Deletion of the SH3 Domain of Src Interferes with Regulation by the Phosphorylated Carboxyl-terminal Tyrosine*

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A current model for the regulation of the Src protein-tyrosine kinase proposes that the COOH-terminal phosphoryrosine, Tyr-527, binds to the Src homology 2 (SH2) region in an intramolecular interaction that represses the kinase domain. This model is consistent with the activation of Src by mutations in the SH2 domain or COOH terminus. Mutations in the SH3 domain also activate Src, although this region is not thought to bind phosphoryrosine. Seidel-Dugan et al. (Seidel-Dugan, C., Meyer, B. E., Thomas, S. M., and Brugge, J. S. (1992) Mol. Cell. Biol. 12, 1835–1845) have shown that Src mutants with deletions in the SH2 or SH3 domain transform chicken embryo fibroblasts and have increased kinase activity. These mutant proteins are underphosphorylated at Tyr-527, a change that could in itself activate the mutants. Therefore, it is not possible to distinguish whether the SH2 and SH3 domains are needed for phosphorylation of Tyr-527 or for Src to adopt or maintain the repressed state. We have artificially increased the level of Tyr-527 phosphoryrosine by deleting or substituting mutations by coexpressing them with the Tyr-527 kinase, Csk, in yeast cells. We find that both the SH2 and SH3 domains are needed for inhibition of Src by Csk. The SH2 domain is needed for efficient phosphorylation by Csk, both in yeast cells and in vitro. The SH3 domain is needed for Src to be inhibited when Tyr-527 is phosphorylated by Csk. This suggests that the SH3 domain cooperates with the SH2 domain and phosphorylated Tyr-527 to inhibit the kinase domain. Dephosphorylation of SH3 domain mutants at Tyr-527 in fibroblasts could be a consequence of a failure of the proposed SH2/phosphoryrosine interaction.

The cytoplasmic protein-tyrosine kinase Src is normally phosphorylated at Tyr-527, close to its carboxyl terminus (reviewed in Ref. 2). Removal of this phosphate stimulates Src kinase activity. In the cell, blockade of phosphorylation by deletion or substitution of Tyr-527 stimulates Src kinase activity and causes malignant transformation. Therefore the maintenance of high levels of phosphorylation of Tyr-527 is important to prevent transformation.

A tyrosine kinase, known as Csk, was identified that phosphorylates Tyr-527, but not other residues, in Src (3). Purification and cloning of Csk showed that it binds to Src, but it lacks a Tyr-527 homolog (4, 5). Purified Csk (reviewed in Refs. 11–13) does not autophosphorylate, and it lacks an equivalent of Tyr-416 of Src, the residue that is phosphorylated in activated forms of Src and is the primary site of autophosphorylation in vitro. Csk can phosphorylate Src at Tyr-527 to an extent that inhibits Src kinase activity significantly in vitro (3). Csk also phosphorylates close relatives of Src (6, 7). Recent evidence indicates that Csk performs a vital role in repressing Src activity in the developing embryo.1 Mouse embryos that do not express Csk die at neurulation, and their cells have high levels of phosphoryrosine due to activation of Src and at least two of its close relatives, Fyn and Lyn. Other protein kinases, besides Csk, also phosphorylate Tyr-527, but at inadequate levels to keep Src inactive.¹

Specific mutations of Src outside the COOH terminus also can activate the kinase domain and cause transformation (reviewed in Ref. 10). Such activating mutations have been detected in the SH2,² SH3, and kinase domains. The SH2 domain is capable of binding peptides that contain phosphoryrosine in vitro, and it presumably is the site where activated forms of Src bind to phosphotyrosyl proteins in the cell (reviewed in Refs. 11–13). These protein-protein interactions may be important for cell transformation, because certain mutations in the SH2 domain interfere with transformation by activated Src mutants without decreasing kinase activity (10, 14). The SH2 domain is also required for activated Src to bind to specific proteins and the cytoskeleton (15, 16). Src has to be catalytically activated in order to bind phosphopeptides (17). The inability of wild-type Src to bind, despite the presence of a functional SH2 domain, has led to the hypothesis that the SH2 domain is occupied by phosphoryrosine 527, and that this interaction inhibits the kinase domain (12, 18). This would explain the activation of Src by certain SH2 domain mutations (19, 20).

The role of the SH3 domain is less clear. SH3 domains are found in a number of cytoskeletal proteins and probably mediate protein-protein interactions (reviewed in Ref. 21).

3 The abbreviations used are: SH2 and SH3, Src homology 2 and 3, respectively; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.
Two proteins that bind to the Src SH3 domain have been identified and both have proline-rich regions required for binding (22, 23). Genetic evidence indicates that the SH3 domains of certain proteins are required for transformation and signal Ras activation (24–27). In the case of Src, the SH3 domain may also play a role in transformation, since some mutations that alter this region decrease both transforming activity (14, 28) and the binding of specific cell proteins (16), although total deletion of the SH3 domain does not nullify transformation (1). The SH3 domain is also important for inhibition of kinase activity, since several mutations in SH3 domain activate Src kinase and transforming activity (10, 19, 29). One activating point mutation in the SH3 domain is at residue 95, a residue that apparently contacts bound proline-rich peptide ligands (29–31), suggesting that repression of Src kinase activity by the SH3 domain requires such an interaction.

It should be simple to determine whether the SH2 and SH3 domains of Src inhibit kinase activity through inter- or intramolecular interactions by testing whether wild-type and mutant forms of Src retain their expected relative kinase activities when purified. Indeed, SH2 and SH3 mutants are more active tyrosine kinases than wild-type Src in vitro. However, the situation is complicated because a small reduction in phosphorylation of Tyr-527 could account for the activation of the mutants. Most SH2 and SH3 domain mutants appear to be hypophosphorylated at Tyr-527 relative to wild-type Src. The effect is most noticeable with mutants containing large deletions in the SH2 and SH3 domains (1). These mutants are highly active and conspicuously underphosphorylated at Tyr-527. Therefore, the mutations could reduce the level of Tyr-527 phosphorylation (e.g. by preventing protein-protein interactions with a kinase such as Csk) and indirectly activate the kinase domain. The alternative, that the SH2 and SH3 domains are needed for the intramolecular interactions that allow phosphoryrosine 527 to inhibit the kinase domain and only indirectly regulate the phosphorylation state of Tyr-527 (e.g. by protecting Tyr-527 from dephosphorylation), has not been tested.

To try to resolve this question, we have expressed SH2 and SH3 domain mutants of Src in yeast together with Csk. We used two simple in-frame deletion mutants, AA and AB, removing the entire SH3 domain (residues 86–137) and the NH2-terminal portion of the SH2 domain (residues 148–187), respectively (Fig. 1). These mutants also impinge on a region, residues 137–157, that may interact with the kinase domain and repress kinase activity unless Tyr-416 is phosphorylated (32). They were constructed by Seidel-Dungan et al. (1) and shown to be activated and hypophosphorylated at Tyr-527 when expressed in chicken fibroblasts. As controls, we expressed the mutants and wild-type Src in the absence of Csk. Because yeast lacks a Csk homolog, phosphorylation of Src at Tyr-527 in yeast is inefficient and the enzyme is active (33). We found that phosphorylation of wild-type Src by Csk was inhibited, but phosphorylation of the ΔA and ΔB mutants was not. This indicates that the SH3 domain is involved in intramolecular interactions that inhibit the enzyme, as proposed previously for the SH2 domain. Surprisingly, we also found that the SH3 domain and, especially, the SH2 domain are needed for efficient phosphorylation by Csk in vitro, so the reduced phosphorylation of the ΔA and ΔB mutants in chicken fibroblasts may be partly due to a decreased rate of phosphorylation by Csk.

MATERIALS AND METHODS

Src and Csk Expression in Yeast—Src mutants were expressed using the vector pL, described elsewhere (34). The nondeleted K+ and K' (R295) mutants have also been described previously (34). Plasmids encoding the ΔA, ΔB, and ΔC mutants (1) were used as templates for polymerase chain reactions to amplify the Src region, using a 5’ primer with a BamH1 restriction site and a 3’ primer with a Xhol restriction site at codons 516/517. The products were cut with BamH1 and MluI, and the 5’ portions subcloned into plasmids that encode wild-type or R295 mutant Src adjacent to the bacterial chloramphenicol transference gene on a common BamH1/BglII restriction fragment (33). The respective BamH1/BglII fragments were cloned into the BamH1 site of pL and recombinants selected on chloramphenicol. Orientation was checked by Xhol digestion. Csk was expressed from pHMCSK (4). Plasmids were introduced into yeast using lithium acetate and selected on media lacking leucine (pL derivative) or leucine and tryptophan (pLMCSEK). Expression levels in several independent transformants were compared, and clones with similar expression levels studied further.

SDS-polyacrylamide gel electrophoresis used 10% acrylamide and 0.27% bis-acrylamide gels to increase the separation between Src deletion mutants. The protein contents of the extracts (assessed using Coomassie Blue) were equalized before electrophoresis. Immunoprecipitated samples were kept at 0 °C before electrophoresis to minimize reduction of IgG. Western blot analysis was performed as described previously (4), using monoclonal antibodies PY20 (antiphosphotyrosine, ICN), or SCRF 35.4 (antibody to residues 2 through 17 of SCRF, Microbiological Associates), alkaline phosphatase-conjugated mouse IgG, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (except for Fig. 2, lower, where we used peroxidase-conjugated anti-mouse IgG and enhanced chemiluminescence (Amer sham Corp.). Csk was detected using affinity-purified rabbit antisera raised to a synthetic peptide corresponding to the COOH-terminal 13 residues of Csk (conjugated through an NH2-terminal lysine to key hole limpet hemocyanin using glutaraldehyde) and alkaline phosphatase-conjugated anti-rabbit IgG. Src immunoprecipitates were prepared from approximately 3 × 106 yeast that had been grown for 2 days in galactose. The yeast were harvested by centrifugation, washed, and broken by vortexing with glass beads (five times, 30 s each) at 4 °C in 0.5 ml of radioimmunoprecipitation buffer containing 2 mM PMSF, 20 μg/ml aprotinin, and 0.2 mM Na3VO4. A high speed supernatant was precipitated with 20 μl of 327 or SCRF 35.4 antibody (1:2000 dilution in rabbit serum), followed by 5 μl of rabbit anti-mouse IgG (30 μl, 0.27% bis-acrylamide gels to increase the separation between Src and repressed kinase activity unless Tyr-416 is phosphorylated (32). They were constructed by Seidel-Dungan et al. (1) and shown to be activated and hypophosphorylated at Tyr-527 when expressed in chicken fibroblasts. As controls, we expressed the mutants and wild-type Src in the absence of Csk. Because yeast lacks a Csk homolog, phosphorylation of Src at Tyr-527 in yeast is inefficient and the enzyme is active (33). We found that phosphorylation of wild-type Src by Csk was inhibited, but phosphorylation of the ΔA and ΔB mutants was not. This indicates that the SH3 domain is involved in intramolecular interactions that inhibit the enzyme, as proposed previously for the SH2 domain. Surprisingly, we also found that the SH3 domain and, especially, the SH2 domain are needed for efficient phosphorylation by Csk in vitro, so the reduced phosphorylation of the ΔA and ΔB mutants in chicken fibroblasts may be partly due to a decreased rate of phosphorylation by Csk.

Preparation and Assay of Glutathione S-transferase-Csk Fusion Protein—A SalI fragment containing the Csk open reading frame was cloned from pBRCSK (4) into the SP64polyA+ vector (Promega), so that a BamH1 site was added to the coding region. The plasmid was digested with BglII, which cuts 2 codons 5’ to the Csk initiation codon, and BamH1, and cloned into the BamH1 site of the pGEX-2T vector (AmRad Corp.). The predicted translation product contains

![Fig. 1. Domain structure of Src. The locations of the homology domains, the kinase domain, the A, B, and C boxes, and of residues K295, Y416 and Y527 are diagrammed. The residues deleted from Src in the AA and AB mutants are indicated.](image-url)
glutathione S-transferase attached to the NH2-terminal methionine of Csk. A 6-ml overnight culture of bacteria was diluted to 100 ml, grown for 1 h at 37 °C, supplemented with isopropyl-thio-β-D-galactopyranoside (0.1 mM) for 2 h, and harvested. Bacteria were resuspended in 10 ml of phosphate-buffered saline with 1% Triton X-100, 1 mM PMSF, and 20 µg/ml aprotinin and sonicated at 0 °C for 40 s each. After centrifugation at 10,000 × g, the supernatant was mixed with 0.2 ml of glutathione-agarose for 10 min at 4 °C. The agarose was washed three times by centrifugation with phosphate-buffered saline, 1 mM PMSF, 20 µg/ml aprotinin, and eluted with 0.5 ml of 50 mM Tris-HCl, pH 8.0, 0.5 mM reduced glutathione. The eluate was dialyzed against buffer A overnight at 4 °C and stored at −20 °C.

GST-Csk (2 µl, approximately 6 ng) was incubated with 3 µl of immunoprecipitated Src or 20 ng of purified M295 mutant Src in a buffer containing 10 mM PIPES, pH 7.0, 10 mM MnCl2, 0.75 µM [γ-32P]ATP (3,000 Ci/mmol), for 20 m at 30 °C (total volume, 20 µl). Reactions were stopped with an equal volume of double-concentration gel loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis. M295 mutant Src was made using a Baculovirus expression system and purified as described elsewhere (36), with minor modifications.

**RESULTS**

The ΔA and ΔB mutants of Src were cloned behind a galactose-regulated promoter in a S. cerevisiae vector. This vector carries the LEU2 marker for selection in media lacking leucine. We prepared both active (K+)

inactive (lysine 295 to arginine mutants, K−) forms of both deletion mutants. We also made use of a yeast vector carrying the Csk cDNA behind a galactose-regulated promoter with a TRP2 marker for selection on media lacking tryptophan. By growing the yeast in galactose, we could induce the expression of both Csk and Src.

We have shown before that Csk does not phosphorylate any yeast proteins detectably, but co-expression of Csk with Src leads to Src phosphorylation and decreases the phosphorylation of yeast proteins on tyrosine (4). To test whether Csk could also phosphorylate ΔA and ΔB, we used kinase-inactive forms of these mutants. The expression levels were determined by immunoblotting with anti-Src antibodies (Fig. 2, upper). In the absence of Csk, nondeleted K+ Src, ΔAK−

and ΔBK− Src all lacked detectable phosphotyrosine (Fig. 2, lower). This was expected because yeast lack kinases able to phosphorylate either Tyr-416 or Tyr-527 of Src (34, 37). Co-expression with Csk induced the tyrosine phosphorylation of both nondeleted K+ and ΔAK− Src to similar levels. The ΔBK− mutant Src was also phosphorylated by Csk, but to a lower level (Fig. 2).

We next expressed active forms of ΔA and ΔB, in the absence or presence of Csk. Expression levels were estimated by immunoblotting (Fig. 3, upper). The activities of these kinases in the yeast cells was evident by the extensive phosphorylation of many yeast proteins, detected by immunoblotting into antiphosphotyrosine antibodies (Fig. 3, lower). Proteins that co-migrated with Src were also phosphorylated, presumably a sign of autophosphorylation. In the presence of Csk, nondeleted K+ Src was inhibited significantly. The only phosphotyrosine detected was in K+ Src itself, presumably because it is phosphorylated at Tyr-527 by Csk (Fig. 3, lane 6). In contrast, phosphorylation of cell proteins by ΔAK+ Src and ΔBK+ Src proceeded unchecked by Csk (Fig. 3, compare lanes 3, 4, 7, and 8).

The inhibition of nondeleted K+ Src in yeast cells expressing Csk was also evident when it was recovered by immunoprecipitation and assayed in vitro (Fig. 4). Phosphorylation of an exogenous substrate was inhibited 7–8-fold. In contrast, the activities of the ΔAK+ and ΔBK+ mutants were not affected by co-expression with Csk (Fig. 4). Equal quantities of Src were used for these assays, as estimated by immunoblotting.
To test whether the ∆A and ∆B mutations directly affected phosphorylation by Csk, we performed in vitro phosphorylation assays. As a source of Csk we used a fusion protein, GST-Csk, purified from Escherichia coli. We determined whether this fusion protein had the characteristic property of Csk purified from brain or spleen, that is, specificity for Tyr-527 in Src (3, 6). GST-Csk was incubated with nondeleted K− Src that had been immunoprecipitated from yeast (K+, Fig. 5), and with K− Src having a phenylalanine mutation at Tyr-527, prepared similarly (K− F527, Fig. 5). The phenylalanine mutation completely blocked phosphorylation, indicating that Tyr-527 is absolutely required for phosphate incorporation. This enzyme was able to phosphorylate a Met-295 mutant Src purified from baculovirus-infected insect cells (M295, Fig. 5), indicating that the results are not an artefact of either immunoprecipitation of the substrate or the arginine substitution of residue 295 in K− Src.

GST-Csk was incubated with immunoprecipitates containing kinase-inactive forms of nondeleted, ∆A or ∆B Src (Fig. 6). All three forms of Src were phosphorylated by GST-Csk. The phosphorylation rates using ∆AK− and ∆BK− Src were decreased respectively to approximately 20% and 2% the rate of nondeleted K− Src, however (Fig. 6). We could not determine whether the decrease was due to a change in the affinity or the maximal rate of the reaction using immunoprecipitated Src as substrate. Increasing the amount of GST-Csk in the reaction 10-fold increased the phosphorylation of all three proteins equally (approximately 7-fold).

**Fig. 4.** Kinase activities of Src proteins immunoprecipitated from yeast. Similar yeast cultures to those described in Fig. 3 were lysed and immunoprecipitated using antibody to Src (SCRF35.4). The activity of Src in each immunoprecipitate was assayed using enolase as an exogenous substrate, and the reaction products analyzed by SDS-PAGE and quantified using a PhosphorImager (Molecular Dynamics). The upper panel shows the image of the screen, and the lower panel shows radioactivity of enolase, in arbitrary units.

**Fig. 5.** Specificity of GST-Csk. GST-Csk was purified from bacteria as described (“Materials and Methods”) and incubated with various Src proteins and [γ-32P]ATP. The Src proteins were: K−, Arg-295 mutant Src immunoprecipitated from yeast; K-F527, Arg-295 → Phe, Arg-527 → Phe double mutant Src immunoprecipitated from yeast; M295, Met-295 mutant Src purified from insect cells (M. Broome and T. Hunter, unpublished data). Left panel, reaction products were analyzed by SDS-PAGE and detected by autoradiography. Note low levels of autophosphorylation of GST-Csk and a breakdown product, and of M295 Src. Right panel, immunoblot of Src preparations used for assays. The bands above Src are partially reduced immunoglobulin.

**Fig. 6.** Phosphorylation of kinase-inactive nondeleted and ∆A and ∆B Src by GST-Csk. Nondeleted K−, ∆AK−, and ∆BK− Src were immunoprecipitated from yeast, and the quantities of Src protein in the immunoprecipitates normalized, as estimated by immunoblotting (left panel). Different volumes of immunoprecipitate (0, 0.5, 1, 2, and 5 μl) were incubated with approximately 6 ng of GST-Csk and [γ-32P]ATP, as described under “Materials and Methods.” Reaction products were analyzed by SDS-PAGE and visualized and quantified using a PhosphorImager. The lower panel shows phosphorylation of nondeleted K− (■), ∆AK− (▲), and ∆BK− (●), in arbitrary units.
SH2 and SH3 Deletions and Regulation of Src by Csk

DISCUSSION

We have found that ΔA mutant Src, containing a deletion of most of SH3 domain of Src, becomes phosphorylated when co-expressed with Csk in yeast (Fig. 2), but it is not inhibited (Figs. 3 and 4), suggesting that the SH3 domain is required for the Src kinase domain to be repressed. Because the ΔA mutant was more active than wild-type Src after COOH-terminal phosphorylation, both in an immunoprecipitate and in yeast, we conclude that the SH3 domain participates in intramolecular interactions to adopt or maintain the repressed state. Since the SH2 domain is the best candidate for binding phosphorylated Tyr-527, the SH3 domain probably inhibits the kinase domain through interactions with other parts of the molecule. The SH3 domain could stabilize the binding of the SH2 domain to phosphotyrosine 527. SH3 and SH2 domains are often found adjacent to each other in a polypeptide, and the ability of SH2 domains to change conformation in response to phosphopeptide binding provides a potential mechanism whereby SH3 and SH2 domains could communicate (38, 39). Alternatively, the SH3 domain could interact directly with the kinase domain. The energies of SH2/Tyr-527 and SH3/kinase domain interactions may need to be combined to counteract a tendency of the kinase domain to become active. The exact mechanism whereby the SH3 domain inhibits Src may be apparent when the atomic structure of Src is known.

The ΔB mutant of Src, lacking approximately one-half of the SH2 domain, was also not inhibited when co-expressed with Csk in yeast (Figs. 3 and 4). This protein was only phosphorylated poorly by Csk, however (Fig. 2). In vitro, the rate of phosphorylation of ΔB mutant Src by GST-Csk was less than 2% of the rate of phosphorylation of wild-type Src (Fig. 6). After 2 days co-expression of ΔB mutant Src with Csk in yeast, tyrosine phosphorylation was detected, but only at 10–20% of the level of wild-type Src (Fig. 2). Similar results for ΔC mutant Src, lacking the COOH-terminal portion of the SH2 domain (1), were obtained when it was phosphorylated in vitro by GST-Csk or co-expressed with Csk in yeast (unpublished results). This suggests that an intact SH2 domain is needed for recognition of Src by Csk. This is surprising, because Csk lacks phosphotyrosine, and phosphorylation is needed for the best understood binding reactions of SH2 domains. Csk appears not to bind to Src (40). The Src SH2 domain may not bind Csk, but affect phosphorylation by Csk indirectly, for example, by altering the conformation of the kinase domain or COOH-terminal region. Deletion of the SH3 domain also reduced phosphorylation of the ΔA mutant by Csk, but less dramatically, and after 2-day co-expression with Csk in yeast, the ΔA mutant was phosphorylated at similar level to wild type. Possibly, Csk recognizes Tyr-527 in the context of its binding site in the SH2 domain, and phosphorylation stabilizes the binding. An effect of SH3 domain deletion on phosphorylation by Csk would be predicted by this model, because our results indicate that the SH3 domain may be needed for efficient binding of the COOH terminus to the SH2 domain.

Our studies did not demonstrate whether the SH2 domain is required for inhibition of Src by phosphorylated Tyr-527, because we could not achieve sufficient levels of phosphorylation of the ΔB or ΔC mutants. If the SH2 domain is the binding site for Tyr-527, however, then the ΔB and ΔC mutants may be active even if high levels of Tyr-527 phosphorylation were achieved.

Activated mutant forms of Src are generally hypophosphorylated at Tyr-527 in the cell (1, 8, 20, 41–43). This may be a consequence of their activated state, in that binding of the COOH terminus to the SH2 domain may be needed to protect phosphotyrosine 527 from cellular phosphatases (17). Increased dephosphorylation would lower the steady state level of phosphorylation. Our results indicate that additional factors may be at play. Deletion of the SH2 domain of Src interferes with phosphorylation by Csk in vitro. This would reduce the level of Tyr-527 phosphorylation in the cell, provided that Csk is limiting for phosphorylation of Tyr-527 in vitro. Recent experiments with csk- animals indicate that Csk is essential to inhibit Src in embryonic fibroblasts. However, other kinases do phosphorylate Tyr-527. Src is still detectably phosphorylated at Tyr-527 in cells from csk- mice, and some mutant forms of Src that are poor in vitro substrates for Csk are nonetheless phosphorylated extensively at Tyr-527 in the cell (9). The reduced phosphorylation of the SH2 deletion mutant in chick fibroblasts (1) may thus be a consequence of a combination of factors: reduced phosphorylation by Csk, reduced phosphorylation by other kinases, and increased accessibility to phosphatases.

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SH2 and SH3 Deletions and Regulation of Src by Csk