MOLECULAR AND CELLULAR BIOLOGY, Apr. 1992, p. 1835–1845
0270-7306/92/041835-11$02.00/0

Vol. 12, No. 4

Effects of SH2 and SH3 Deletions on the Functional Activities of Wild-Type and Transforming Variants of c-Src

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Received 21 October 1991/Accepted 23 December 1991

The amino-terminal, noncatalytic half of Src contains two domains, designated the Src homology 2 (SH2) and Src homology 3 (SH3) domains, that are highly conserved among members of the Src family of tyrosine kinases. The SH2 domain (which can be further divided into the B and C homology boxes) and the SH3 domain (also referred to as the A box) are also found in several proteins otherwise unrelated to protein tyrosine kinases. It is believed that these domains are important for directing specific protein-protein interactions necessary for the proper functioning of Src. To determine the importance of the SH2 and SH3 domains in regulating the functions of c-Src, we evaluated mutants of c-Src lacking the A box (residues 88 to 137), the B box (residues 148 to 187) or the C box (residues 220 to 231). Each of these deletions caused a 14- to 30-fold increase in the in vitro level of kinase activity of c-Src. Chicken embryo fibroblasts expressing the deletion mutants displayed a transformed cell morphology, formed colonies in soft agar, and contained elevated levels of cellular phosphotyrosine-containing proteins. Src substrates p36, p85, p120, p125, the GTPase-activating protein (GAP), and several GAP-associated proteins were phosphorylated on tyrosine in cells expressing the A, B, or C box deletion mutant. p110 was highly phosphorylated in cells expressing the C box mutant, was weakly phosphorylated in cells expressing the B box mutant, and was not phosphorylated in cells expressing the A box mutant. Expression of the mutant proteins caused a reorganization of the actin cytoskeleton similar to that seen in v-Src-transformed cells. In addition, deletion of the A, B, or C box did not diminish the transforming or enzymatic activity of an activated variant of c-Src, E378G. These data indicate that deletion of the A, B, or C homology box causes an activation of the catalytic and transforming potential of c-Src and that while these mutations caused subtle differences in substrate phosphorylation, the homology boxes are not required for many of the phenotypic changes associated with transformation by Src.

The transforming gene of Rous sarcoma virus, v-*src*, encodes a 60-kDa protein tyrosine kinase that is necessary and sufficient to initiate and maintain oncogenic transformation (7, 48). The normal cellular counterpart, c-*src*, is not oncogenic even when expressed at high levels (20–22, 47). The kinase activity of c-Src is regulated by the phosphorylation of Tyr-527, the major in vivo site of tyrosine phosphorylation in c-Src (7, 48). A number of single amino acid changes in c-Src can activate its tyrosine kinase activity and transforming potential (19, 27, 36, 46, 50); however, the mechanism by which tyrosine phosphorylation mediates the events leading to cellular transformation has not been clearly elucidated.

c-Src can be divided into several distinct functional domains (Fig. 1). The catalytic domain is contained within the carboxy-terminal half of the protein (5, 33). Mutations within the catalytic domain of c-Src can activate the oncogenic potential of the protein (27, 36). The regulatory site of tyrosine phosphorylation, Tyr-527, is located just outside the catalytic domain in the 11 carboxy-terminal amino acids of c-Src. Deletion of this region or mutation of Tyr-527 results in activation of kinase activity and transforming potential (6, 28, 49, 56). Sequences necessary for myristylation and membrane association are contained in the first 15 amino acids of Src (9, 26, 53, 54). The amino acid sequences immediately downstream (approximately residues 18 to 83) are not conserved within the Src family of tyrosine kinases and have been termed the unique domain. This domain may be important for directing the interaction of Src with either specific cellular effectors or membrane receptors. The association of the unique domain of the Src-like kinase c-Lck with the membrane receptors CD4 and CD8 provides evidence for this proposed function (62, 65).

Finally, the remaining portion of the amino-terminal half of Src contains three regions of amino acid homology found in members of the Src kinase family as well as in a growing number of proteins otherwise unrelated to protein tyrosine kinases. The Src homology 2 (SH2) domain (which can be further subdivided into the B and C homology boxes) has been identified in phospholipases C-γ1 and -2, the v-Crk oncoprotein, a Ras GTPase-activating protein (GAP), the p85 subunit of phosphatidylinositol 3′ kinase (PI3K), tensin, and the oncoproteins Nck and Vav (29). Src-related kinases and most of these proteins also contain a distinct motif termed the Src homology 3 (SH3) domain (the A homology box). Interestingly, the SH3 domain alone (without SH2) has also been identified in a variety of cellular proteins, most of which associate with the cellular membranes or the cytoskeleton, including two neutrophil NADPH oxidase-associated proteins, an actin-binding protein, myosin 1B, nonerythroid alpha spectrin, and the *Saccharomyces cerevisiae* proteins FUS1, CDC25, and IRA1 (29). The presence of these domains in such a wide variety of proteins suggests that they may be important for directing protein-protein interactions necessary for protein function or cellular localization (29, 57).

Several lines of evidence demonstrate that these homology domains may be important in modulating Src function. Mutational analyses of the SH3 domain of v-Src and c-Src

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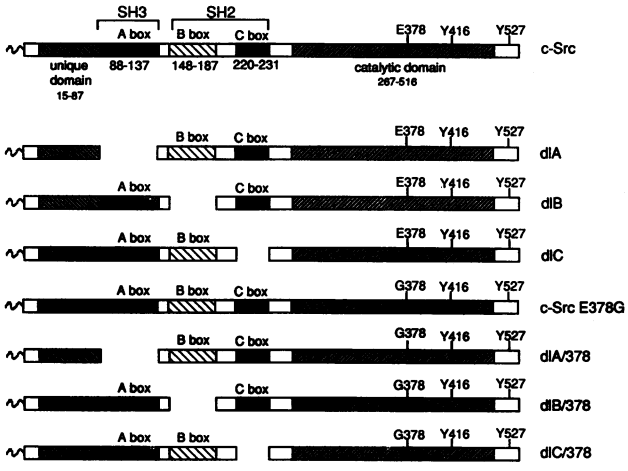

FIG. 1. Structures of c-Src, c-Src E378G, and the deletion mutants. The domain structure of Src is diagrammed, including the locations of the unique domain, the homology domains containing the A, B, and C boxes, and the catalytic domain. The positions of major sites of in vivo tyrosine phosphorylation, as well as the point mutation at residue 378, are also indicated. The actual amino acid sequences deleted from Src are identified underneath the appropriate homology domains.

suggest a role for this domain in regulating kinase activity and in inducing the morphological changes associated with transformation (8, 19, 23, 27, 44, 50). Interestingly, the neuronal form of c-Src contains either a 6- or 17-amino-acid insert within SH3 that may be important for the specific function of c-Src in neuronal cells (35, 37, 51). Mutations within the SH2 domain often render v-Src entirely, conditionally, or partially defective for transformation (8, 11, 18, 19, 52, 66, 67). Several mutations in this region confer a host range phenotype (11, 18, 66). These results suggest that the SH2 domain may direct the interaction of Src with cellular proteins necessary for maintenance of transformation. Recently, it has been shown that SH2 domains may mediate these interactions by binding to phosphotyrosine residues of target proteins (1, 38–40, 42). In addition, the SH2 domain of c-Src may specifically interact with phosphorylated Tyr-527 to repress the kinase activity of this molecule. Activation of c-Src by dephosphorylation of Tyr-527 would then release the SH2 domain to interact with the appropriate target proteins (29).

To determine the importance of the SH2 and SH3 domains in regulating the function of c-Src, we constructed viral vectors encoding mutants of c-Src and an activated variant of c-Src (E378G) which lack the A, B, or C box. Deletion of the A, B, or C box in c-Src activated the kinase activity of this protein. Chicken embryo fibroblasts (CEFs) overexpressing each of the deletion mutants exhibited a transformed phenotype. In addition, deletion of these homology boxes did not diminish the transforming or enzymatic activity of c-Src E378G. These data suggest that the A, B, and C boxes are not required for many of the phenotypic changes normally associated with transformation by Src. We also examined the in vivo phosphorylation of cellular proteins known to be phosphorylated on tyrosine in v-Src-transformed cells and found that although cells expressing the deletion mutations displayed high levels of tyrosine phosphorylation, there were several differences in the substrates phosphorylated by each set of mutants.

MATERIALS AND METHODS

Cells, plasmids, viruses, and antibodies. CEFs were prepared from 9- or 10-day-old gs^- embryos (SPAFAS, Inc., Norwich, Conn.). The Rous sarcoma virus expression vectors pRSVBH v-Src, pRSVBH c-Src, and pRSVBH c-Src E378G have been described previously (44). Tertiary CEFs were transfected with plasmid DNA by a modification of the DEAE-dextran method (44). Virus stocks were prepared and used to infect CEFs. All biochemical assays were performed when approximately 90% of the cells were infected.

A monoclonal antibody (MAb), LA074, that recognizes amino acids 2 to 17 of Src was purchased from Microbiological Associates (Bethesda, Md.; now available from Quality Biotech, Inc., Camden, N.J.). An antiphosphotyrosine MAb, 4G10, was provided by T. Roberts (Dana Farber Cancer Institute). MAbs specific for the Src substrates p85 (4F11), p110 (4C3), p120 (2B12), and p125 (2A7) were obtained from T. Parsons (University of Virginia) (24). Rabbit anti-GAP antibodies prepared against residues 521 to 988 of human GAP expressed in bacteria were supplied by S. Decker (Parke-Davis, Pharmaceutical Research Division) (4).

Mutant constructions. A *Cla*I fragment containing the complete coding region of wild-type c-Src was cloned into a pBluescript II phagemid (Stratagene Cloning Systems, La Jolla, Calif.) to prepare single-stranded DNA for mutagenesis. Deletion mutations were introduced into the c-Src gene by oligonucleotide-directed mutagenesis (31), and clones were screened by restriction enzyme analysis and DNA sequencing. After confirmation of the mutation, the *Cla*I fragment was introduced into pRSVBH and plasmid DNA was sequenced across the junction of the deletion to confirm that the mutagenesis was accurate and that the coding region remained in frame.

We also introduced each deletion mutation into the gene encoding an activated variant of c-Src, E378G (20, 36). A *Cla*I-*Mlu*I fragment containing the amino-terminal half of each of the deletion mutants was cloned into pRSVBH containing an *Mlu*I-*Cla*I fragment that in turn contained the carboxy-terminal half of c-Src E378G (including the activating mutation at 378).

Colony formation in soft agar. Anchorage-independent growth was assayed by seeding cells in Dulbecco's modified Eagle's medium containing 0.4% Bacto Agar, 11% calf serum, 1% chick serum, 10% tryptose phosphate broth, and 1% dimethyl sulfoxide as described previously (23).

In vitro kinase assay. Infected CEFs were rinsed with phosphate-buffered saline (PBS), and lysates were prepared in RIPA buffer (158 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100). Equal amounts of protein from each lysate were immunoprecipitated with LA074, and immune complexes were adsorbed to Formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). The immunoprecipitates were divided in half, and one-half was eluted from the immunoabsorbent and used in a protein immunoblot with ^{125}I-anti-mouse immunoglobulins (Amersham Corp., Arlington Heights, Ill.) to quantitate Src protein levels (44). The other half of the immunoprecipitates was incubated for 2 min at room temperature in kinase buffer with activated enolase as exogenous substrate (34). The amount of Src-specific radioactivity (from the immunoblot) and the amount of [^{32}P]enolase radioactivity were quantitated on a Molecular Dynamics PhosphorImager. The

specific activity of each Src mutant was determined relative to the activity for wild-type c-Src.

Antiphosphotyrosine immunoblotting. Phosphotyrosine-containing proteins in whole-cell lysates were detected by immunoblotting as described previously (44). Briefly, whole-cell lysates were normalized for protein concentration, separated by electrophoresis on SDS–7.5% polyacrylamide gels, and transferred to nitrocellulose. The filters were blocked with 5% bovine serum albumin (ICN Pharmaceuticals, Irvine, Calif.) and probed with antiphosphotyrosine antibody 4G10 and then with horseradish peroxidase-coupled secondary antibody, and immunoreactivity was detected by enhanced chemiluminescence reagents (Amersham Corp.).

To detect tyrosine phosphorylation of individual substrates of Src, infected cell lysates were prepared in either a Triton X-100-containing buffer (1% Triton X-100, 10 mM Tris, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride [pH 7.6], 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μM aprotinin) or a modified RIPA buffer (158 mM NaCl, 10 mM Tris [pH 7.2], 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μM leupeptin, 1% aprotinin). The protein concentrations of the lysates were normalized, and equal amounts of protein were immunoprecipitated with the designated antibody and either Pansorbin or protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.). The immunoprecipitates were then separated by electrophoresis, transferred to nitrocellulose, and probed with 4G10 as described above.

Cyanogen bromide cleavage of Src proteins. Infected cells were labeled with 1 mCi of carrier-free $^{32}P_i$ (ICN Pharmaceuticals) per ml for 3 h in phosphate-free Dulbecco's modified Eagle's medium. The cells were washed in PBS, lysates were prepared in RIPA buffer, and immunoprecipitated proteins were separated by electrophoresis on SDS–10% polyacrylamide gels. Src bands were excised from the dried gel, and proteins were eluted from the gel pieces, cleaved with CNBr, and subjected to electrophoresis on a Tris borate–27.5% polyacrylamide gel (61).

Immunofluorescence microscopy. Cells grown on glass coverslips were fixed for 20 min at room temperature with 2% paraformaldehyde, rinsed with 0.15 M glycine in PBS to block reactive groups, permeabilized for 10 min with 1% Nonidet P-40 in PBS, and blocked for 1 h in PBS with 5% goat serum (Cappel, Malvern, Pa.) to block nonspecific binding. After 1 h of incubation with LA074, the cells were rinsed in PBS and incubated with biotin-conjugated goat anti-mouse immunoglobulin G followed by fluorescein isothiocyanate-conjugated avidin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). For actin staining, the same coverslips were also stained with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, Oreg.). The cells were rinsed in PBS and mounted in Elvanol.

RESULTS

Construction of c-Src deletion mutants. To examine the functional significance of the SH2 and SH3 domains of c-Src, mutations which resulted in deletion of the A box (SH3; amino acids 88 to 137), the B box (SH2; amino acids 148 to 187), or the C box (SH2; amino acids 220 to 231) were introduced into the c-Src gene (Fig. 1). The B and C boxes represent two subdomains within the SH2 domain which are conserved within phospholipase C-γ and other SH2 domain-containing proteins (63). The B and C boxes were analyzed separately so that we could evaluate the function of each subdomain of conserved sequences within the SH2 domain. Since c-Src has little intrinsic transforming activity, we also examined the effect of each deletion on an activated, transforming variant of c-Src, E378G (20, 36). The c-Src variants were cloned into an avian retroviral vector, and virus stocks were used for infection of CEFs.

Morphology of infected cells and anchorage-independent growth. CEFs expressing the A, B, or C deletion mutations in wild-type c-Src displayed altered morphologies compared with uninfected CEFs or CEFs overexpressing the wild-type c-Src protein (Fig. 2). Overexpression of wild-type c-Src caused only subtle changes in cell morphology (Fig. 2B). In contrast, cells expressing c-Src dlA (Fig. 2D) or c-Src dlC (Fig. 2F) exhibited a fusiform, highly refractile morphology, with cell rounding at areas of higher cell density. Cells expressing c-Src dlB (Fig. 2E) also displayed a fusiform, refractile morphology yet tended to align in parallel clusters with less cell rounding than that seen with the dlA or dlC mutants. The morphology of cells expressing dlA, dlB, and dlC was distinct from that induced in cells expressing v-Src (Fig. 2C) or c-Src E378G (Fig. 2G), since the latter cells were more refractile and rounded and were more loosely attached to the dish. Deletion of the A, B, or C box in the E378G background did not diminish the ability of this activated c-Src variant to induce a transformed morphology (Fig. 2H through J).

For each mutant, the induction of colony formation in soft agar was assayed as a measure of anchorage-independent growth. Although uninfected CEFs did not form colonies in soft agar, cells expressing high levels of c-Src were able to form detectable colonies (Fig. 3). The efficiency of c-Src-induced colony formation was approximately threefold lower than that of c-Src E378G-expressing cells, and the colonies induced by c-Src were smaller than those induced by the activated c-Src variant. Cells expressing dlA formed colonies in soft agar as efficiently as c-Src E378G, whereas cells expressing dlB, dlC, dlB/378, or dlC/378 formed colonies more efficiently than c-Src E378G-expressing cells, and these colonies were generally larger than those induced by expression of c-Src E378G. The numbers of colonies formed by cells expressing dlA/378 were consistently intermediate between those of c-Src and c-Src E378G, suggesting that an A box deletion may reduce the colony-forming ability of c-Src E378G. This difference in colony formation does not reflect a difference in viral titer as assayed by focus formation or production of *gag* gene products 16 h after infection. In addition, these results were still observed when the cells were placed in soft agar 4 days after infection (to allow virus spread through the culture). Taken together, these results suggest that the A, B, and C boxes are not required for colony formation by Src, although in the E378G background, removal of the A box may possibly decrease the ability of that activated variant to form colonies in soft agar.

In vitro kinase activity. To determine whether deletion of the A, B, or C box had an effect on the protein tyrosine kinase activity of c-Src or c-Src E378G, we examined the specific activity of each of the mutant proteins in an in vitro immunocomplex kinase assay with enolase as exogenous substrate. The phosphorylation of enolase was quantitated relative to Src protein levels, and the values from three separate assays were used to calculate relative levels of kinase activity. A representative assay is shown in Fig. 4.

The steady-state levels of each of the mutant Src proteins were similar in infected CEFs (Fig. 4A), with each mutant protein expressed at levels 40 to 70% of that of the steady-

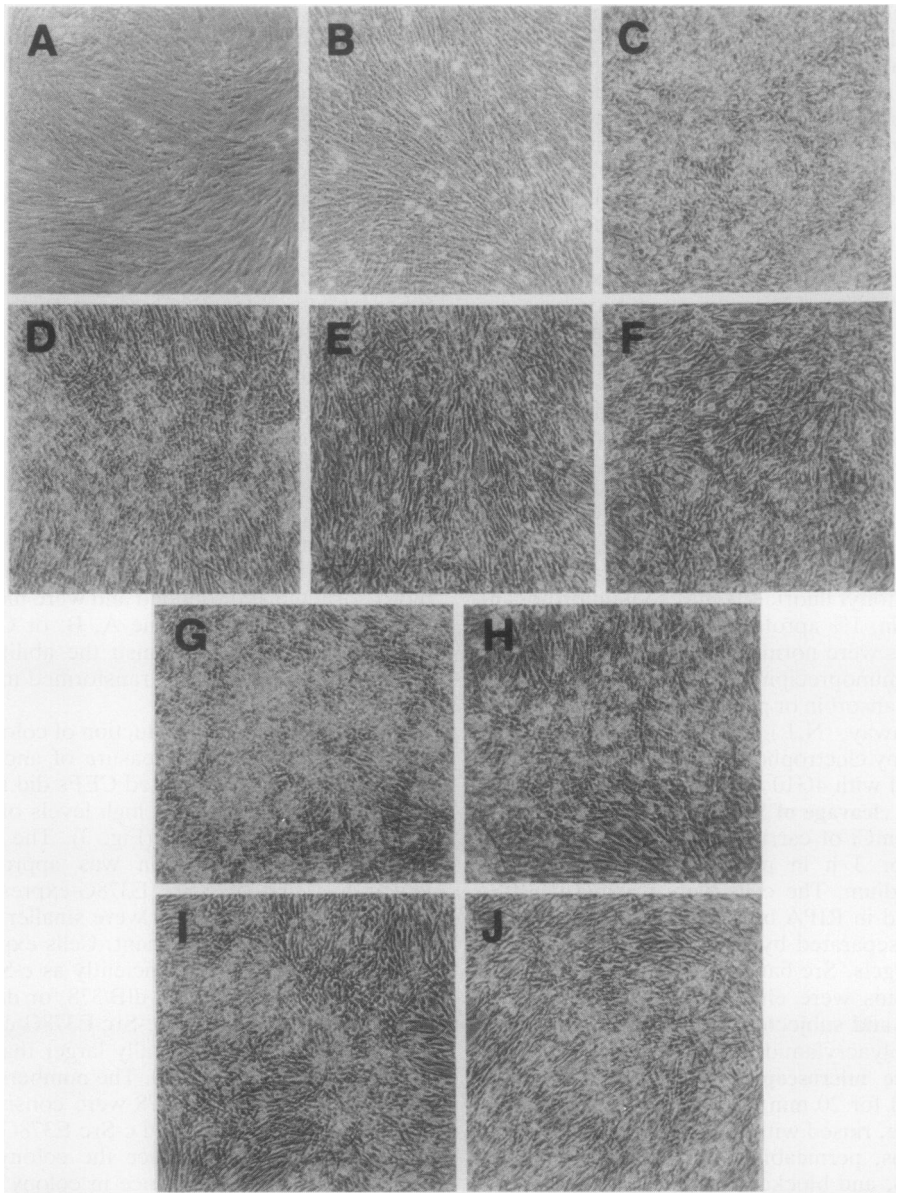

FIG. 2. Photographs of infected cells in monolayers. Shown are uninfected CEFs (A) and CEFs infected with c-Src (B), v-Src (C), dlA (D), dlB (E), dlC (F), c-Src E378G (G), dlA/378 (H), dlB/378 (I), or dlC/378 (J). The photographs were taken 4 days postinfection.

state levels of wild-type c-Src. The slight variations in expression levels of the mutants indicated in Fig. 4A were not consistently observed in replicate experiments. Although DNA sequencing confirmed that the deletions were accurate and that the coding regions remained in frame, the mobilities of dlB and dlB/378 were slower than was predicted from the size of the deletion and may reflect differences in the folding or posttranslational processing of the protein.

In in vitro kinase assays, as shown in Fig. 4B, the specific activities of the mutant proteins averaged 14- to 30-fold higher than that of wild-type c-Src. Although there were slight differences in the specific activity of the mutant Src proteins in individual assays, none of the mutant proteins consistently displayed a higher kinase activity than the others. The same deletions in the c-Src E378G background did not diminish enolase phosphorylation by this activated mutant. In the presence of enolase, the in vitro autophosphorylation of dlA/378 was reproducibly approximately half that of wild-type c-Src, whereas the autophosphorylating activity of the other activated mutants was two- to threefold higher than that of wild-type c-Src. The significance of this variation is not understood.

Mapping of in vivo phosphorylation sites. The tyrosine kinase activity of c-Src is negatively regulated by phosphorylation at tyrosine residue 527 (7, 48). Wild-type c-Src is phosphorylated on tyrosine 527 in vivo, whereas v-Src and activated variants of c-Src are phosphorylated on tyrosine 416 (7, 48). To examine how deletion of the A, B, or C homology box affects in vivo sites of phosphorylation, peptide maps were prepared by cyanogen bromide cleavage of each Src variant to map the phosphorylation sites. Cleavage of wild-type c-Src with cyanogen bromide yields a

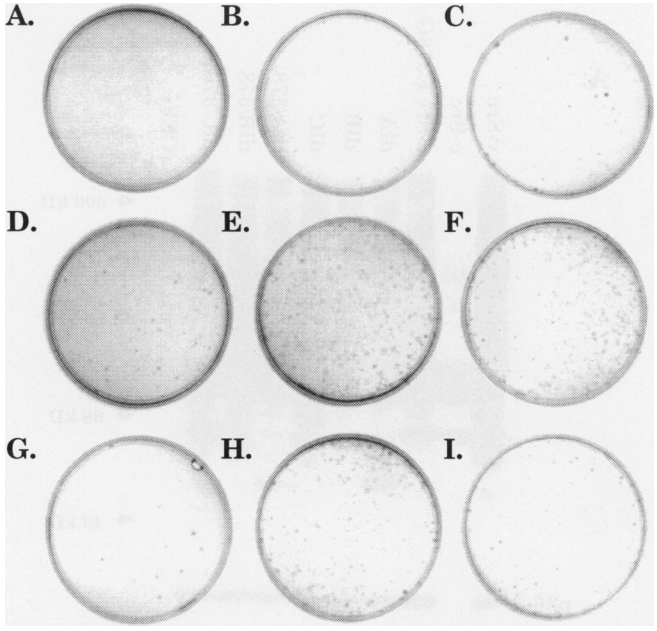

FIG. 3. Colonies formed in soft agar. The photographs were taken 3 weeks after seeding infected cells in agar. (A) CEFs; (B) c-Src; (C) c-Src E378G; (D) dlA; (E) dlB; (F) dlC; (G) dlA/378; (H) dlB/378; (I) dlC/378. This experiment is representative of five independent assays. Strict quantitation of the results was not included, since the absolute number of colonies varied in each experiment and since the size distribution of the colonies precluded precise quantitation.

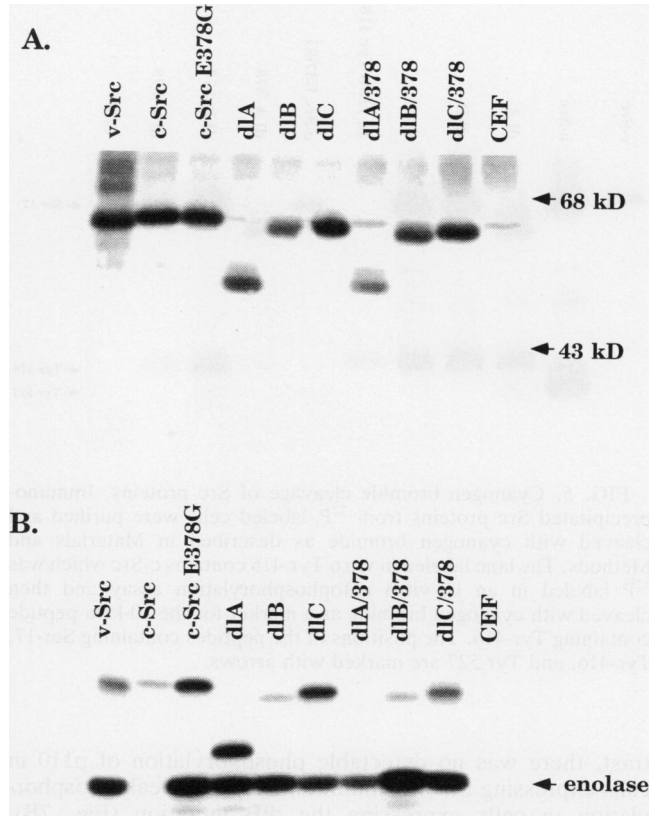

FIG. 4. Determination of the specific activity of the mutant Src proteins. Src was immunoprecipitated from equal amounts of total-cell protein with anti-Src MAb LA074. (A) One-half of the immunoprecipitate was used to quantitate the levels of Src protein by immunoblotting with MAb LA074; (B) the other half of the immunoprecipitate was used in an in vitro kinase assay with enolase as an exogenous substrate. The amount of Src-specific radioactivity (from the immunoblot) and the amount of [^{32}P]enolase radioactivity were quantitated, and the specific activity of each Src mutant relative to that of wild-type c-Src was determined.

32-kDa peptide containing Ser-17, a 10-kDa peptide containing Tyr-416, and a 4-kDa peptide containing Tyr-527. As shown in Fig. 5, c-Src was phosphorylated in vivo on the 4-kDa peptide containing Tyr-527. v-Src was phosphorylated exclusively on a 10-kDa peptide containing Tyr-416. The activated variant c-Src E378G was phosphorylated at Tyr-416 as well as at Tyr-527, although some of the Tyr-527 peptide may be derived from endogenous c-Src which could not be separated from c-Src E378G for mapping. All of the deletion mutants were phosphorylated on Tyr-416, consistent with their activated phenotype, although c-Src dlA did contain a small amount of phosphorylation on Tyr-527.

Tyrosine phosphorylation of cellular proteins. Since the SH2 and SH3 domains may be involved in critical protein-protein interactions necessary for Src function, we determined whether deletion of the A, B, or C box altered tyrosine phosphorylation of cellular proteins detected in Src-transformed cells.

To examine the profile of total cellular phosphotyrosine-containing proteins, whole-cell lysates were probed with a MAb against phosphotyrosine (Fig. 6A). As previously reported (44), overexpression of wild-type c-Src caused an increase in tyrosine phosphorylation of several proteins, including c-Src itself and proteins of 80 to 85 kDa and 120 to 130 kDa. Expression of each of the deletion mutations resulted in an increase in proteins phosphorylated on tyrosine; the pattern of phosphorylated proteins was similar to that seen in cells expressing either v-Src or the transforming variant c-Src E378G. In addition, the deletions in c-Src E378G did not significantly alter this profile of phosphotyrosine-containing proteins.

Although the pattern of phosphorylated proteins was similar for each of the mutants, there were some variations in the profile (Fig. 6B). For example, there were differences in the extent of phosphorylation of multiple protein bands that migrate within the 80- to 85-kDa and 120- to 130-kDa regions of the gel in cells expressing the A, B, or C box mutation. The degree of induction of tyrosine phosphorylation of p36, the calpactin I heavy chain, was elevated by deletion of the A, B, or C box (Fig. 6A and unpublished results from immunoprecipitates of p36 from ^{32}P-labeled cells).

To further investigate substrate phosphorylation by the mutant Src proteins, we used antibodies to immunoprecipitate specific Src substrates and probed the immunoprecipitates with antibodies against phosphotyrosine. Proteins of 85, 110, 120, and 125 kDa were previously identified as substrates for tyrosine phosphorylation in Src-transformed cells by using a panel of MAbs prepared against phosphorylated proteins in v-Src-infected cells (24). As shown in Fig. 7, tyrosine phosphorylation of p85 (panel A) and p120 (panel C) was not altered in cells transformed with dlA/378, dlB/378, or dlC/378, indicating that the homology boxes are not necessary for phosphorylation of these substrates. In con-

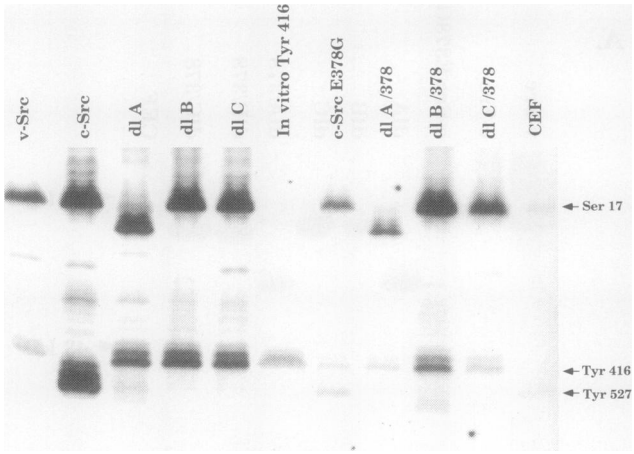

FIG. 5. Cyanogen bromide cleavage of Src proteins. Immunoprecipitated Src proteins from $^{32}P_i$-labeled cells were purified and cleaved with cyanogen bromide as described in Materials and Methods. The lane labeled in vitro Tyr-416 contains c-Src which was ^{32}P labeled in an in vitro autophosphorylation assay and then cleaved with cyanogen bromide as a marker for the 10-kDa peptide containing Tyr-416. The positions of the peptides containing Ser-17, Tyr-416, and Tyr-527 are marked with arrows.

trast, there was no detectable phosphorylation of p110 in cells expressing the dlA mutation and only weak phosphorylation in cells expressing the dlB mutation (Fig. 7B), supporting previous studies indicating that two domains are critical for tyrosine phosphorylation of p110 by Src (25). Deletion of the C box had no effect on tyrosine phosphorylation of p110. Finally, although p125 was constitutively phosphorylated on tyrosine in normal CEFs, there was a significant increase in the level of tyrosine phosphorylation in c-Src E378G-transformed cells, and p125 exhibited a characteristic shift in mobility in transformed cells (Fig. 7D). Expression of each of the deletion mutations induced an increase in the level of tyrosine phosphorylation of p125 compared with the constitutive phosphorylation seen with CEFs, but the extensive mobility shift observed with c-Src E378G-transformed cells was seen only with cells overexpressing the dlA mutation.

Transformation of CEFs by Src results in tyrosine phosphorylation of the p21^{ras} GAP as well as two GAP-associated proteins, p62-68 and p190 (2, 13, 43). The functional significance of this phosphorylation is unclear, but it may be important in altering the function of GAP and linking the Src and Ras signal transduction pathways. The results shown in Fig. 8 demonstrate that GAP is phosphorylated on tyrosine in cells expressing v-Src, c-Src E378G, and the three deletion mutants. No detectable phosphorylation of GAP was observed with CEFs infected with the c-Src vector. The GAP-associated protein p190 was constitutively phosphorylated on tyrosine in CEFs; however, this level of phosphorylation was increased in cells expressing c-Src, v-Src, and each of the activating variants of c-Src. Additional GAP-associated proteins of 65 to 70 kDa and 55 kDa were also detected in CEFs transformed with v-Src. The proteins of 65 to 70 kDa, which may be related to the 62- to 68-kDa GAP-associated proteins previously identified in transformed mammalian cells (2, 13, 43), were partially resolved as two diffuse bands (marked with brackets in Fig. 8). Both bands were phosphorylated in CEFs expressing v-Src, c-Src

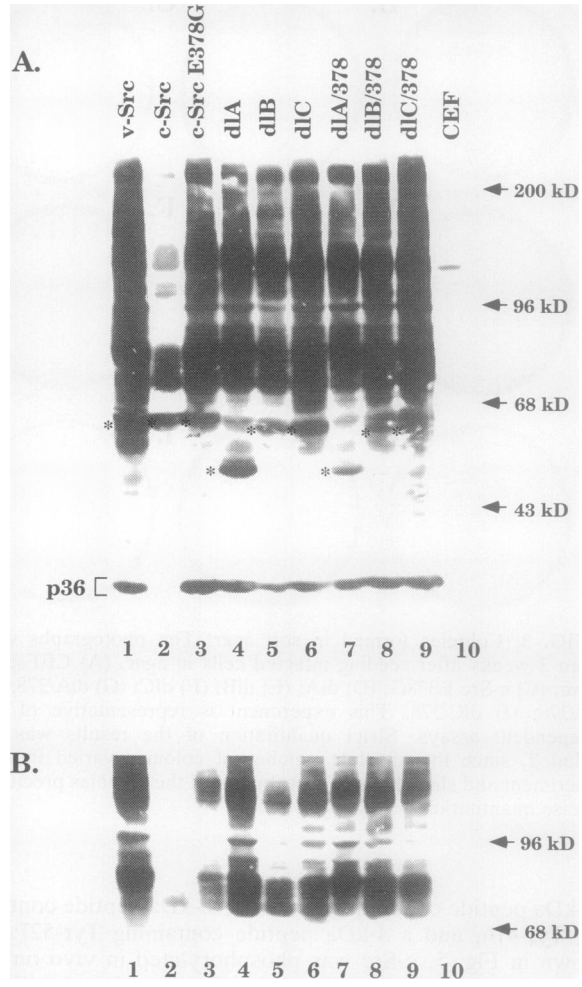

FIG. 6. Identification of phosphotyrosine-containing proteins in CEFs expressing the mutant proteins. A total of 100 μg of whole-cell lysates were analyzed by immunoblotting with antiphosphotyrosine MAb 4G10 as described in Materials and Methods. (A) Representative immunoblot. Stars denote the Src proteins, and the bracket indicates p36, the calpactin I heavy chain. (B) A portion of a complete blot between 68 and 140 kDa is shown to demonstrate the differences in levels of tyrosine phosphorylation of substrates of 120 to 130 kDa and 80 to 85 kDa.

E378G, and dlA, although the lower band contained more detectable phosphotyrosine than the upper band in v-Src- and dlA-transformed cells. In contrast, expression of dlB or dlC induced higher levels of tyrosine phosphorylation in the upper band. There was no detectable phosphorylation of the 65- to 70-kDa proteins in either CEFs or CEFs expressing c-Src. The relationship of these two bands to each other has not yet been examined; however, it is possible that the more slowly migrating bands represent more highly phosphorylated forms of the lower-molecular-mass protein. Finally, the 55-kDa protein, constitutively phosphorylated on tyrosine in CEFs and CEFs expressing c-Src, displayed variable increases in phosphotyrosine levels in cells transformed with v-Src, c-Src E378G, dlA, dlB, or dlC.

Recently, Brott et al. reported that GAP is stably associated in complexes with the Src protein in cell lysates (3, 4). Interestingly, phosphorylated proteins with the same mobilities as those of v-Src, c-Src E378G, dlA, dlB, and dlC were

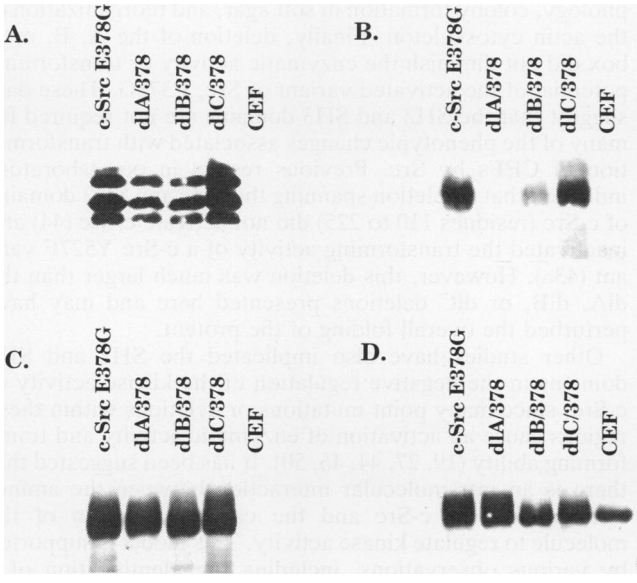

FIG. 7. Tyrosine phosphorylation of the Src substrates p85, p110, p120, and p125. Lysates prepared with a Triton X-100 buffer were normalized for protein content, and each substrate was immunoprecipitated with the designated antibody and Pansorbin. The immunoprecipitates were then separated by electrophoresis, transferred to nitrocellulose, and probed with antiphosphotyrosine MAb 4G10 as described in Materials and Methods. (A) p85; (B) p110; (C) p120; (D) p125.

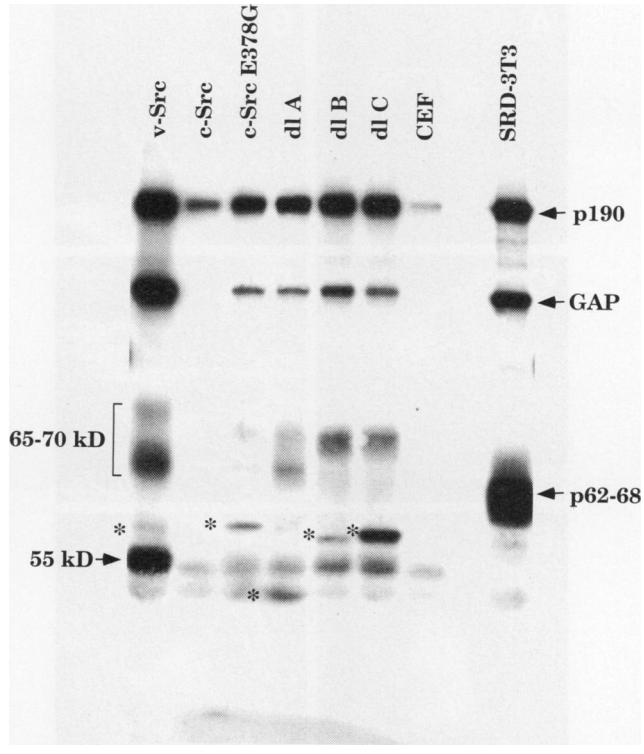

FIG. 8. Tyrosine phosphorylation of p21^{ras} GAP and GAP-associated proteins. Lysates prepared with a modified RIPA buffer were normalized for protein content, and p21^{ras} GAP was immunoprecipitated with antiserum to p21^{ras} GAP and protein A-Sepharose. After electrophoresis, the samples were immunoblotted with antiphosphotyrosine MAb 4G10 as described in Materials and Methods. GAP and the GAP-associated proteins identified in Src-transformed mouse cells (SRD-3T3) are labeled. Brackets denote the 65- to 70-kDa and 55-kDa GAP-associated proteins seen in CEFs. Stars indicate the Src proteins present in the anti-GAP immunoprecipitates.

detected in GAP immunoprecipitates (shown by stars in Fig. 8). This result suggests that the homology boxes may not be required for stable association with GAP, but further experiments are necessary to establish the basis for the detection of Src in these immunoprecipitates.

Reorganization of actin cytoskeleton. Transformation by v-Src induces a reorganization of the actin cytoskeleton into structures referred to as rosettes or podosomes (10, 64). Coimmunostaining with antibodies against Src indicates that the Src protein is also localized in these rosettes (45, 58). We used a double-label immunofluorescence assay to determine whether c-Src variants lacking the A, B, or C box were able to reorganize the actin cytoskeleton and colocalize with the actin rosettes. CEFs infected with v-Src exhibited a complete rearrangement of actin filaments into actin rosettes (Fig. 9B) that was not observed in cells expressing c-Src (Fig. 9D). Consistent with their transformed phenotype, expression of c-Src dlA (Fig. 9F), dlB (Fig. 9H) and dlC (Fig. 9J) also resulted in rosette formation similar to that seen with v-Src. This actin reorganization depended on Src expression, since rosette formation was not observed in cells not expressing v-Src, dlA, dlB, or dlC. This finding demonstrated that the SH2 and SH3 domains of Src are not required for the characteristic actin reorganization seen in Src transformed cells. In addition, the colocalization of Src to the rosettes evident in v-Src-transformed cells (Fig. 9A) was not altered by deletion of the A, B, or C domain (Fig. 9E, G, and I); however, the staining pattern for the Src protein was more diffuse than the pattern seen with the actin staining. These results suggest that the SH2 and SH3 domains are not absolutely necessary for the association of Src with these cytoskeletal elements in transformed cells.

DISCUSSION

The SH2 and SH3 domains of c-Src are believed to be necessary for modulating the kinase activity of Src and for directing protein-protein interactions necessary for Src-induced changes in cell behavior. We constructed a panel of c-Src mutants that lacked either the SH3 domain (A box) or one of the two SH2 subdomains (B or C box) to study the role of these domains in regulating Src function. The wild-type c-*src* gene was chosen for this study rather than the Rous sarcoma virus v-*src* gene, since v-Src contains multiple mutations throughout the gene that contribute to its potent biological activity and could complicate analysis of the specific importance of SH2 and SH3 domain deletions. We also examined the effect of each of the mutations on a transforming variant of c-Src, E378G, to determine whether these deletions diminished the biological and biochemical activity of this protein.

By several criteria, deletion of the A, B, or C homology box caused an activation of the kinase activity of wild-type c-Src. The in vitro specific activity of each of the mutant proteins was 14- to 30-fold higher than that of c-Src. In vivo, cells expressing the mutant proteins displayed qualitative and quantitative increases in tyrosine phosphorylation of multiple cellular proteins. In addition, the major in vivo site

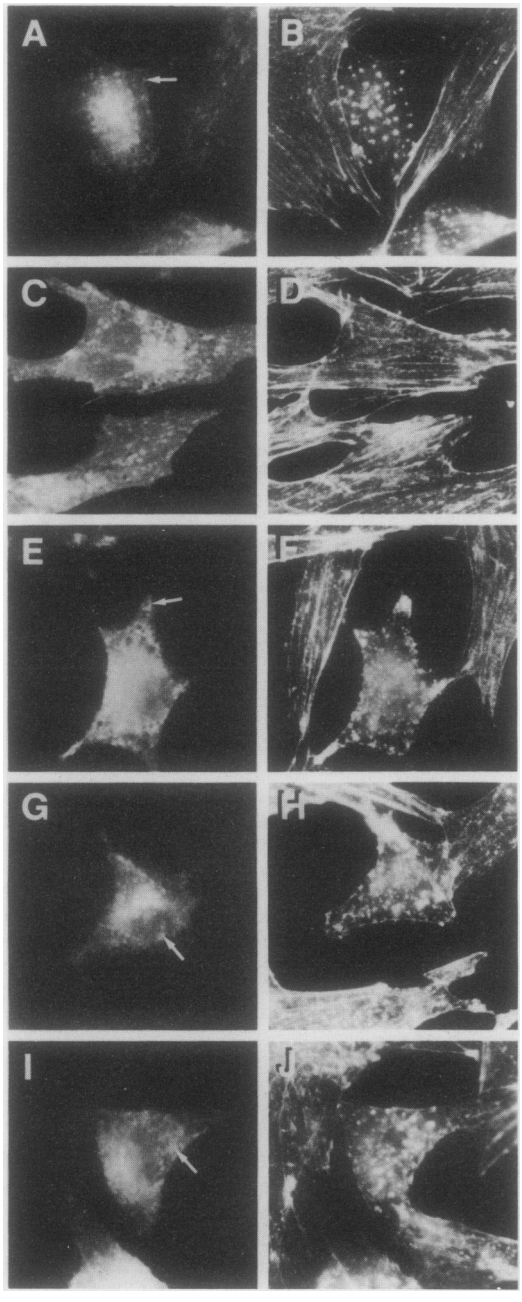

FIG. 9. Immunofluorescence microscopy of CEFs expressing the Src mutations. CEFs expressing v-Src (A and B), c-Src (C and D), dlA (E and F), dlB (G and H), or dlC (I and J) were grown on coverslips and stained for Src and actin. Cells stained for Src with anti-Src MAb LA074, biotin-conjugated goat anti-mouse immunoglobulin G, and fluorescein isothiocyanate-conjugated avidin are shown in panels A, C, E, G, and I. The identical cells stained for actin with rhodamine-conjugated phalloidin are shown in panels B, D, F, H, and J.

of tyrosine phosphorylation in dlA, dlB, and dlC was Tyr-416, further supporting the conclusion that the level of catalytic activity of these proteins is elevated. This enhancement of catalytic activity correlated with an activation of the transforming potential of Src as demonstrated by the ability of the deletion mutants to induce a transformed cell morphology, colony formation in soft agar, and reorganization of the actin cytoskeleton. Finally, deletion of the A, B, or C box did not diminish the enzymatic activity or transforming potential of the activated variant of Src, E378G. These data suggest that the SH2 and SH3 domains are not required for many of the phenotypic changes associated with transformation of CEFs by Src. Previous results in our laboratory indicated that a deletion spanning the SH2 and SH3 domains of c-Src (residues 110 to 225) did not activate c-Src (44) and inactivated the transforming activity of a c-Src Y527F variant (43a). However, this deletion was much larger than the dlA, dlB, or dlC deletions presented here and may have perturbed the overall folding of the protein.

Other studies have also implicated the SH2 and SH3 domains in the negative regulation of the kinase activity of c-Src, since many point mutations or deletions within these regions cause an activation of enzymatic activity and transforming ability (19, 27, 44, 46, 50). It has been suggested that there is an intramolecular interaction between the amino-terminal half of c-Src and the catalytic domain of the molecule to regulate kinase activity. This model is supported by various observations, including the identification of a MAb which recognizes an epitope formed from amino- and carboxy-terminal sequences of Src (41), the demonstration that a peptide containing residues 137 to 157 can specifically inhibit Src kinase activity (60), and the demonstration of an increased level of enzymatic activity of a proteolytic fragment of Src lacking the amino-terminal half of the protein (5). One possibility is that the SH2 domain interacts with the regulatory phosphotyrosine at residue 527 in c-Src to inhibit enzymatic activity. Several lines of evidence support this model. First, the stable interaction of phosphorylated PI3K and Src, presumably because of the binding of phosphorylated PI3K to the SH2 domain, depends on removal of the phosphotyrosine at residue 527, therefore making the SH2 domain available for binding PI3K (14). Second, a carboxy-terminal peptide phosphorylated at Tyr-527 can bind to c-Src mutants lacking the Tyr-527 phosphorylation site, whereas only traces of wild-type c-Src bound to the phosphorylated peptide. Peptide binding depended on the presence of an intact SH2 domain (59). In addition, the SH3 domain is also likely to play a significant role in regulating kinase activity, possibly by interacting directly with the catalytic domain. One model proposes that kinase activity is inhibited by binding to substratelike sequences found in the SH3 domain, particularly tyrosines 90 and 92 (17). The data presented here are consistent with both of these models of regulation of Src activity, since deletion of either the SH2 or the SH3 domain is sufficient to activate c-Src.

Since the SH2 and SH3 domains have been implicated in the recognition of and interaction with substrates necessary for cellular transformation, it is surprising that deletion of these domains did not significantly alter the total profile of phosphotyrosine-containing proteins in infected CEFs. When tyrosine phosphorylation of individual substrates of Src was examined, p36, p85, p120, GAP, and the GAP-associated protein p190 were each phosphorylated in CEFs expressing the deletion mutants to levels similar to those in cells transformed by wild-type Src. The GAP-associated proteins with M_rs of 65,000 to 75,000 were also phosphorylated on tyrosine in cells transformed by the deletion mutants, although the relative level of phosphorylation of each of the bands in this region was not identical. In addition, there was an increase in tyrosine phosphorylation of p125 in CEFs expressing each of the mutations, although expression of dlB and dlC did not result in the characteristic mobility

shift seen with Src-transformed cells. Of the individual substrates examined thus far, only p110 phosphorylation required the A and B boxes, since this protein was not tyrosine phosphorylated in cells expressing dlA and was only weakly phosphorylated in cells expressing dlB. These results extend previous observations by Kanner et al. (25) that a panel of three amino acid deletions within the SH3 domain of c-Src or the deletion of amino acids 155 to 157 or 175 to 177 in the SH2 domain prevents p110 phosphorylation. Mutations within other portions of the SH2 domain did not affect p110 phosphorylation. These results, together with the results presented here, suggest that the SH3 domain is necessary for stable phosphorylation of p110 by Src, while the SH2 domain can contribute to but is not absolutely required for phosphorylation of this substrate.

These data suggest that the SH2 and SH3 domains are not required for the phosphorylation of the majority of tyrosine-phosphorylated proteins seen with Src-transformed cells. Although there are differences in the ability of each mutant to phosphorylate several specific substrates, phosphorylation of these specific proteins is not likely to be essential for many of the phenotypic changes associated with transformation, since CEFs expressing dlA, dlB, or dlC displayed dramatic alterations in cell morphology and formed colonies in soft agar. It is possible that the SH2 and SH3 domains are necessary for phosphorylation of Src substrates not evaluated in this study, such as PI3K (14). Alternatively, the SH2 and SH3 domains may be required for phosphorylation of a protein(s) which is not essential for transformation but is involved in signal transduction by Src in the normal cell.

Recently, it has become clear that Src can form stable associations with several molecules in transformed cells, including GAP, PI3K, p110, and p130 (4, 15, 55). c-Src also forms a transient complex with the platelet-derived growth factor receptor after platelet-derived growth factor stimulation of quiescent fibroblasts (32). The SH2 and SH3 domains are likely to be involved in formation of these complexes (1, 15, 25). In addition, a number of mutations in the SH2 domain express a host range phenotype; that is, they are capable of transforming CEFs but are transformation defective in mammalian cells (11, 18, 66). These results have been interpreted to indicate a requirement for the SH2 region in directing a critical interaction between Src and a protein(s) necessary for transformation in the mammalian cells. The mutants described here will be useful for a further understanding of the molecular interactions between Src and these cellular proteins.

Kanner et al. recently showed that deletion of residues 155 to 157 or 175 to 177 in c-Src Y527F caused a loss of the transforming potential of an activated c-Src variant (25). It is difficult to reconcile the loss of transforming activity of these small deletions with the negligible effects caused by the complete deletion of the SH2 domain in this study. It is possible that deletion of residues 155 to 157 or 175 to 177 results in the stabilization of an interaction between the mutant Src and a cellular protein(s) which interferes with the transforming ability of c-Src Y527F. Presumably, deletion of the entire B box would prevent an interaction with SH2 domain-binding proteins so that Src molecules lacking the B box would still be able to exert any biological effects not directly dependent on the SH2 domain itself.

The actin cytoskeleton of cells transformed by Src is reorganized into new structures named rosettes. The results presented here indicate that the SH2 and SH3 domains are not necessary for this reorganization. This result was of particular interest, since the SH3 domain has been identified in a number of cellular proteins associated with the cytoskeleton, and it has been suggested that effects on the cytoskeleton seen with transformed cells may be mediated by the SH3 domain (12). Although results with the dlA mutant do not rule out a role for the SH3 domain in directing protein-protein interactions in the cytoskeleton, it remains to be determined which proteins might be involved in such interactions or what effect such interactions would have on the organization of the cytoskeleton. Although Fukui et al. have suggested a role for the SH2 domain in targeting Src to a detergent-insoluble cellular matrix containing cytoskeletal proteins (16), the results presented here indicate that the localization of Src in actin rosettes does not appear to be directed by either the SH2 or the SH3 domain.

The results presented in this paper support the conclusion that the induction of a transformed phenotype by activated variants of Src does not depend on either the SH2 or the SH3 domain. This conclusion was surprising, since it is generally assumed that these domains are responsible for directing the protein interactions necessary for the phenotypic changes associated with transformation. The question of how these variant Src proteins, which lack a functional A, B, or C box, induce transformation thus remains. One possibility is that the two remaining boxes in each of the mutants are able to compensate for the loss of the third; we feel, however, that it is unlikely that the homology boxes are functionally redundant. Another possibility is that the activated kinase activity of dlA, dlB, or dlC constitutively activates either endogenous c-Src or another Src-like kinase in the infected CEFs and that these activated proteins, with their intact SH2 and SH3 domains, carry out the functions usually associated with the SH2 and SH3 domains of Src. However, we have not detected activation of endogenous c-Src in cells expressing the deletion mutants (results not shown). Alternatively, since there appear to be multiple pathways which lead to the same end point of cell transformation, induction of just one of these redundant pathways by activated Src variants could be sufficient to compensate for the absence of the SH2 and SH3 domains. For example, there is recent evidence that the receptors for insulinlike growth factor I and the epidermal growth factor are phosphorylated on tyrosine in cells expressing v-Src and that the signaling activity of the epidermal growth factor receptor (as assayed by phospholipase C-γ phosphorylation) was activated by this Src-induced phosphorylation (30, 68). The authors suggest that v-Src-mediated changes in the phosphorylation and activity of growth regulatory receptors could be responsible for some of the phenotypic changes associated with transformation. Therefore, although the SH2 and SH3 domains may be important for mediating protein-protein interactions of Src, the redundancy of the pathways leading to transformation could complicate investigations of the functional role of these domains in the normal functioning of c-Src.

ACKNOWLEDGMENTS

We thank M. Trahey and B. Haimovich for helpful discussions; S. Decker, T. Parsons, and T. Roberts for antibodies; H. Hanafusa for the c-Src E378G variant; S. Hughes for vectors; and J. Thomas for critical reading of the manuscript.

This work was supported by grant CA27951 from the National Institutes of Health.

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