain and the N-lobe of the catalytic domain. The linker binds to the SH3 domain and also forms hydrophobic interactions with the N-lobe of the catalytic domain. In the catalytic domain, the α-helix C and the activation loop assume conformations that are consistent with inactive kinases. The structure of one active form of Src has been determined (12), in which the SH3 and SH2 domains and the 2-C linker act as one structural unit and flip to the other side of the kinase domain. In the active structure, the SH3 domain still binds to the 2-C linker, and the α-helix C and the activation loop assume conformations characteristic of active kinases.

Kinetic studies (13) demonstrate that Src phosphorylated on Tyr527 (p-Src) exists in equilibrium between a closed form where the SH2 domain binds to Tyr(P)527 (~98%) and an open form where the SH2 domain is not occupied by Tyr(P)527.
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Plasmid Construction and Protein Purification—The construction of human Csk expression plasmid (pGEX-Csk) (20), kdSrc expression plasmid (pRSET-kdSrc) (21, 22), was previously described. The inserted DNA fragments were sequenced to ensure that the sequences were correct. Site-specific kdSrc mutants were generated by introducing specific mutations into pRSET-kdSrc. For expression of the kdSrc-ΔSH3, the fragment encoding the SH3 domain was deleted from the kdSrc coding region in pRSET-kdSrc. For expression of the fusion proteins, bacterial cells containing recombinant plasmids were used to inoculate 600 ml of Luria-Bertani culture medium. Bacterial cell culture, induction of fusion protein expression, cell lysis, and protein purification were performed as described previously (22). All purification steps were carried out at 4 °C. Protein concentrations were determined by Bradford protein assay using bovine serum albumin as a standard.

Preparation of kdSrc and Mutants Phosphorylated on Tyr527—For kdSrc and most kdSrc mutants used in this study, the phosphorylated form was prepared by co-expressing kdSrc with Csk in Escherichia coli. A separate plasmid pCDF-Csk was introduced to BL21 (DE3) cells harboring the Src expression plasmid. The pCDF-Csk plasmid directs the synthesis of Csk that is not affinity-tagged. kdSrc and kdSrc mutant proteins purified from such cells were phosphorylated on Tyr527 and could not be further phosphorylated by Csk in vitro. Exceptions to this general pattern were kdSrc-W260A and kdSrc-L255A, which were not fully phosphorylated upon purification and could be further phosphorylated by Csk. These two proteins were further phosphorylated in vitro by excess amounts of Csk and then repurified. Such in vitro phosphorylated proteins were used for binding analyses. The lack of full phosphorylation in bacteria by Csk for these two proteins was likely because of the fact that Tyr(P)527 did not bind to the SH2 domain and thus exposed to dephosphorylation by general phosphatase activities in bacterial cells.

Expression of Active Src Kinase—Active human Src kinase was expressed in bacteria as a fusion protein with maltose-binding protein (MBP) and protein-Tyr phosphatase 1B as described elsewhere (36). Briefly, recombinant expression of active Src in bacteria causes extensive degradation to Src and very low yield of expression and purification. Such a problem is associated with the intrinsic kinase activity of Src, likely because of Src phosphorylation of bacterial proteins. Addition of a protein-tyrosine phosphatase to the expression construct helps stabilize the plasmid and Src protein during expression and purification. A thrombin cleavage site was incorporated between the protein-tyrosine phosphatase and Src. Upon purification of the fusion protein, Src was separated from MBP and

MATERIALS AND METHODS

Reagents and Chemicals—All reagents used for bacterial culture and protein expression were purchased from Fisher. Chromatographic resins were purchased from Sigma. DNA primers were synthesized by Integrated DNA Technologies. Phosphopeptides were synthesized by solid phase synthesis and purified by high pressure liquid chromatography. Their molecular weights were confirmed by electrospray mass spectrometry.

FIGURE 1. Structure of inactivated Src. Structural motifs and domains relevant for the current study are labeled. This figure was generated using the Protein Data Bank coordinates of code 2SRC.
the phosphatase by thrombin digestion and purification on fast protein liquid chromatography.

Fluorescence Polarization Assay and \( K_d \) Determination—The binding of a fluorescent phosphopeptide, Flu-GpYEEI, to kdSrc and kdSrc mutants was determined by the fluorescence polarization assay as described previously (23). To determine the binding of an unlabeled phosphopeptide to the SH2 domain, fluorescence polarization of 80 nM Flu-GpYEEI as the probe in the presence of 80 nM SH2 domain and variable concentrations of the unlabeled phosphopeptide was determined. Fluorescence polarization as a function of the total unlabeled phosphopeptide concentration was curve-fitted using the following equation: fluorescence polarization = \( A \times ([\text{SH2}]_p \times [\text{probe}]) / (K_{d,1} \times [\text{SH2}]_p + K_{d,2} \times [\text{probe}] + [\text{peptide}] + [\text{SH2}]_p \times K_{d,2}) \), where \( K_{d,1} \) was the dissociation constant of Flu-GpYEEI binding to the SH2 domain, which was pre-determined by the direct binding assay, and \( K_{d,2} \) was the dissociation constant of unlabeled peptide binding to the SH2 domain. \( A \) is a conversion factor between the concentration of probe-SH2 complex and the fluorescence polarization value. \([\text{SH2}])_p \) and \([\text{probe}] \), were total concentrations of the SH2 domain and the fluorescent probe, Flu-GpYEEI, respectively. \([\text{Peptide}] \), was the total concentration of the unlabeled phosphopeptide. All binding assays were performed in duplicate and repeated at least twice with similar results.

Kinase Activity Assay—To quantify the kinase of Csk and Src, poly(E,Y) (1 mg ml\(^{-1}\)) was used as the phosphate-accepting substrate at 0.2 mM \([\gamma-3^2P]ATP \sim 1000 \text{ dpm pmol}^{-1}\). The kinase reactions included both substrates and appropriate amounts of the kinase in the kinase assay buffer (75 mM EPPS, pH 8.0, 12 mM MgCl\(_2\), 5% glycerol, 0.005% Triton X-100) (19, 22). The kinase reaction was for 20 min, and the reaction was linear with reaction time and enzyme concentration under these conditions. To determine the inactivation of Src by Csk phosphorylation, the indicated amounts of Src or Src mutants were preincubated with varying amounts of Csk in the presence of 0.2 mM ATP in the kinase assay buffer for 30 min at 30 °C. At the end of the preincubation, poly(E,Y) and \([\gamma-3^2P]ATP \sim 1000 \text{ dpm pmol}^{-1}\) were added to the reaction. After an incubation at 30 °C for another 20 min, the reaction mixtures were spotted onto filter paper strips, which were then washed in 5% trichloroacetic acid (three times for 10 min each time). Phosphorylated and unphosphorylated poly(E,Y) was precipitated onto the filter paper, and the phosphate precipitated onto the filter paper was determined by liquid scintillation counting. The residual Src activity was determined by subtracting the Csk activity from the total kinase activity.

Relationship between the Fraction of p-Src in the Closed Form and the Affinity between the Src SH2 Domain and Tyr(P)\(^{527}\)—The following outlines the derivation of the quantitative relationship between the fraction of p-Src in the closed form and the affinity between the Src SH2 domain and Tyr(P)\(^{527}\). According to the binding equation, \( K_{d,\text{intra}} = (c\text{losed form})/\text{open form}^2 \). If the fraction of Src in the closed form is \( c \), then \( K_{d,\text{intra}} = ([\text{Src}] \times C) / ([\text{Src}] \times (1 - C))^2 \). Because \( K_{d,\text{intra}} = K_{d,\text{intra}} \times \text{CEF} \), and CEF = 0.059/[Src], then \( K_{d,\text{intra}} \times C) / ([\text{Src}] \times (1 - C))^2 \). This equation can be simplified as the following: \( K_{d,\text{inter}} = C / (0.059 \times (1 - C))^2 \) (\( M^{-1} \)).

RESULTS

A Phosphopeptide Mimicking the Phosphorylated C-terminal Tail of Src Has Weak Affinity for the Src SH2 Domain—The C-terminal tail of Src has the sequence of Pro-Gln-Tyr527-Pro-Gly, which is significantly different from the optimal phosphopeptide ligand for the Src SH2 domain, pYEEI (15). Furthermore, when the phosphorylated C-terminal tail binds to the SH2 domain in the closed form of Src (10) and Hck (11), only Tyr(P)\(^{527}\) interacts with the SH2 domain, whereas the other tail residues have very little contact with the SH2 domain. These observations suggest that the phosphorylated C-terminal tail is not a high affinity ligand for the SH2 domain. We characterized this interaction by measuring the binding of the phosphopeptide, Pro-Gln-Tyr(P)\(^{527}\)-Gln-Pro-Gly, to the SH2 domain in a kinase-defective mutant of Src (kdSrc). kdSrc contains an inactivating point mutation (K295M) and also lacks the myristoylation motif and the unique region at the N terminus. It retains the structural dynamics related to Tyr(P)\(^{527}\)-triggered inactivation (13, 22). Binding between the phosphopeptide and kdSrc was determined by a fluorescence polarization competition assay using fluoresceinated GpYEEI (Flu-GpYEEI) as a probe (\( K_d = 25 \text{ nM} \)). The unfluoresceinated GpYEEI bound to the kdSrc SH2 domain tightly with a \( K_d \) value of 321 nM (Fig. 2). The phosphopeptide PQPYGQ bound to kdSrc with a \( K_d \) value of 167 ± 19 \( \mu M \), weaker than GpYEEI by a factor of 500. To confirm the lack of contribution by the three side chains following Tyr(P)\(^{527}\), we determined the binding of PQpYAAA to the SH2 domain in kdSrc, which yielded a \( K_d \) of 87 ± 7 \( \mu M \). To determine whether the additional three Src residues following PQpYQG might contribute to the SH2-Tyr(P)\(^{527}\) binding, we determined the binding of GpYEEIENL and PQpYGQGENL to kdSrc. GpYEEIENL had a \( K_d \) of 0.3 ± 0.02 \( \mu M \), identical to that of GpYEEI. PQpYGQGENL had a \( K_d \) of 24 ± 1 \( \mu M \), ~6 times better than PQpYQG but still ~80-fold worse than GpYEEI. These data
demonstrated that the Src C-terminal tail phosphopeptide represented a very weak ligand for the Src SH2 domain.

Inactivated Src Exists in Equilibrium between the Closed and Open Forms—By two independent methods, Wang et al. (13) determine that about 98% of p-Src exists in the closed form and 2% in the open form. To confirm this finding in our hands, we prepared kdSrc that was phosphorylated by Csk on Tyr527 and compared the accessibility of the SH2 domain before and after Tyr527 phosphorylation. In the unphosphorylated kdSrc, the SH2 domain was readily accessible to Flu-GpYEEI, with an apparent $K_d$ of 25 ± 4 nM (Fig. 3). In contrast, in kdSrc that was phosphorylated on Tyr527, the SH2 domain was much less accessible with an apparent $K_d$ of 1.25 μM. This indicated that Tyr527 phosphorylation reduced the accessibility of the SH2 domain by a factor of 50. This difference in the SH2 domain accessibility was in a good agreement with the previously (13) determined ratio of closed versus open forms at ~98 to 2.

The weak affinity between the SH2 domain and the tail phosphopeptide and the high portion of p-Src in the closed form indicated that the SH2-Tyr(P)527 affinity could not possibly account for the SH2-Tyr(P)527 binding in Src. For example, at 1 μM concentration for both the SH2 domain and the phosphopeptide, the $K_d$ of 24 μM would dictate that only 4% of the SH2 domain would be bound to the phosphopeptide, whereas 96% would be free. This large disparity of 4 versus 98% indicated that the SH2-Tyr(P)527 binding was not accounted for by the affinity and suggested that a conformational contribution played a predominant role in the SH2-Tyr(P)527 binding.

Intramolecular Binding of Tyr(P)527 to the SH2 Domain Is Largely Independent of the Amino Acid Sequence of the C-terminal Tail—To test the finding that the C-terminal residues following Tyr(P)527 did not contribute to the SH2-Tyr(P)527 binding, we generated two kdSrc mutants: kdSrc-3A and kdSrc-6A. kdSrc-3A had the three residues following Tyr527 mutated to Ala, and kdSrc-6A had three residues on each side of Tyr527 mutated into Ala. We determined whether the phosphorylated tails in these mutants could still bind to the SH2 domain intramolecularly. The SH2 domain in the unphosphorylated form of both mutants was readily accessible, with an apparent $K_d$ for Flu-pYEEI of 80 nM (Fig. 3, B and C). Although the mutations decreased the rate of phosphorylation by Csk somewhat, Csk could still phosphorylate both mutants on Tyr527 to completion (24). Phosphorylation by Csk significantly reduced the accessibility of the SH2 domain in both mutants, increasing the apparent $K_d$ to 2 μM for kdSrc-3A and 1.3 μM for kdSrc-6A. This result demonstrated that the intramolecular SH2-Tyr(P)527 binding was largely independent of the C-terminal residues surrounding Tyr(P)527.

Inactivating Mutation to the SH2 Domain Does Not Abolish Src Inactivation by Tyr527 Phosphorylation—The above data suggested that the SH2-Tyr(P)527 binding was largely not driven by affinity. To test the idea further, we introduced an inactivating mutation to the SH2 domain of kdSrc and determined how the mutation affected the SH2-Tyr(P)527 binding. Arg175 is a highly conserved residue as part of the Tyr(P) binding pocket in all SH2 domains. R175A mutation in the Src SH2 domain is reported to decrease its affinity for pYEEI by a factor of 200 (25). When this mutation was introduced to kdSrc SH2 domain, the $K_d$ value of the mutant kdSrc for Flu-GpYEEI was increased to 1.28 μM, ~50-fold higher than that of kdSrc. In the competition fluorescence polarization assay, this mutant kdSrc displayed undetectable binding for the Src tail phosphopeptide, PQpYQGENL. Assuming that the mutation decreased the SH2 domain affinity for the tail phosphopeptide also by 50-fold, the mutant would have a $K_d$ of 1.2 mM for PQpYQPG. As shown in Fig. 4A, phosphorylation of kdSrc-R175A by Csk further reduced its affinity for Flu-GpYEEI, indicating that the mutant SH2 domain was still able to bind to Tyr(P)527.
To further test this conclusion, we introduced the mutation to the SH2 domain of active Src, and we determined whether this mutant (Src-R175A) was still inactivated by Csk phosphorylation. The mutant, side by side with WT Src and an Src mutant with no C-terminal tail (Src-NT), was incubated with different concentrations of Csk in the presence of ATP and MgCl₂ in the kinase assay buffer for 30 min, and the residual activity of Src was determined (Fig. 4B). As expected, WT Src was inactivated with increasing amounts of Csk, although Src-NT was not inactivated, indicating that phosphorylation on Tyr₅²⁷ was responsible for the inactivation. Src-R175A was also inactivated by incubation with Csk. Although WT Src was inactivated to ~15% of Src incubated without Csk, the inactivated Src-R175A mutant displayed ~30% of the control activity. The difference in the residual Src activity suggested either that a higher portion of the phosphorylated Src-R175A was in the open form or the closed form of the mutant had a high level of kinase activity. The former interpretation is consistent with the idea that a 50-fold reduction in the SH2 domain affinity for a phosphopeptide resulted in a higher portion of this mutant in an open form. It is important to note that the residual activity may not directly correlate to the portion of the closed form, because even the closed form may still possess a certain amount of activity. Overall, these data indicated that even an SH2 domain with 2% of the WT function was still able to mediate the SH2-Tyr(P)₅²⁷-triggered inactivation of Src to a large extent. This result further demonstrated the importance of a conformational contribution, relative to the affinity, to the SH2-Tyr(P)₅²⁷ binding.

Unphosphorylated Mutant Tail Containing YEEI Motif Inactivates Src by Binding to the SH2 Domain—The above results suggested that very weak affinity was required for Tyr(P)₅²⁷ to bind to the SH2 domain to inactivate Src. To further confirm this conclusion, we determined whether an unphosphorylated mutant tail could also bind to the SH2 domain to inactivate Src.

**FIGURE 4.** Effect of R175A mutation on the SH2-Tyr(P)₅²⁷ binding and Src inactivation. A, effect of the Arg¹⁷⁵ mutation on the SH2-Tyr(P)₅²⁷ binding. Binding of the SH2 domain to Flu-GpYEEI in kdSrc-R175A before and after phosphorylation by Csk was compared with the binding of the probe to kdSrc. B, inactivation of Src, an Src mutant lacking the C-terminal tail (Src-NT), and an Src mutant containing an R175A point mutation. The details of the experiments are described in the text and Experimental Procedures.

**FIGURE 5.** Effect of a C-terminal tail containing YEEI sequence on the accessibility of the SH2 domain in kdSrc and on Src activity in the WT Src. A, binding of kdSrc and a kdSrc mutant containing the EEI motif after Tyr₅²⁷. B, activation of WT Src and Src-EEI mutant by an SH3 domain ligand VSLARRLPPLL.
that with the help of the conformational contribution, the EEI sequence had sufficient interaction with the SH2 domain to reduce its accessibility. Substitution of Gln-Pro-Gly by EEI in the active Src reduced Src kinase activity about 7-fold, from a specific activity of 34 min\(^{-1}\) for WT Src to 5 min\(^{-1}\) for the mutant Src-EEI, consistent with the idea that the EEI tail suppressed Src activity.

To confirm that the EEI tail was indeed suppressing Src activity by binding to the SH2 domain, we determined whether this mutant could be activated by an SH3 domain peptide ligand, VSLARRPPLP. The WT Src was activated by the SH3 domain ligand \(\sim 100\%\), increasing from 34 min\(^{-1}\) in the absence of the ligand to 61 min\(^{-1}\) in the presence of 500 \(\mu M\) of the SH3 domain ligand (Fig. 5B). In contrast, Src-EEI was activated by the SH3 domain ligand \(\sim 8\)-fold in a concentration-dependent manner, increasing from 5 min\(^{-1}\) in the absence of the ligand to 46 min\(^{-1}\) in the presence of 500 \(\mu M\) of the ligand. This result indicated that Src-EEI activity was suppressed in an SH3 domain-dependent manner, and the nearly full activity can be recovered by abolishing the suppression. These results demonstrated that the unphosphorylated YEEI tail was sufficient to bind to the SH2 domain to cause significant Src inactivation. This conclusion further supports the conclusion that extremely weak affinity between the SH2 domain and the C-terminal tail was sufficient to cause Src to assume the closed form.

**Conformational Contribution to the SH2-Tyr(P)\(^{527}\) Binding Can Be Estimated by the Effective Concentrations**—Binding between a ligand and a receptor is determined by the following two factors: the affinity between the receptor and the ligand and their respective concentrations. In intramolecular binding, however, although the actual concentrations of the ligand and the receptor are the same as that of the whole molecule, the effective concentrations can be much higher because of their attachment to the same molecule and possibly tertiary arrangement that brings the ligand and the receptor together. The effective concentration is an overall measure of the conformational contribution to an intramolecular binding. The fact that extremely weak affinity is sufficient to cause significant SH2-tail binding suggested a large conformational contribution. Understanding this conformational contribution and its structural basis may hold the key to understanding molecular mechanisms of Src inactivation and possibly activation.

To determine the conformational contribution to the SH2-Tyr(P)\(^{527}\) binding, we calculated the effective concentration of the tail and the SH2 domain in the intramolecular complex. Based on the 98:2 ratio for the closed to open forms, the concentration of the free SH2 domain and the phosphorylated C-terminal tail at a given concentration of Src would be [Src] \(\times 0.02\), whereas concentration of the receptor-ligand complex (the closed form) is [Src] \(\times 0.98\). The apparent \(K_a\) \(\left(K_a\right)_{\text{inter}} = \left([\text{Src}] \times 0.98\right)/\left([\text{Src}] \times 0.02\right)^2\) \(= 2.5 \times 10^7/[\text{Src}]\). A comparison between the \(K_a\) \(\left(K_a\right)_{\text{intra}}\) with the determined \(K_a\) value for the intermolecular binding \(\left(K_a\right)_{\text{inter}}\) would reveal the extent of the conformational contribution, termed conformational enhancement factor (CEF): CEF = \(K_a\) \(\left(K_a\right)_{\text{intra}}/\left(K_a\right)_{\text{inter}}\). The \(K_a\) \(\left(K_a\right)_{\text{intra}} = 1/\left(K_a\right) = 1/24 \mu M = 4.2 \times 10^{-6} m^{-1}\). Thus, CEF for Src tail-SH2 binding can be calculated as follows: CEF = \(2.5 \times 10^7/[\text{Src}]\)\(/(4.2 \times 10^{-6}) = 0.059/\)\([\text{Src}]\). This value indicates that the CEF is inversely related to the Src concentration. The effective concentration of the SH2 domain and the tail can be calculated as \([\text{Src}] \times CEF = 0.059/\)\([\text{Src}] \times [\text{Src}] = 0.059 m\). These calculations indicate that the equilibrium between the closed and open forms of Src depends on two factors as follows: the effective concentration of 59 mM and an affinity between the tail and the SH2 domain. The high effective concentration of 59 mM provides sufficient compensation for the low affinity to ensure the SH2-Tyr(P)\(^{527}\) binding. Modification to either one of these two factors would alter the equilibrium and the Src activation level.

The CEF value dictates a specific relationship between the fraction of p-Src in the closed form and the affinity between the SH2 and Tyr(P)\(^{527}\). The relationship can be described by the following equation: \(K_{a\text{-inter}} = C/(0.059 \times (1 - C)^2)\), where \(C\) is the fraction of p-Src in the closed form. The derivation of this equation is given under “Materials and Methods.” The relationship can also be graphically presented (Fig. 6). An examination of the relationship indicates that a \(K_{a\text{-inter}}\) of 24 \(\mu M\) is sufficient to cause 98% of p-Src to be in the closed form (Fig. 6, arrow 1). Increasing the affinity 100-fold would lead to 99.8% of p-Src in the closed form (Fig. 6, arrow 2). Thus, increasing the affinity will result in only a marginal effect in the activity level of p-Src. On the other hand, tight binding between the SH2 domain and Tyr(P)\(^{527}\) may lock Src in an inactive form that cannot be readily dephosphorylated or activated by other mechanisms. If the affinity is decreased 100-fold (Fig. 6, arrow 3), only 82% of p-Src will be in the closed form. With 18% of p-Src in the open form, this may not be a sufficient level of Src inactivation for physiological purposes. Thus, it appears likely that the affinity between the SH2 domain and Tyr(P)\(^{527}\) is an evolutionary optimization for both achieving sufficient Src inactivation and allowing for ready activation. It is noted that 50% of Src would be in the closed form when \(K_{a\text{-inter}}\) is 29.5 mM (Fig. 6, arrow 4). This may explain that even unphosphorylated WT Src appears suppressed and can be activated by the SH3 domain ligand about 100%. This graph also provides an explanation for the fairly minor effect on Src inactivation by the
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R175A mutation. It predicts that a $K_d$ of 1.2 mM, as in the R175A mutant, would be sufficient to cause 87% of phosphorylated Src to be in the closed form (Fig. 6, arrow 5). The increase in the fraction of the open form for this mutant (13%) versus that in WT Src (2%) is consistent with the higher residual Src-R175A activity upon Csk phosphorylation.

**SH3 Domain Is Not Required for the SH2-Tyr(P)\textsuperscript{527} Binding but Is Essential for Src Inactivation**—To explore the structural basis for the conformational contribution, we examined the structure of inactivated Src to identify any structural features that may help bring the SH2 domain and the C-terminal tail together. The SH3 domain is required for Csk inactivation of Src (26, 27), and it has been suggested that the SH3 domain through its interaction with the 2-C linker stabilizes the closed form (10, 11). We determined whether the SH3 domain was required for the SH2-Tyr(P)\textsuperscript{527} binding. The SH3 domain was deleted from kdSrc, and we determined whether the phosphorylated C-terminal tail could still bind to the SH2 domain. The SH2 domain in this mutant bound to Flu-GpYEEI peptide with an apparent affinity of 25 ± 6 nM (Fig. 7A), which was comparable with the SH2 domain in full-length kdSrc, indicating that deletion of the SH3 domain did not affect the function of the SH2 domain. Upon Tyr\textsuperscript{527} phosphorylation by Csk, the binding of the SH2 domain to the exogenous ligand is greatly reduced, resulting in a $K_d$ of 3 ± 0.2 μM for Flu-GpYEEI. This result indicated that the phosphorylated C-terminal tail bound to the SH2 domain well in this mutant, and therefore, the SH2-Tyr(P)\textsuperscript{527} binding was not dependent on the presence of the SH3 domain. In light of this surprising result, we generated an active Src mutant that lacked the SH3 domain. This mutant was indeed not inactivated by Csk phosphorylation (Fig. 7B). These results together demonstrated that the Src SH3 domain was required for Src inactivation but not required for the SH2-Tyr(P)\textsuperscript{527} binding. This also demonstrated that the SH2-Tyr(P)\textsuperscript{527} binding was not sufficient for Src inactivation, which also required the action of the SH3 domain.

**Trp\textsuperscript{527} and Leu\textsuperscript{255} Are Key Residues for the Conformational Contribution to the SH2-Tyr(P)\textsuperscript{527} Binding**—Two residues, Trp\textsuperscript{260} and Leu\textsuperscript{255}, appear to be in crucial positions to contribute to the SH2-Tyr(P)\textsuperscript{527} binding (28–30). Both residues are located on the 2-C linker and interact with the N-lobe of the catalytic domain in the inactive Src structure. The interactions would locate the linker to one side of the catalytic domain and help position the SH2 domain near the base of the molecule, where the C-terminal tail is located. Furthermore, both residues are required for Src inactivation by Tyr\textsuperscript{527} phosphorylation, although the mechanisms for their requirement are not clear. We tested if the residues Trp\textsuperscript{260} and Leu\textsuperscript{255} were required for the SH2-Tyr(P)\textsuperscript{527} binding. We generated kdSrc-W260A and kdSrc-L255A mutants and determined the effects of the respective mutations on the SH2-Tyr(P)\textsuperscript{527} binding. Both mutants had similar binding to Flu-GpYEEI as kdSrc, indicating that the mutations themselves did not affect the function of the SH2 domain (Fig. 7C). Efficient phosphorylation of both mutants by Csk on Tyr\textsuperscript{527} was independently confirmed. Upon phosphorylation by Csk, the W260A mutant bound to the probe with affinity identical to that of the unphosphorylated form, indicating the Tyr\textsuperscript{527} in this mutant could not occupy the SH2 domain to reduce its accessibility. The accessibility of L255A mutant is reduced slightly by Csk phosphorylation but to a much less extent than in kdSrc, indicating that Tyr\textsuperscript{527} could bind to the SH2 domain, but much less effectively as in kdSrc. These results suggested that Trp\textsuperscript{260} and Leu\textsuperscript{255} played important roles in the conformational contribution that strengthened the SH2-Tyr(P)\textsuperscript{527} binding.

**Discussion**

Central to the regulation of the Src family kinases is the binding between the SH2 domain and Tyr(P)\textsuperscript{527} on the C-terminal tail. This binding triggers the formation of a closed conformation where the SH3 and SH2 domains pack against the catalytic domain to cause its inactivation. This inactive structure is subject to activation by numerous mechanisms, such as Tyr(P)\textsuperscript{527} dephosphorylation, autophosphorylation on Tyr\textsuperscript{416}, exogenous phosphotyrosine containing proteins binding to the Src SH2 domain, the α-subunit of the G-protein binding to the catalytic domain, or a ligand to the SH3 domain. Because the SH2-Tyr(P)\textsuperscript{527} binding triggers Src inactivation, and controls Src
activation, it is important to understand the mechanistic basis and the dynamics of this binding.

In this study, we quantified the factors contributing to the SH2-Tyr(P)^{527} binding for Src inactivation. First, the SH2-Tyr(P)^{527} binding was not driven by the affinity between the SH2 domain and the phosphorylated C-terminal tail. Several independent experiments demonstrated that the binding was largely dependent on a conformational contribution. Second, we quantified the conformational contribution by determining the effective concentrations of the SH2 domain and the C-terminal tail in their binding at 59 mM. Third, several substructures are known to be required for Src inactivation. We determined whether they were also required for the SH2-Tyr(P)^{527} binding. Trp^{260} and Leu^{255} were essential for the conformational contribution. Surprisingly, the SH3 domain, although essential for Src inactivation and suggested to assist SH2-Tyr(P)^{527} binding based on inactivated Src structure, was not required for the SH2-Tyr(P)^{527} binding.

Insights into Src Regulation—These results offer the following insights into Src regulation. First, the SH2-Tyr(P)^{527} binding is not sufficient to inactivate Src. This is clearly demonstrated by the SH3 deletion mutants, in which Tyr(P)^{527} still binds to the SH2 domain, but the binding does not inactivate Src. Second, the SH3 domain, through its interaction with the 2-C linker, does not contribute to the SH2-Tyr(P)^{527} binding but is essential for Src inactivation. Because the SH3 binding to the 2-C linker is not sufficient for Src inactivation (12, 31), this suggests that the role of the SH3 domain in inactivating Src is dependent on SH2-Tyr(P)^{527} binding. Third, the SH2-Tyr(P)^{527} binding is dependent on the side chains of Trp^{260} and Leu^{255}. In the inactivated Src structure, the side chain of Trp^{260} binds to a pocket formed by the residues of Ala^{259}, Glu^{261}, Leu^{308}, Gln^{312}, Lys^{315}, and Leu^{325}. Leu^{255} binds to a hydrophobic pocket formed by Ile^{262}, Trp^{286}, Thr^{290}, Val^{292}, Tyr^{326}, and Val^{337} in the N-lobe of Src. The interactions of Trp^{260} and Leu^{255} appear to orient the SH2 domain toward the base of the catalytic domain where the C-terminal tail is. Such a spatial arrangement brings the SH2 domain and the C-terminal tail together to increase their effective concentrations for the SH2-Tyr(P)^{527} binding.

These mechanistic elements together suggest the following sequence of events for Src inactivation upon Tyr(P)^{527} phosphorylation. 1) The interactions of Trp^{260} and Leu^{255} with the N-lobe bring the C-terminal tail near the SH2 domain. 2) Tyr(P)^{527} binds to the SH2 domain. The SH2-Tyr(P)^{527} affinity is low but sufficient for the binding because of the high effective concentrations of the SH2 domain and the C-terminal tail. 3) The SH3 domain binds to the linker, which results in Src inactivation. It is not clear how the SH3 domain inactivates Src. There are at least two possibilities. First, the binding of the SH3 domain to the 2-C linker alters the interactions of Trp^{260} and Leu^{255} with the N-lobe, thus leading to Src inactivation. Because the binding environment of Trp^{260} consists of several residues from the α-helix C, any changes to the conformation of Trp^{260} can be readily communicated to the α-helix C to alter Src activity. It is conceivable that binding of the SH3 domain to the 2-C linker could affect the conformation of Trp^{260}, which can be communicated to the α-helix C. It is also possible that coordinated changes to the interactions of Trp^{260} and Leu^{255} could lead to Src inactivation. Second, the inactivation may be due to coordinated actions of both the SH2 and the SH3 domain. In the inactivated Src structure, the SH2 and SH3 domain become one rigid structural unit. It is proposed that this structure unit may act as a “clamp” to alter the conformation of the catalytic domain.

The Ratio between the Closed and Open Forms—The ratio of 98:2 between closed and open forms of Src is likely the compromise of multiple functional demands of Src regulation. First, the 98:2 ratio dictates the level of Src activity upon Tyr^{527} phosphorylation. It can be envisioned that a certain level of basal activity may be required for Src cellular function. Second, the presence of 2% of the p-Src in the open form may also control the rate of Tyr(P)^{527} dephosphorylation. When Tyr(P)^{527} is bound to the SH2 domain, it is protected from dephosphorylation (13), and only the free form is accessible to the phosphatase. Thus the equilibrium between the closed and open forms would dictate the dynamics of Src Tyr^{527} dephosphorylation. Third, the ratio may also affect Src activation by phosphorylated receptor PTKs and other phosphorytrosine-containing proteins binding to the Src SH2 domain.

Contribution of Affinity Versus Conformation to the SH2-Tyr(P)^{527} Binding—Two parameters, the SH2-Tyr(P)^{527} affinity and conformational contribution, dictate the SH2-Tyr(P)^{527} binding and the ratio between the closed and open form. However, the 98:2 ratio can be achieved by a continuum of combinations of affinity and effective concentrations. Thus, the combination of 24 μM and 59 mM is likely selected based on factors other than the requirement of maintaining the correct closed-open ratio. Several factors likely contribute to this choice. First, by using a high effective concentration and low affinity combination, it ensures that the SH2-Tyr(P)^{527} binding only occurs intramolecularly. A more affinity-based binding would result in a higher probability of intermolecular SH2-Tyr(P)^{527} binding, forming Src dimers or even oligomers. Second, the conformation-driven SH2-Tyr(P)^{527} binding also allows conformation-based regulatory mechanisms. If the SH2-Tyr(P)^{527} binding were affinity-driven, activation of Src would have to rely on reducing the affinity, by regulating the conformation/structure of either the SH2 domain or the C-terminal tail. On the other hand, in the conformation-driven binding, any mechanisms that can alter the conformational contribution would be able to regulate the SH2-Tyr(P)^{527} binding and Src activity. This provides a platform for regulatory mechanisms that do not directly affect the affinity between the SH2 domain and Tyr(P)^{527}. Autophosphorylation on Tyr{416} and G-protein binding to the catalytic domain are two such mechanisms. It is plausible that either autophosphorylation or G-protein binding could trigger a conformational change to Src, abolishing the conformational contribution to the SH2-Tyr(P)^{527} binding. This hypothesis awaits experimental testing.

SH2-Tyr(P)^{527} Binding Versus Src Inactivation—To our knowledge, this study provides the first piece of direct evidence that the binding between the SH2 domain and Tyr(P)^{527} does not directly lead to Src inactivation, which also requires the action of the SH3 domain. This raises the possibility that in certain activated forms of Src, the SH2 domain may be occupied.
by pTy527. For example, when p-Src is activated by a ligand to the SH3 domain, it may just disrupt the interaction between the SH3 domain and the 2-C linker, and leaves the SH2-Tyr(P)527 intact. In this scenario, Tyr(P)527 is still protected from rapid dephosphorylation, and the Src can return to the inactivated state once the SH3 domain ligand is removed. Further research would be required to fully understand if such implications are relevant to Src regulation.

**Intermolecular Versus Intramolecular Binding in Protein Function**—Our results demonstrated that conformational contribution played a large role in the SH2-Tyr(P)527 binding, and further quantified the contribution by determining the effective concentration of the SH2 domain and Tyr(P)527 in the intramolecular binding. This approach may apply to understanding the regulation of many other molecular systems. Several kinase families are regulated by similar intramolecular bindings. c-Abl is autoinhibited by the interaction between the N-terminal myristoylation motif and a hydrophobic pocket in the C-lobe of the catalytic domain (32, 33). Glycogen synthase kinase 3 is inhibited by the interaction of a phosphorylated Ser residue located near the N terminus with a phosphate binding pocket near the active site of the kinase domain (34, 35). In each case, the intramolecular interaction will depend on both the affinity between the two interacting components and the conformational contribution that re-enforces the binding. Full understanding of one molecular system such as Src may be helpful for investigating other enzyme systems.

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