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Suppression of c-Src Activity by C-Terminal Src Kinase Involves the c-Src SH2 and SH3 Domains: Analysis with *Saccharomyces cerevisiae*

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The kinase activity of c-Src is normally repressed in vertebrate cells by extensive phosphorylation of Y-527. C-terminal Src kinase (CSK) is a candidate for the enzyme that catalyzes this phosphorylation. We have used budding yeast to study the regulation of c-Src activity by CSK in intact cells. Expression of c-Src in *Saccharomyces cerevisiae*, which lacks endogenous c-Src and Y-527 kinases, induces a kinase-dependent growth inhibition. Coexpression of CSK in these cells results in phosphorylation of c-Src on Y-527 and suppression of the c-Src phenotype. CSK does not fully suppress the activity of c-Src mutants lacking portions of the SH2 or SH3 domains, even though these mutant proteins are phosphorylated on Y-527 by CSK both in vivo and in vitro. These results suggest that both the SH2 and SH3 domains of c-Src are required for the suppression of c-Src activity by Y-527 phosphorylation.

c-Src is a member of a family of protein-tyrosine kinases that are associated with the cytoplasmic face of cellular membranes (6, 49). The physiological role of c-Src is unclear. In a variety of cell types, c-Src is associated with endosomes (16) and specialized secretory vesicles (12, 22, 33), suggesting a role in the regulation of vesicular transport. Mice containing a homozygous disruption of the c-*src* gene exhibit impaired osteoclast function (24, 47), perhaps as a result of defective secretory processes. In addition, c-Src has been implicated in platelet activation (4, 52) and in the control of mitosis in fibroblasts (49). Finally, mutant forms of c-Src (e.g., v-Src) induce unrestrained cell proliferation, perhaps indicating that c-Src is involved in cell growth control (6).

Members of the Src family share a structural framework (Fig. 1) that includes a C-terminal kinase domain and three N-terminal regions that appear to be involved in controlling kinase activity and association with other proteins (3, 10, 11, 13–15, 17, 38, 44, 49, 53). These N-terminal regions include a variable region whose sequence is not conserved among Src family members, as well as two conserved segments known as the Src homology 2 (SH2) and SH3 domains. SH2 domains are found in many signal transduction proteins and facilitate certain protein-protein interactions by binding to phosphotyrosine residues in specific contexts (36). SH3 domains are also found in a variety of signalling molecules (often adjacent to SH2 domains), but their function is unclear (28, 36).

c-Src is extensively regulated by phosphorylation. The N-terminal variable region is phosphorylated by a variety of protein kinases (6). For example, CDC2 phosphorylates c-Src in this region, possibly contributing to the increase in c-Src activity that is observed during mitosis (49). c-Src also contains an autophosphorylation site at Y-416 which may be necessary for full c-Src activity (19, 20, 37). The phosphorylation site with the clearest regulatory role is Y-527, a negative regulatory site near the C terminus of the protein (6). In vertebrate cells, the kinase activity of c-Src is normally inhibited by extensive phosphorylation at this site (7). The kinase activity of c-Src is increased approximately 10-fold when Y-527 is dephosphorylated or mutated (Y-527→F [Y527F]) (2, 8, 20, 37). Expression of the Y527F mutant causes a transformed phenotype in cultured vertebrate cells (2, 20, 37, 39] and tumors in nude mice (20). Thus, it is of critical importance that the phosphorylation state of this residue be tightly regulated.

Several lines of evidence suggest that Y-527 is phosphorylated by a separate protein kinase. c-Src does not autophosphorylate extensively at Y-527 in vitro (8) and is poorly phosphorylated on Y-527 when expressed in *Saccharomyces cerevisiae* (5, 21). When kinase-deficient c-Src is expressed in a cell line lacking wild-type c-Src, it is highly phosphorylated at Y-527 (50). A candidate for the Y-527 kinase is C-terminal Src kinase (CSK) (29, 31). CSK is a 50-kDa protein-tyrosine kinase that exhibits many of the structural characteristics of a Src family member, including a C-terminal kinase domain and N-terminal SH3 and SH2 domains. CSK has been shown to specifically phosphorylate c-Src and two other Src family members in vitro on Y-527 or the analogous residue (1, 29, 30). It remains unclear whether CSK is responsible for the high level of Y-527 phosphorylation seen in vivo.

It has been proposed that phosphorylation of Y-527 causes an interaction between the C-terminal region of c-Src and its SH2 domain. Thus, Y-527 phosphorylation may cause c-Src to adopt a closed conformation that inhibits its kinase activity (26, 49). Support for this model comes from studies showing that a phosphopeptide based on the region surrounding Y-527 binds the c-Src Y527F mutant but does not bind wild-type c-Src (as predicted if the SH2 domain is unoccupied in the Y527F mutant) (40). Additional evidence comes from studies showing that certain point mutations or deletions in the SH2 domain cause an increase in the kinase activity of c-Src (14, 44).

We have developed a system in which it is possible to analyze the regulation of c-Src by CSK in an intact cell. We find that c-Src causes a kinase-dependent growth inhibition

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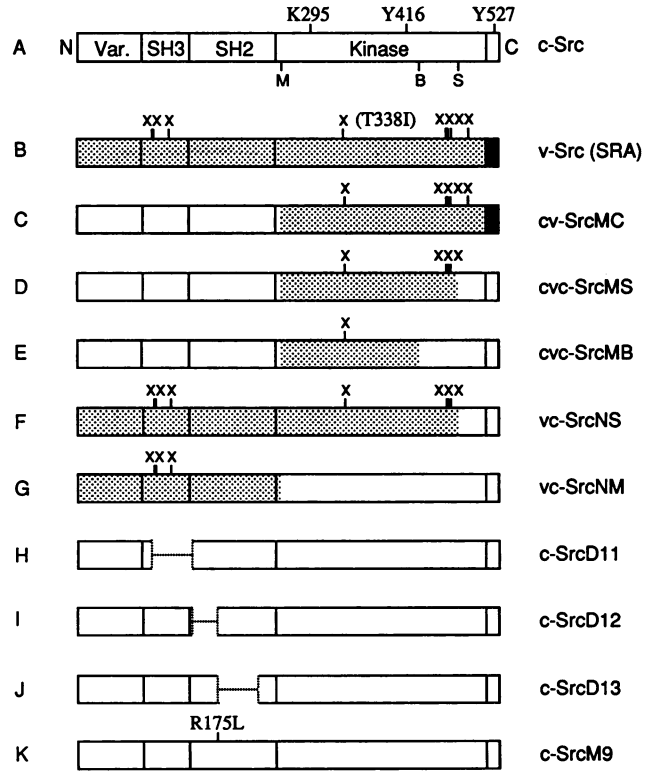

FIG. 1. Src proteins used in this work. (A) Diagram of c-Src, indicating the boundaries of the variable (Var.), SH3, SH2, and kinase domains. Also shown are the locations of the negative regulatory phosphorylation site (Y-527), the major autophosphorylation site (Y-416) and the conserved lysine in the ATP binding site (K-295). M, B, and S, *Mlu*I, *Bgl*I, and *Sph*I restriction sites, respectively, used to make v-*src*/c-*src* chimeras. (B) Diagram of v-Src, indicating the amino acid changes (marked by X) and the C-terminal sequence (dark shading) that differ from c-Src (see text). (C to G) v-Src/c-Src chimeras. Lightly shaded portions represent sequences derived from v-Src. The c-Src/v-Src chimeras are named according to the v-*src* restriction fragments they contain (e.g., cvc-SrcMS is a chimera in which a *Mlu*I-*Sph*I fragment of v-*src* is flanked by c-*src* sequences; see Materials and Methods). (H to K) c-Src proteins containing SH2 and SH3 mutations. Deletions are indicated by a single dotted line connecting the boundaries of the deletion (see Materials and Methods).

when expressed in the budding yeast *S. cerevisiae*. When CSK is coexpressed in these cells, c-Src is highly phosphorylated on Y-527 and the c-Src phenotype is suppressed. Complete inhibition of c-Src activity by CSK is not observed when c-Src is mutated in its SH2 or SH3 domains, suggesting that both of these domains are involved in the control of c-Src activity by Y-527 phosphorylation.

MATERIALS AND METHODS

Yeast procedures. Strains used in this work were made by transforming *S. cerevisiae* SM362A with various plasmids by electroporation. SM362A (W303, *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-Δ99 ura3-1*) was made by deleting *trp1-1* from RD360 (kindly provided by R. Deshaies).

Yeast cells were grown in standard minimal medium lacking the appropriate amino acids for plasmid selection (45). Noninduction medium contained either raffinose or dextrose as the carbon source, while induction medium contained galactose. Amino acid dropout mixes were purchased from BIO 101 (La Jolla, Calif.).

To assess the effects of various plasmids on cell growth, yeast cells were grown in noninducing (raffinose) liquid minimal medium at 30°C until the cultures reached saturation (approximately 2 days). Cultures were serially diluted (in minimal medium without amino acids or a carbon source), and equal amounts of saturated, 1/10-saturated, and 1/100-saturated cultures were spotted onto noninduction (dextrose) and induction (galactose and 1 mM Na_3VO_4) plates. Plates were incubated at 30°C until the highest dilution cultures were clearly visible in the control strain. The growth inhibition induced by the various Src proteins was slightly enhanced by the addition of Na_3VO_4 to the induction plates but was also observed in its absence (data not shown). The enhancement of the growth inhibition by Na_3VO_4 was dependent on the kinase activity of c-Src, since it had no apparent effect on the growth of strains carrying kinase-defective c-Src (c-SrcM) or vector control (data not shown).

Plasmids. The yeast expression vectors used in this work are YpRS314GAL1-10, YpRS424GAL1-10, and YpAB23 BXN. YpRS314GAL1-10 (kindly provided by P. Sorger) contains the galactose-inducible GAL1-10 promoter in the polylinker of YpRS314 (46), a low-copy-number plasmid containing a centromere sequence (CEN), autonomously replicating sequences (ARS), and the *TRP1*$^+$ gene. YpRS424 GAL1-10 was constructed by cloning the GAL1-10 promoter from YpRS314GAL1-10 into the polylinker of YpRS424 (46), a high-copy-number plasmid containing the 2 μm origin of replication and the *TRP1*$^+$ gene. YpAB23BXN (kindly provided by A. Brake) is a high-copy-number plasmid containing the 2 μm origin of replication, the *URA3* gene, and the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (42).

The human CSK cDNA (35) was placed under the control of the constitutive GAP promoter in YpAB23BXN. Chicken *src* alleles were placed under the control of the GAL1-10 promoter in YpRS314GAL1-10 (low copy) or YpRS424 GAL1-10 (high copy) as indicated in the text.

Src chimeras and mutants were constructed as follows (Fig. 1). cv-SrcMC, the sequence 3′ of the *Mlu*I site in c-*src* was replaced with that of v-*src* (SRA [9], kindly provided by M. McMahon); cvc-SrcMB, an internal *Mlu*I-*Bgl*I fragment of c-*src* was replaced with the equivalent fragment from v-*src*; cvc-SrcMS, an *Mlu*I-*Sph*I fragment of c-*src* was replaced with the equivalent fragment from v-*src*; vc-SrcNS, sequence 5′ of the *Sph*I site in c-*src* was replaced with that of v-*src*; vc-SrcNM, sequence 5′ of the *Mlu*I site in c-*src* was replaced with that of v-*src*. c-Src point mutants (c-SrcM [K295M], and c-SrcF [Y527F]) were constructed as previously described (27). SH2 and SH3 deletion mutants (c-SrcD11[Δ93-143], c-SrcD12[Δ144-175], c-SrcD13[Δ176-226], and c-SrcM9[R175L]) have been described [14]. Kinase-deficient versions of the c-Src SH2 and SH3 deletion mutants (c-SrcMD11, c-SrcMD12, and c-SrcMD13) were constructed by replacing the sequence 3′ of the *Mlu*I site in c-*srcD11*, c-*srcD12*, and c-*srcD13* with the corresponding sequence from c-*srcM*.

Lysate preparation and immunoblotting. *S. cerevisiae* strains were grown in noninducing (dextrose) liquid minimal medium until the cultures reached saturation. Cultures were diluted 1:50 into 10 ml of fresh noninducing (dextrose) medium and incubated (in a roller drum) at 30°C for 12 h (to mid-log phase). Cells were harvested by centrifugation, resuspended in 10 ml of induction medium (galactose plus 1 mM Na_3VO_4), and incubated (in a roller drum) for 8 h at

30°C. Cells were harvested and resuspended in 700 μl of cold (4°C) modified radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM EDTA, 1 mM dithiothreitol (DTT), 2 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4). Cells were lysed in the cold by adding approximately 1 ml of glass beads (0.5-mm diameter) and then pulsing twice (80 s each) in a minibeadbeater (Biospec Products, Bartlesville, Okla.). Lysates were clarified by centrifugation (10 min, 15,000 × *g*), and equal amounts of lysate were analyzed on an SDS–10% polyacrylamide gel. Proteins were electrophoretically transferred to 0.2-μm-pore-size nitrocellulose filters and blocked in 1% gelatin (anti-phosphotyrosine 4G10), 1% bovine serum albumin (anti-Src EC10 or 327) or 4% nonfat dry milk (anti-CSK). Blots were incubated with one of the following: a 1:1 dilution of culture medium containing the monoclonal anti-phosphotyrosine antibody 4G10 (kindly provided by D. Morrison), 0.5 μg of the monoclonal anti-Src antibody 327 (kindly provided by J. Brugge [23]) per ml, a 1:2,000 dilution of ascites fluid containing the monoclonal anti-Src antibody EC10 (kindly provided by S. Parsons [34]), or a 1:2,000 dilution of rabbit antiserum directed against the N terminus or C terminus of CSK (1). Following an additional incubation with alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibody (Promega, Madison, Wis.), immunoreactive proteins were visualized with alkaline phosphatase substrates (Sigma Chemical Co., St. Louis, Mo.).

Phosphorylation of Src proteins in vivo. Yeast strains were induced to express various c-Src proteins as described for immunoblotting. Cells were harvested by centrifugation and resuspended in 10 ml of YPE medium (45) from which phosphate had been removed (41). After 3 h at 30°C, cells were pelleted by centrifugation, 5 ml of medium was removed, and 1 mCi of $^{32}P_i$ per ml was added. Cells were resuspended, incubated for 2 h at 30°C, and lysed as described for immunoblotting. An equal amount of each lysate (2 mg) was incubated for 2 h at 4°C with monoclonal antibody EC10 immobilized on protein A-Sepharose. Beads were washed twice with RIPA buffer (150 mM NaCl, 50 mM HEPES [pH 8.0], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), boiled in SDS sample buffer, and analyzed on an SDS-polyacrylamide gel. The gel was lightly stained with Coomassie blue in water, and the Src bands were excised, eluted, precipitated with 25% trichloroacetic acid, and incubated overnight with 30 μl of 70-mg/ml cyanogen bromide in 70% formic acid (43). Cleavage products were analyzed on a 27.5% polyacrylamide gel as previously described (43), except that 0.1% SDS was added to the gel.

Baculovirus construction, expression, and purification of CSK. The human CSK cDNA was cloned into the baculovirus transfer vector pVL1392 (32). This vector was cotransfected with baculovirus genomic DNA into Sf9 insect cells, and recombinant baculoviruses were isolated and plaque purified as previously described (27, 48). CSK makes up approximately 10 to 20% of soluble protein in insect cells infected with the CSK virus (see Fig. 7A).

To prepare purified CSK protein, a 150-ml culture of Sf9 cells (3×10^8 cells) was infected with the CSK baculovirus and incubated for 2 days as previously described (27). Cells were harvested by centrifugation, resuspended in 7 ml of hypotonic lysis buffer (10 mM NaCl, 10 mM HEPES [pH 7.4], 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml), and lysed by Dounce homogenization. The lysate was clarified by centrifugation (100,000 × *g*, 1 h, 4°C), and the supernatant was diluted to 4 mg of protein per ml with HN25EG (10 mM HEPES [pH 7.4], 25 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% glycerol). The diluted lysate was injected onto a DEAE-Sepharose Fast Flow column (1 by 8 cm, 6 ml) preequilibrated with HN25EG. After washing with HN25EG, bound proteins were eluted with a linear 100-ml salt gradient (25 to 500 mM NaCl). Fractions containing CSK (approximately 150 mM NaCl) were pooled, diluted fivefold with HEG (same as HN25EG but lacking NaCl) and injected onto an ATP affinity column preequilibrated with HN25EG. This column was composed of ATP coupled through ribose hydroxyls to 4% agarose (Sigma; 1 by 2.5 cm, 2 ml). After being washed, bound proteins were eluted with a 40-ml linear salt gradient (25 to 500 mM NaCl). The fractions containing CSK (approximately 150 mM NaCl) were pooled and diluted fourfold in HEG and injected onto an S-Sepharose Fast Flow column (1 by 6.4 cm, 5 ml). After being washed, the bound proteins were eluted with a 50-ml linear salt gradient (25 to 500 mM NaCl). Fractions containing CSK were pooled, frozen in liquid nitrogen, and stored at −80°C.

Phosphorylation of Src proteins by purified CSK. To measure CSK activity toward Src, purified Src proteins were immunoprecipitated on Sepharose beads and washed to remove detergents that inhibit CSK activity. Purified c-SrcM (K295M) or c-SrcMF (K295M and Y527F) were prepared as previously described (27) and immunoprecipitated with monoclonal antibody EC10 immobilized on protein A-Sepharose in RIPA buffer. Beads were washed twice with HBS (150 mM NaCl, 50 mM HEPES [pH 7.4]) and once with kinase buffer (20 mM Tris [pH 7.4], 1 mM DTT, 10 mM $MnCl_2$, 10 μM ATP). Kinase reactions were performed by incubating purified CSK (20 ng) with immunoprecipitated c-SrcM (1.2 μg) or c-SrcMF (1.2 μg) plus 20 μl of kinase buffer containing 2.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham, Arlington Heights, Ill.) for 10 min at room temperature. We determined that under these reaction conditions, the incorporation of phosphate is linear with time (data not shown). Reaction products were analyzed on an SDS–10% polyacrylamide gel and then autoradiographed.

To measure phosphorylation of various mutant Src proteins by purified CSK, yeast strains carrying galactose-inducible *src* alleles (without CSK) were grown in galactose medium, and cell lysates were prepared as described for immunoblotting. An equal amount of each lysate (4 mg) was incubated for 1.5 h at 4°C with monoclonal antibody EC10 immobilized on protein A-Sepharose. Beads were washed as described above, divided, and incubated either in the presence or absence of 20 ng of purified CSK in 20 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP. After 10 min at room temperature, reaction products were separated on an SDS–10% polyacrylamide gel, and Src bands were analyzed by cyanogen bromide cleavage as described above.

RESULTS

c-Src induces a kinase-dependent growth inhibition in *S. cerevisiae*. Previous reports have shown that c-Src is poorly phosphorylated at Y-527 and exhibits elevated kinase activity when expressed in yeast cells (5, 21). It has also been reported that expression of Src proteins in *S. cerevisiae* inhibits cell growth (21). We hypothesized that Src-induced growth inhibition in *S. cerevisiae* is kinase dependent and might therefore provide a useful measure of c-Src kinase activity in vivo. To explore this possibility, we placed

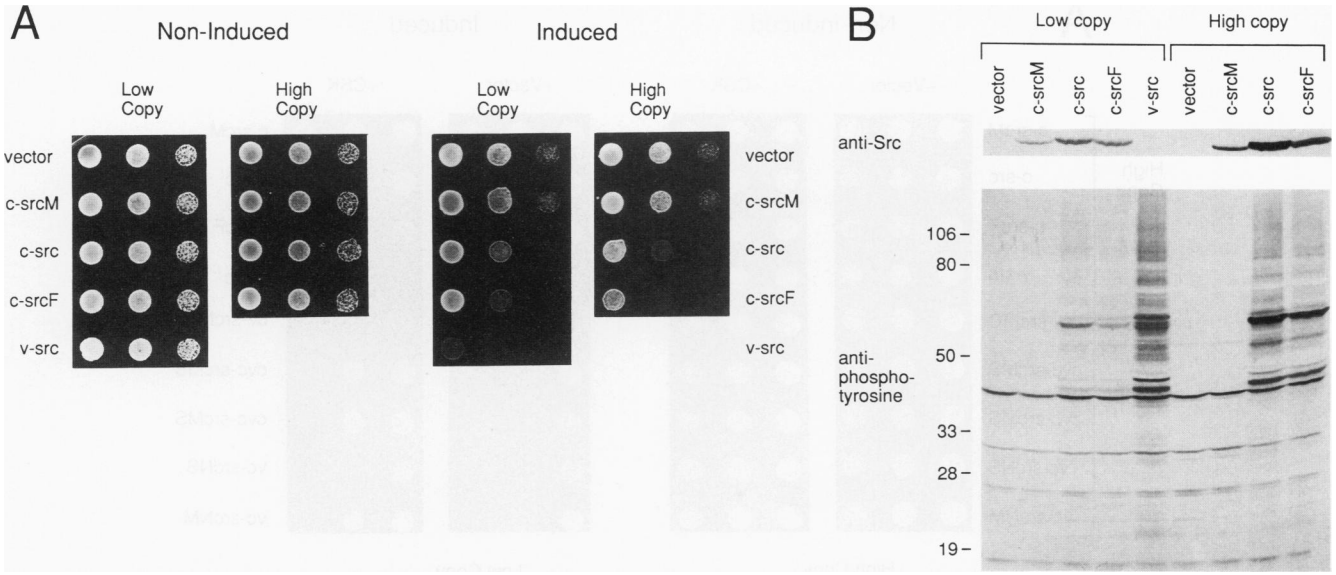

FIG. 2. Expression of c-Src in *S. cerevisiae* causes a kinase-dependent inhibition of growth. (A) *S. cerevisiae* was transformed with low- or high-copy-number vectors containing *src* alleles under the control of the GAL1-10 promoter. The resulting strains were grown to saturation in noninducing liquid culture under selection for the plasmid and then serially diluted (10-fold dilutions beginning with saturated on the left). Equal amounts of each culture were spotted onto noninduction (glucose) and induction (galactose) plates and grown at 30°C. The *src* allele carried by each strain is indicated. (B) The same strains were grown in noninducing liquid culture to mid-log phase and then switched to inducing medium and grown for 8 h. Cells were harvested, and cell lysates were subjected to immunoblotting with anti-c-Src monoclonal antibody 327 or anti-phosphotyrosine monoclonal antibody 4G10. c-SrcF contains a Y527F mutation; c-SrcM contains a K295M mutation. The apparent molecular masses (in kilodaltons) of prestained marker proteins (Bio-Rad) are indicated.

various c-*src* alleles under the control of the galactose-inducible GAL1-10 promoter in low (CEN/ARS)- and high (2 μm)-copy-number yeast vectors.

The expression of c-Src in *S. cerevisiae* caused a growth inhibition phenotype whose severity was proportional to the level of c-Src kinase activity (measured by antiphosphotyrosine immunoblots) (Fig. 2). Expression of c-Src from a low-copy-number plasmid caused a modest growth inhibition and increase in phosphotyrosine levels (Fig. 2). Phosphotyrosine levels and the strength of the growth inhibition were both increased when c-Src was expressed from a high-copy-number plasmid (Fig. 2 and 3). Integration of one, two, or three copies of c-*src* into the yeast genome (under GAL control) also induced growth inhibition phenotypes whose severity was proportional to the level of c-Src protein and kinase activity (data not shown). The growth inhibition induced by c-Src is dependent on its kinase activity, since the expression of a kinase-deficient mutant (c-SrcM, K295M) did not inhibit growth or stimulate tyrosine phosphorylation (Fig. 2).

Mutation of the C-terminal tyrosine (Y527F, c-SrcF) only slightly enhanced the c-Src phenotype, consistent with previous observations that Y-527 is poorly phosphorylated in yeast *S. cerevisiae* (5, 21). We also tested the effect of the viral v-Src protein, whose kinase activity is higher than that of c-SrcF (14). v-Src caused a very strong growth inhibition and increase in total phosphotyrosine levels (Fig. 2).

Multiple mutations contribute to the strong phenotype of v-Src. The amino acid sequence of v-Src (SRA; Schmidt-Ruppin subgroup A strain) is identical to that of c-Src except for eight single-amino-acid changes (R95W, T96I, D117N, T338I, V467G, R469G, Q474R, and K501R) and a replacement of the last 19 amino acids with 12 amino acids of nonrelated sequence (9) (Fig. 1). To identify the mutations that are responsible for the strong phenotype of v-Src, v-*src*/c-*src* chimeras were constructed and cloned into low-copy-number vectors under the control of the GAL1-10 promoter (Fig. 1). Kinase domain mutations, SH3 mutations, and the C-terminal deletion of v-Src each enhanced the effect of c-Src on cell growth. When expressed from a low-copy-number vector, c-Src mutants containing the kinase domain mutation T338I (cvc-SrcMB) or the SH3 mutations (vc-SrcNM) induced a slightly stronger phenotype than wild-type c-Src expressed from a high-copy-number vector (Fig. 3A, +Vector). Expression of a c-Src mutant containing multiple kinase domain mutations (cvc-SrcMS) resulted in a slightly stronger phenotype than the single T338I mutation; this phenotype was not enhanced by the addition of the SH3 domain mutations (vc-SrcNS) (Fig. 3A, +Vector). Growth inhibition similar to that of v-Src was displayed by a c-Src mutant containing all of the v-Src mutations except those in the SH3 domain (cv-SrcMC) (Fig. 3A, +Vector). These subtle differences were consistently observed in multiple experiments with several different transformants and became more apparent after longer periods of growth (data not shown). In most cases, the intensity of the phosphotyrosine profile paralleled the strength of the phenotype, although in one case (vc-SrcNS) the level of phosphotyrosine was higher than that expected from the strength of the growth inhibition (Fig. 3B, +Vector).

CSK suppresses the c-Src phenotype. To test whether CSK can suppress the kinase-dependent phenotype of c-Src, we cloned the human CSK cDNA into a high-copy-number vector under the control of the strong constitutive GAP promoter. The expression of CSK alone had no detectable effect on growth (Fig. 3A, uninduced). Coexpression of CSK with c-Src completely suppressed the growth inhibition phenotype (Fig. 3A) and the kinase activity of c-Src (Fig.

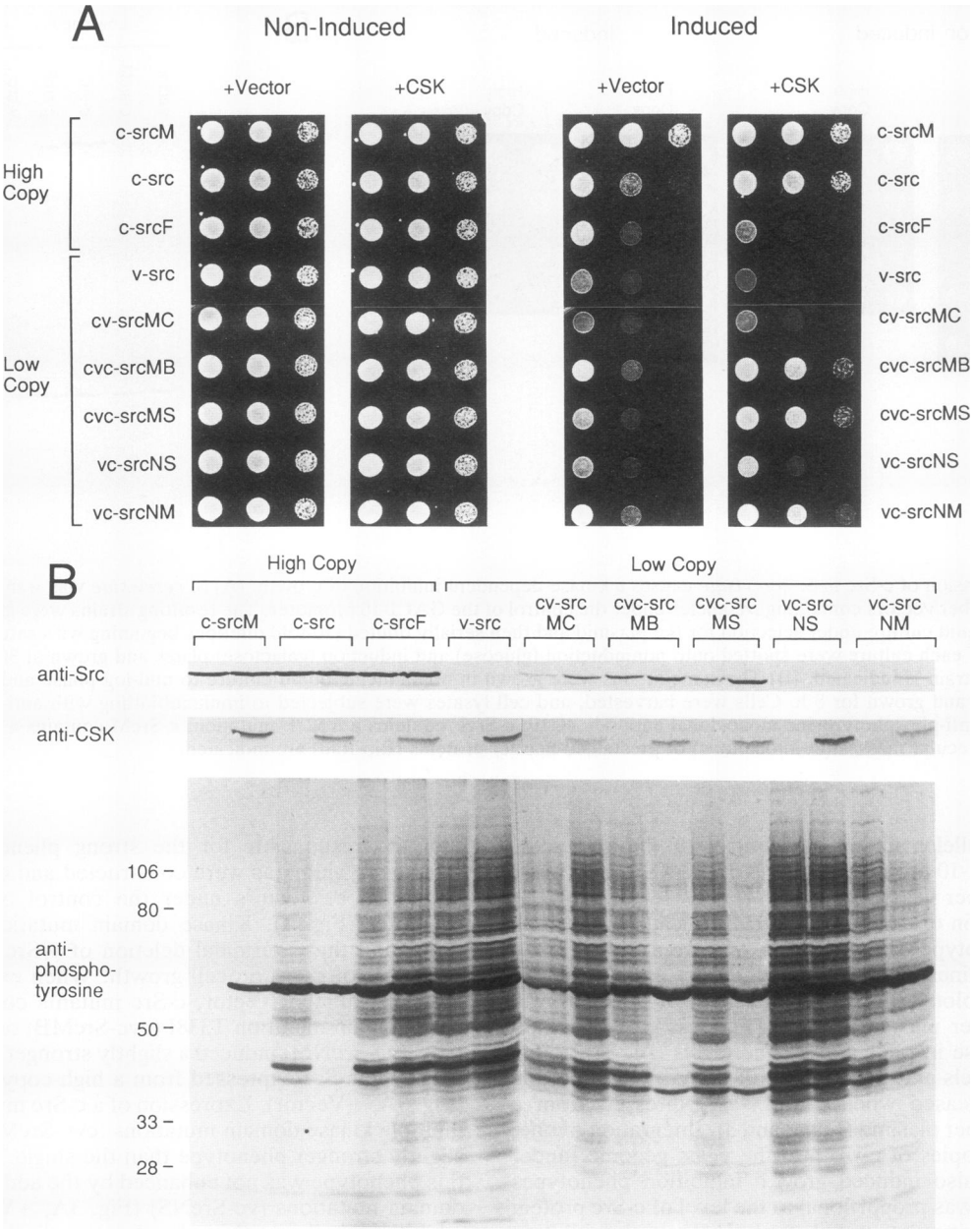

FIG. 3. CSK suppresses wild-type c-Src and certain c-Src/v-Src chimeras. (A) *S. cerevisiae* strains carrying galactose-inducible *src* alleles in low- or high-copy-number vectors were transformed with a high-copy-number vector containing no insert (+Vector) or the CSK cDNA (+CSK) under the control of the constitutive GAP promoter. The resulting strains were grown under selection for both plasmids and spotted onto noninduction and induction plates as described in the legend to Fig. 2. (B) The same strains were grown in liquid medium and induced as described in the legend to Fig. 2. Cell lysates were subjected to immunoblotting with anti-c-Src monoclonal antibody 327, anti-CSK polyclonal antibody, or anti-phosphotyrosine monoclonal antibody 4G10. The decreased level of Src protein and phosphotyrosine in the lysate of cells expressing cv-srcMC without CSK resulted from a loading error, since no difference was observed upon reevaluation of these lysates or lysates from other transformants. The apparent molecular masses (in kilodaltons) of prestained marker proteins (Bio-Rad) are indicated.

3B). CSK did not suppress the growth phenotype or the kinase activity of c-SrcF (Y527F), as expected if CSK were suppressing c-Src by phosphorylating Y-527 (Fig. 3). Indeed, the coexpression of CSK and c-SrcF caused a slightly stronger phenotype than c-SrcF alone, suggesting that CSK may induce some subtle growth inhibition (Fig. 3A).

A single major phosphotyrosine band (~60 kDa) was detectable in lysates of cells coexpressing c-Src and CSK (Fig. 3B). This band probably represents CSK-dependent phosphorylation of Y-527 in c-Src, since it is also present when CSK is coexpressed with a kinase-deficient mutant (c-SrcM, K295M) (Fig. 3B) but is not present when CSK is coexpressed with c-SrcMF (K295M and Y527F) (Fig. 4). The substrate specificity of CSK appears to be more limited than that of c-Src, since CSK detectably phosphorylates only the Y-527 residue of c-Src in the yeast cell (Fig. 4), whereas

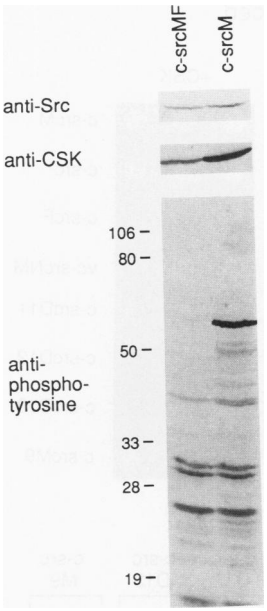

FIG. 4. CSK specifically phosphorylates Y-527 on c-Src. *S. cerevisiae* strains carrying galactose-inducible c-*srcM* (K295M) or c-*srcMF* (K295M and Y527F) on high-copy-number vectors were transformed with a high-copy-number vector containing the CSK cDNA under the control of the constitutive GAP promoter. The resulting strains were grown under selection for both plasmids in liquid medium and induced as described in the legend to Fig. 2. Cell lysates were analyzed by immunoblotting with anti-c-Src monoclonal antibody 327, anti-CSK polyclonal antibody or antiphosphotyrosine monoclonal antibody 4G10. The apparent molecular masses (in kilodaltons) of prestained marker proteins (Bio-Rad) are indicated.

c-Src induces the phosphorylation of many proteins (Fig. 3B).

CSK can regulate some but not all v-Src/c-Src chimeras. In order to gain insight into the mechanisms by which the various mutations of v-Src increase the kinase activity of c-Src, we coexpressed v-Src/c-Src chimeras with CSK or a vector control. As expected, the growth inhibition and phosphotyrosine profiles induced by v-Src and vc-SrcMC (which do not contain Y-527) are not suppressed by CSK (Fig. 3). CSK did suppress the growth inhibition and phosphotyrosine profiles induced by chimeras containing only the kinase domain point mutations (cvc-SrcMB and cvc-SrcMS), although not as well as it suppressed the phenotypes induced by c-Src (Fig. 3). The ability of CSK to almost completely suppress these chimeras suggests that these kinase domain mutations cause a general increase in kinase activity but do not significantly interfere with the basic mechanism by which CSK suppresses c-Src kinase activity.

CSK only moderately suppressed the growth inhibition and phosphotyrosine profile induced by a c-Src mutant containing SH3 mutations (vc-SrcNM), suggesting that the SH3 domain may be involved in the mechanism by which Y-527 phosphorylation suppresses kinase activity. This conclusion is further supported by the inability of CSK to suppress the growth inhibition and phosphotyrosine profile induced by the c-Src mutant containing the kinase domain mutations (which alone are largely suppressible) and the SH3 mutations (vc-SrcNS) (Fig. 3).

The SH2 and SH3 domains of c-Src are necessary for suppression by CSK. To further explore the role of the SH2 and SH3 domains of c-Src in the regulation of its kinase activity, we cloned several mutant alleles of c-*src* into high-copy-number vectors under the control of the GAL1-10 promoter and coexpressed them with either CSK or a vector control. CSK did not suppress the phosphotyrosine or growth inhibition phenotypes induced by c-Src proteins lacking either the N- or C-terminal halves of the SH2 domain (c-SrcD12 or c-SrcD13) (Fig. 1 and 5). Similarly, CSK only partially suppressed the kinase activity of a c-Src protein containing a point mutation in a highly conserved region of the SH2 domain (c-SrcM9, R175L) (Fig. 5B), although it did suppress the weak growth inhibition phenotype of this mutant. CSK only partially suppressed the growth inhibition and did not effectively suppress tyrosine phosphorylation induced by c-Src proteins containing either point mutations (vc-SrcNM) or a deletion (c-SrcD11) of the SH3 domain (Fig. 5). These results suggest that the SH2 and SH3 domains of c-Src are both involved in the suppression of its kinase activity by CSK.

It is interesting to note that c-Src proteins lacking portions of the SH2 domain (c-SrcD12 and c-SrcD13) or most of the SH3 domain (c-SrcD11) induce levels of phosphotyrosine that are much higher than those expected from their effect on growth (Fig. 5). In addition, the growth inhibition induced by a point mutation in the SH2 domain (c-SrcM9) is almost completely suppressed by coexpression of CSK (Fig. 5A), although the level of phosphotyrosine is only partially suppressed (Fig. 5B). This may indicate that these domains are involved in interactions with other proteins as well as the regulation of kinase activity.

Phosphorylation of SH2 and SH3 mutants by CSK in vivo. Our results indicate that complete inhibition of c-Src activity by CSK requires intact SH2 and SH3 domains. It is not clear if this requirement is due to an inability of CSK to phosphorylate these proteins or an inability of these proteins to respond properly to Y-527 phosphorylation. To address this issue, we analyzed the ability of CSK to phosphorylate various c-Src mutants.

Yeast cells coexpressing CSK and various forms of c-Src were labeled with $^{32}P_i$, and c-Src proteins were isolated from cell lysates by immunoprecipitation. To determine the relative phosphorylation on Y-416 (autophosphorylation) and Y-527, phosphorylated Src proteins were cleaved with cyanogen bromide and analyzed on a high-percentage polyacrylamide gel. This analysis separates a 4-kDa peptide containing Y-527 from a 10-kDa peptide containing Y-416 (43). In the absence of CSK, Y-416 was the major site of phosphorylation in wild-type and mutant c-Src proteins (Fig. 6A). Coexpression of CSK with wild-type c-Src increased Y-527 phosphorylation and reduced Y-416 phosphorylation (Fig. 6A). A similar increase in Y-527 phosphorylation was seen when CSK was coexpressed with c-Src lacking the SH3 domain (c-SrcD11) or c-Src lacking the N-terminal portion of the SH2 domain (c-SrcD12) (Fig. 6A). Coexpression of CSK with these mutants did not decrease the level of Y-416 phosphorylation, as expected if c-Src kinase activity is not suppressed by Y-527 phosphorylation in these mutants (Fig. 6A).

We also examined the level of CSK-dependent Y-527 phosphorylation by antiphosphotyrosine immunoblotting. As described earlier (Fig. 4), immunoblotting with antiphosphotyrosine antibodies provides a useful measure of Y-527 phosphorylation in kinase-deficient c-Src proteins containing the K295M mutation. Double mutants that contained both the K295M mutation and a deletion in the SH3 domain

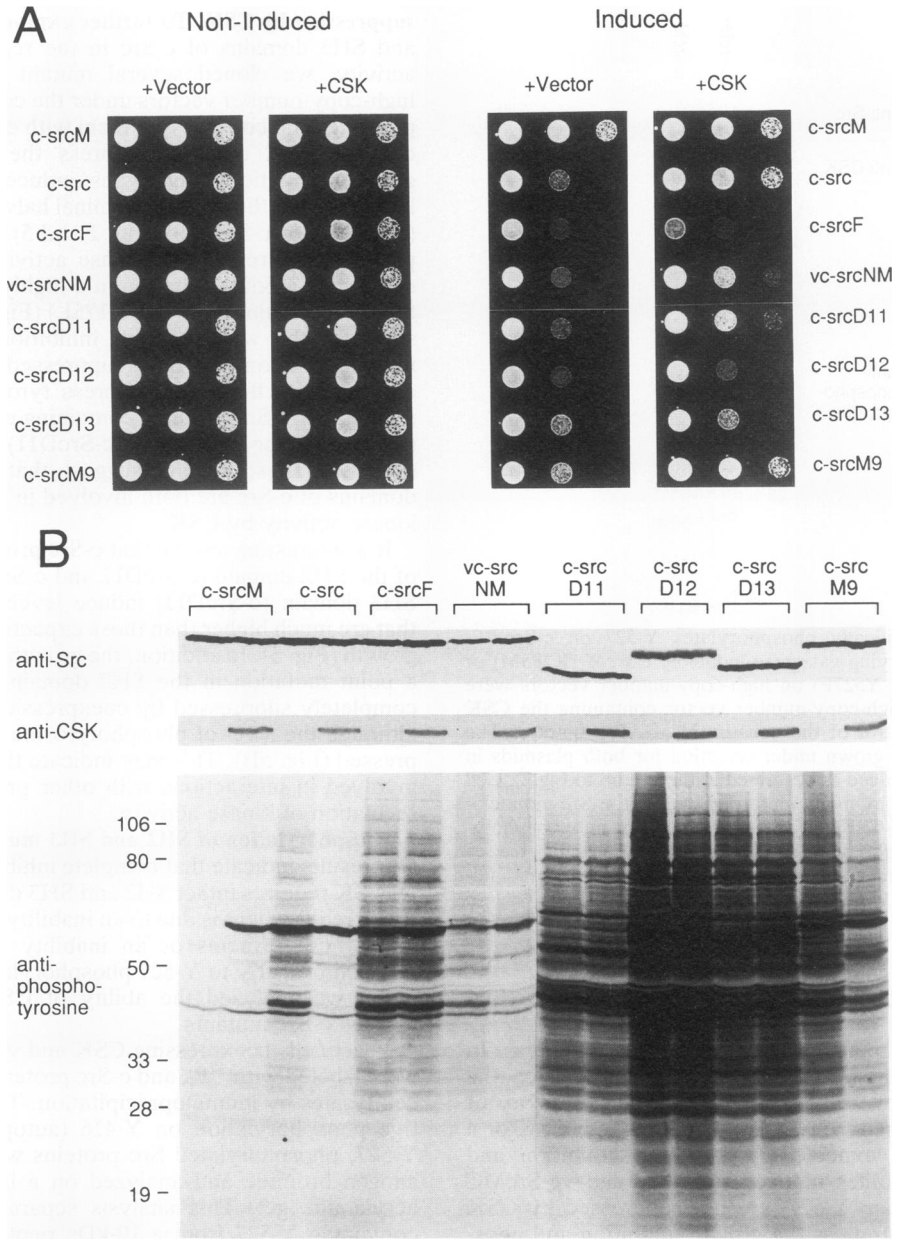

FIG. 5. The SH2 and SH3 domains of c-Src are necessary for suppression by CSK. (A) *S. cerevisiae* strains carrying galactose-inducible *src* alleles in high- or low-copy-number vectors were transformed with a high-copy-number vector containing no insert (+Vector) or the CSK cDNA (+CSK). The resulting strains were grown under selection for both plasmids and were spotted onto noninduction and induction plates as described in the legend to Fig. 2. (B) The same strains were grown in liquid medium and induced as described in the legend to Fig. 2. Cell lysates were subjected to immunoblotting with anti-c-Src monoclonal antibody EC10, anti-CSK polyclonal antibody, or antiphosphotyrosine monoclonal antibody 4G10. vc-*srcNM* is the only allele carried on a low-copy-number plasmid. The apparent molecular masses (in kilodaltons) of prestained marker proteins (Bio-Rad) are indicated.

(c-SrcMD11), the N-terminal portion of the SH2 domain (c-SrcMD12), or the C-terminal portion of the SH2 domain (c-SrcMD13) were constructed. Immunoblotting with antiphosphotyrosine antibodies revealed that the level of CSK-dependent Y-527 phosphorylation was unaffected by SH2 and SH3 deletions (Fig. 6B). These results, combined with the results of ^{32}P labeling in vivo, suggest that the SH2 and SH3 deletion mutants are effectively phosphorylated by CSK in vivo.

Phosphorylation of c-Src mutants by purified CSK. To further explore the ability of CSK to phosphorylate wild-type and mutant c-Src proteins, we analyzed the phosphorylation of c-Src proteins by purified CSK in vitro. Human CSK was overexpressed with the baculovirus system and purified by standard chromatographic techniques to approximately 95% homogeneity (see Materials and Methods) (Fig. 7A). The ability of purified CSK to specifically phosphorylate c-Src on Y-527 was tested by performing in vitro kinase reactions with purified c-SrcM (K295M) or purified c-SrcMF (K295M and Y527F). c-SrcM served as an excellent sub-

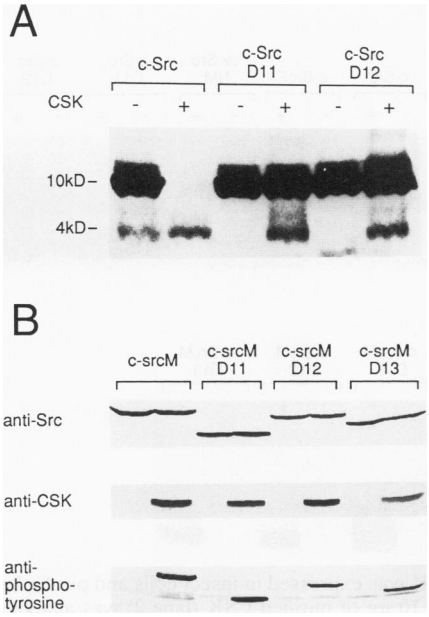

FIG. 6. c-Src SH2 and SH3 mutants are phosphorylated by CSK in vivo. (A) *S. cerevisiae* strains carrying the indicated galactose-inducible *src* alleles and CSK or a vector control were induced and grown in medium containing $^{32}P_i$ (see Materials and Methods). Cell lysates were subjected to immunoprecipitation with monoclonal antibody EC10 immobilized on protein A-Sepharose beads. Beads were washed, boiled in SDS sample buffer, and analyzed on a 10% polyacrylamide gel. Src proteins were excised and subjected to CNBr cleavage (see Materials and Methods). The resulting peptides were analyzed on a 27.5% polyacrylamide gel and then autoradiographed. The inferred positions of the 10-kDa peptide containing Y-416 and the 4-kDa peptide containing Y-527 are indicated. Note that phosphate turnover at Y-527 and Y-416 may be different, and therefore differences in labeling at these two sites may not be significant. (B) *S. cerevisiae* strains carrying the indicated galactose-inducible *src* alleles and CSK or a vector control were induced as described in the legend to Fig. 2. Cell lysates were subjected to immunoblotting with anti-c-Src monoclonal antibody EC10, anti-CSK polyclonal antibody, or antiphosphotyrosine monoclonal antibody 4G10. Only the 45- to 70-kDa regions of the immunoblots are shown. c-SrcMD11, c-SrcMD12, and c-SrcMD13 are kinase-deficient (K295M) versions of c-SrcD11, c-SrcD12, and c-SrcD13, respectively.

strate for CSK, whereas c-SrcMF was not significantly phosphorylated (Fig. 7B). Phosphoamino acid analysis indicated that CSK phosphorylates c-SrcM on tyrosine (data not shown), and analysis of cyanogen bromide cleavage products indicated that CSK-dependent phosphorylation occurs on the 4-kDa fragment that contains Y-527 (data not shown). Our attempts to perform detailed kinetic analyses of CSK activity were hindered by the poor solubility of purified c-SrcM and the sensitivity of CSK to the detergents used to maintain c-SrcM solubility. Thus, saturating concentrations of c-SrcM could not be achieved. However, even at moderate concentrations of c-SrcM, CSK exhibited a specific activity of 0.01 μmol of phosphate incorporated per minute per milligram. Although this rate probably represents an underestimate of CSK activity, it is well within the range of turnover rates reported for other protein-tyrosine kinases (18).

To test the ability of CSK to phosphorylate mutant forms of c-Src, various Src proteins were immunoprecipitated from yeast cell lysates and incubated with purified CSK and [γ-^{32}P]ATP, under conditions in which phosphate incorporation increased linearly with time. Phosphorylated c-Src proteins were subjected to cyanogen bromide cleavage for analysis of phosphorylation at Y-416 and Y-527. CSK-dependent phosphorylation of wild-type c-Src and the kinase-deficient mutant (c-SrcM) occurred mainly on Y-527 (Fig. 7C). CSK-dependent phosphorylation of Y-527 was also observed in c-Src proteins with point mutations in the SH3 domain (cv-SrcNM), a deletion of the SH3 domain (c-SrcD11), or deletions in the SH2 domain (c-SrcD12 and c-SrcD13) (Fig. 7C). Interestingly, some of these mutants appeared to exhibit enhanced autophosphorylation at Y-527. To study CSK-dependent phosphorylation in the absence of autophosphorylation, we analyzed SH2 and SH3 mutants that also contained a K295M mutation (Fig. 7D). These mutants were also phosphorylated by CSK in vitro (although SH2 mutants appeared to be less effective substrates under these conditions). Thus, our studies of CSK activity in vitro, like our previous analyses in vivo (Fig. 6), indicate that CSK is capable of effectively phosphorylating c-Src proteins lacking SH3 or SH2 domains.

DISCUSSION

The kinase activity of c-Src is normally repressed in vertebrate cells by extensive phosphorylation of Y-527. The regulation of this phosphorylation is critical for cells to maintain proper growth control and is probably important in controlling the normal function of c-Src (49). We have developed an *S. cerevisiae* system that allows us to study the regulation of Y-527 phosphorylation and the mechanism by which this phosphorylation inhibits the kinase activity of c-Src. We have found that expression of c-Src in *S. cerevisiae* induces a kinase-dependent growth inhibition that is completely suppressed by coexpression of CSK (Fig. 3). This result, combined with the ability of CSK to efficiently phosphorylate c-Src on Y-527 (Fig. 6 and 7), supports the notion that CSK is the enzyme that catalyzes Y-527 phosphorylation in vertebrate cells. It remains possible that additional Y-527 kinases exist (25), and we are addressing this possibility by screening human cDNA libraries for other kinases capable of suppressing the c-Src growth inhibition phenotype in *S. cerevisiae*.

It has been proposed that the phosphorylation of Y-527 inhibits c-Src activity by promoting an interaction between the C terminus and the SH2 domain (26, 49). Our results support this model. We find that c-Src mutants lacking portions of the SH2 domain can be phosphorylated by CSK on Y-527 (Fig. 6 and 7) but are not inhibited by this phosphorylation (Fig. 5). In addition, the activity of a c-Src protein containing a point mutation in a highly conserved region of the SH2 domain (c-SrcM9, R175L) is only partially suppressed by CSK in vivo (Fig. 3). The arginine residue affected by this mutation is an important component of the phosphotyrosine binding site, and mutation of this residue is predicted to reduce phosphotyrosine binding (51).

SH2 domains in c-Src and other proteins only bind with high affinity to phosphotyrosine residues in the appropriate sequence context, indicating that binding also involves interactions between the SH2 domain and sequences adjacent to the phosphotyrosine (36, 49). Our results suggest that even in the absence of Y-527 phosphorylation, the C terminus of c-Src may bind to the SH2 domain with low affinity and suppress kinase activity. We found that deletion of the N-terminal half of the SH2 domain (c-SrcD12) increases the

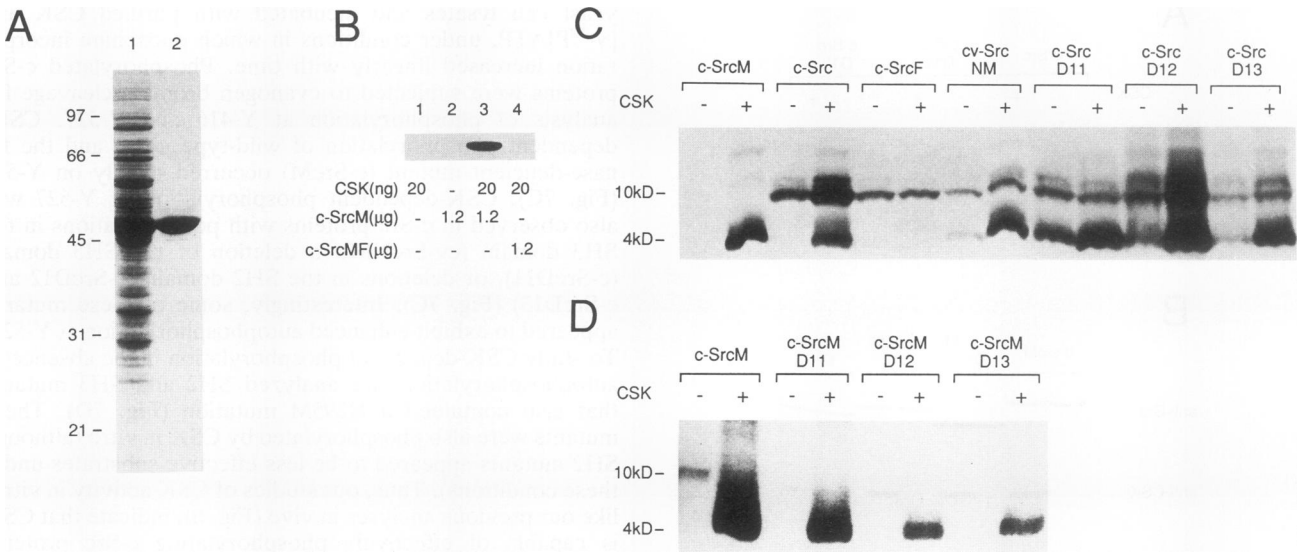

FIG. 7. Phosphorylation of c-Src SH2 and SH3 mutants by CSK in vitro. (A) CSK was expressed in insect cells and purified as described in Materials and Methods. A total of 50 μg of crude insect cell lysate (lane 1) and 10 μg of purified CSK (lane 2) was analyzed on a 10% polyacrylamide gel and stained with Coomassie blue. The molecular masses (in kilodaltons) of marker proteins (Bio-Rad) are indicated. (B) Kinase assays were performed with the indicated amounts of purified CSK and c-Src proteins in the presence of [γ-^{32}P]ATP (as described in Materials and Methods). Reaction products were analyzed on a 10% polyacrylamide gel and then autoradiographed. Only the portion of the autoradiograph containing the labeled c-Src protein is shown; no other bands were visible. (C and D) *S. cerevisiae* strains carrying galactose-inducible c-*src* alleles on high- or low-copy-number vectors were grown under selection in liquid medium as described in the legend to Fig. 2. Cell lysates were prepared, and c-Src proteins were immunoprecipitated from equal amounts of lysates with the monoclonal anti-Src antibody EC10 immobilized on protein A-Sepharose beads. The beads were divided in half and incubated with or without 20 ng of CSK for 10 min at room temperature in the presence of [γ-^{32}P]ATP (see Materials and Methods). Reaction products were analyzed on a 10% polyacrylamide gel, and phosphorylated Src proteins were excised and subjected to cyanogen bromide cleavage. The resulting peptides were analyzed on a 27.5% polyacrylamide gel and then autoradiographed. The inferred positions of the 10-kDa peptide containing Y-416 and the 4-kDa peptide containing Y-527 are indicated. The 10-kDa peptide in the first lane of panel D is overflow from an adjacent lane containing wild-type c-Src. vc-*srcNM* is the only allele carried on a low-copy-number plasmid.

kinase activity of c-Src to levels considerably higher than that of the Y-527F mutant (c-SrcF) (Fig. 5). These results are consistent with previous observations that deletion of the SH2 domain increases the kinase activity of the Y527F mutant in vertebrate cells (14).

Our results suggest that suppression of c-Src activity by CSK involves the SH3 domain as well as the SH2 domain. CSK only weakly suppressed the activity of a c-Src protein lacking the SH3 domain (c-SrcD11) (Fig. 5), even though this protein was extensively phosphorylated on Y-527 by CSK (Fig. 6 and 7). Similarly, CSK only partially suppressed the activity of a version of c-Src containing three point mutations in the SH3 domain (vc-SrcNM [Fig. 3 and 5]). A role for the SH3 domain in the suppression of c-Src activity is also supported by previous observations that certain SH3 mutations elevate the kinase activity of c-Src in vertebrate cells (17, 44). There are several mechanisms by which the SH3 domain could suppress c-Src kinase activity. For example, the SH3 domain may enhance binding of the SH2 domain to the C terminus or may interact directly with the kinase domain.

Increasing the levels of wild-type c-Src activity in yeast cells consistently induces parallel increases in the level of cellular phosphotyrosine and the severity of the growth inhibition phenotype (Fig. 2). In contrast, we found that these two phenotypes became partially dissociated in cells expressing certain c-Src SH2 and SH3 mutants. For example, mutants lacking the SH2 domain (c-SrcD12) induce levels of phosphotyrosine that are greater than those predicted from the strength of the growth inhibition (Fig. 5). In addition, the growth inhibition induced by a point mutant in the SH2 domain (c-SrcM9) is suppressed by CSK, while phosphotyrosine levels are only partially decreased (Fig. 5). A c-Src mutant lacking the SH3 domain (c-SrcD11) induces the same degree of growth inhibition as that induced by a c-Src protein carrying SH3 point mutations (vc-SrcNM), even though the deletion mutant is expressed at higher levels and induces a much greater increase in phosphotyrosine (Fig. 5). Finally, a c-Src protein containing kinase domain and SH3 mutations (vc-SrcNS) induced a much higher level of phosphotyrosine than a c-Src protein containing only the kinase domain mutations (cvc-SrcMS), even though these proteins induced similar growth inhibition (Fig. 3). These observations support previous suggestions that the SH2 and SH3 domains, in addition to regulating catalytic activity, are involved in association with other cellular proteins (3, 10, 14, 44, 53).

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