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Regulation of the Oncogenic Activity of the Cellular *src* Protein Requires the Correct Spacing between the Kinase Domain and the C-Terminal Phosphorylated Tyrosine (Tyr-527)

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Repression of the tyrosine kinase activity of the cellular *src* protein ($pp60^{c\text{-}src}$) depends on the phosphorylation of a tyrosine residue (Tyr-527) near the carboxy terminus. Tyr-527 is located 11 residues C terminal from the genetically defined end of the kinase domain (Leu-516) and is therefore in a negative regulatory region. Because the precise sequence of amino acids surrounding Tyr-527 appears to be unimportant for regulation, we hypothesized that the conformational constraints induced by phosphorylated Tyr-527 may require the correct spacing between the kinase domain (Leu-516) and Tyr-527. In this report, we show that deletions at residue 518 of two, four, or seven amino acids or insertions at this residue of two or four amino acids activated the kinase activity and thus the transforming potential of $pp60^{c\text{-}src}$. As is the case for the prototype transforming variant, $pp60^{527F}$, activation caused by these deletions or insertions was abolished when Tyr-416 (the autophosphorylation site) was changed to phenylalanine. In comparison with wild-type $pp60^{c\text{-}src}$, the *src* proteins containing the alterations at residue 518 showed a lower phosphorylation state at Tyr-527 regardless of whether residue 416 was a tyrosine or a phenylalanine. Mechanisms dealing with the importance of spacing between the kinase domain and Tyr-527 are discussed.

The enzymatic activity of the cellular *src* gene product, $pp60^{c\text{-}src}$, is negatively regulated in vivo by near stoichiometric phosphorylation of a C-terminal tyrosine residue (Tyr-527) (8, 32). This regulation is bypassed in the naturally occurring retroviral *src* proteins (e.g., $pp60^{v\text{-}src}$) because Tyr-527 has been eliminated by replacement of the C-terminal region of $pp60^{c\text{-}src}$ with an unrelated sequence containing no phosphorylation site (19, 45; Fig. 1). Deletion of this entire region also results in oncogenic activation of $pp60^{c\text{-}src}$; in fact, the simple alteration of Tyr-527 to another residue (e.g., Phe or Ser) is sufficient for constitutive protein tyrosine kinase activity and oncogenic transformation (4, 23, 36, 40).

Leu-516 has been identified genetically as the end of the kinase domain on the basis of the following evidence. First, as described above, deletion of the region C terminal to this residue does not result in loss of enzyme activity. However, truncation at Leu-516 or preceding residues abolishes both kinase activity and transformation ability (49, 50). Moreover, Leu-516 is conserved in all Src family members as well as in all other protein-tyrosine kinases (in a few instances, a similar hydrophobic residue is present at the analogous position) (16). Thus, Leu-516 defines the end of the kinase domain, and the residues C terminal to 516, including Tyr-527, function as a negative regulatory sequence.

Whereas Leu-516 and Tyr-527 are conserved in the seven known members of the Src family, the intervening sequence of amino acids shows some divergence and appears not to be important for phosphorylation of Tyr-527 or regulation of kinase activity. For example, the C-terminus of $pp60^{c\text{-}src}$ differs from that of $pp56^{lck}$ at 8 of 17 residues (including three conservative differences between Leu-516 and Tyr-527), yet replacement of the C-terminal 18 residues of $pp60^{c\text{-}src}$ with the corresponding residues of $pp56^{lck}$ reconstitutes a normally regulated protein which is phosphorylated in vivo at Tyr-527 (27). In addition, a substitution in $pp60^{c\text{-}src}$ at residue 525 or 526 or a truncation at 528 has no effect on the phosphorylation of Tyr-527 and hence regulation (6). Taken together, these data indicate that the precise sequence of amino acids within the negative regulatory region is of secondary importance, whereas the spacing between the kinase domain (i.e., Leu-516) and Tyr-527, which is conserved in all Src family members, may be critical.

To test this hypothesis, we constructed a series of mutations in c-*src* which introduced short deletions or insertions designed to alter the spacing between Leu-516 and Tyr-527. The alterations were introduced at residue 518 in order to maintain the structural integrity of both the kinase domain (i.e., Leu-516) and the sequence surrounding Tyr-527 (i.e., the regulatory domain). Characterization of these variant *src* proteins in chicken embryo (CE) cells indicated that even minimal (two-residue) alterations in spacing resulted in biological effects indistinguishable from those produced by expression of the transforming variant $pp60^{527F}$. Perhaps surprisingly, the deletions and insertions caused a significant reduction in the phosphorylation of Tyr-527, even though Tyr-527 and the surrounding sequence was essentially intact. Nevertheless, precise spacing between Leu-516 and Tyr-527 appears to be essential for conformational changes that are important for the mechanism of inhibition.

MATERIALS AND METHODS

Plasmids and mutagenesis. Expression of *src* DNA in CE cells was carried out by using the Rous sarcoma virus (RSV) expression vector pRLc. This construct is a pBR322-based

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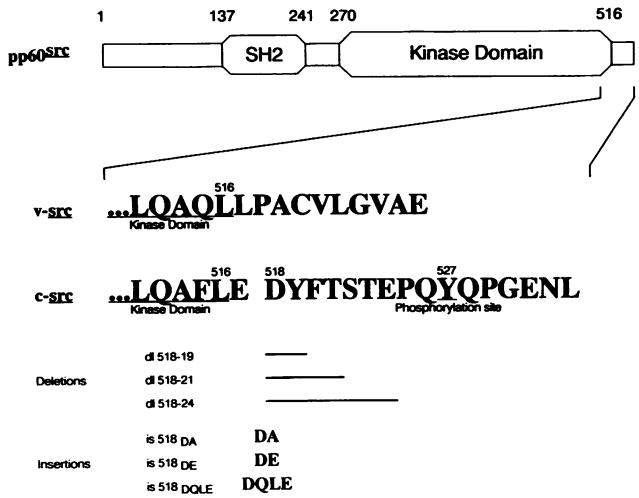

FIG. 1. Structure of the *src* gene product showing the locations of the kinase domain and the SH2 domain (amino acid residues designated above). The carboxy terminus is enlarged to show the amino acid sequences of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. In $pp60^{v\text{-}src}$, the site of regulation, Tyr-527, is not present because of a substitution at the carboxy terminus of 12 unrelated residues for the 19 found in $pp60^{c\text{-}src}$. Below the sequence of $pp60^{c\text{-}src}$ is a schematic showing the alterations introduced by oligonucleotide-directed mutagenesis of the c-*src* gene. The numbers denote the deleted residues. The insertions of two and four amino acids were introduced between residues 517 and 518.

plasmid containing a nonpermuted molecular clone of Prague A RSV in which the v-*src* gene has been replaced by a c-*src* cDNA (40). Oligonucleotide-directed mutagenesis was done as described by Nakamaye and Eckstein (30). M13mp18 which contained the c-*src* cDNA (40) was used as the template DNA for mutagenesis. The mutations were confirmed by sequencing and were cloned into pRLc by using a strategy similar to that described by Reynolds et al. (40). The doubly mutated genes containing 416F were derived by replacing the *MluI-BglI* fragment (encoding residues 259 to 431) of the C-terminally mutated c-*src* gene with the same fragment from c-*src* 416F.

Cell culture. Cultures of primary CE cells were prepared from 10-day-old embryos (SPAFAS, Norwich, Conn.) and maintained in culture as previously described (2). For transfection experiments, DNA (1 to 2 μg) was applied to cells by using standard $CaPO_4$ transfection techniques. After virus expression and infection of the entire cell population (9 to 11 days), virus stocks were harvested and used to infect CE cells for further experiments.

Cell lysate preparation and immunoprecipitation. For immunoprecipitations of *src* proteins, extracts were prepared by using $RIPA^+$ (9). The protein concentrations of individual lysates were determined with the BCA protein assay kit (Pierce Chemical Co., Rockford, Ill.). The lysates were adjusted to equal protein concentration and volume, and then the *src* proteins were immunoprecipitated with the *src*-specific monoclonal antibody (MAb) EC10 (33). Western immunoblotting was performed as described previously (39).

Metabolic labeling and CNBr cleavage. Cells were labeled with 1 mCi of $^{32}P_i$ (Dupont, NEN Research Products, Boston, Mass.) per ml for 3 h in Dulbecco modified Eagle medium supplemented with 10% dialyzed fetal calf serum and with 6.25 μg of $NaHPO_4$ per ml. The ^{32}P-labeled *src* proteins were immunoprecipitated as described above and resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Radiolabeled proteins were located in the wet gel by autoradiography and then were excised and extracted from the gel slice in 200 μl to 1 ml of 0.05% trifluoroacetic acid. Extraction was performed with shaking either at room temperature for 1 h or at 4°C overnight. This method resulted in 80 to 95% recovery of radioactivity. The trifluoroacetic acid extract was transferred to a siliconized glass tube, and 10 μg of bovine serum albumin was added as a carrier to prevent adherence of labeled proteins to the walls of the tube. The samples were reduced to dryness and resuspended in freshly prepared 70% formic acid containing 50 mg of CNBr (Sigma, St. Louis, Mo.) per ml. Cleavage proceeded for 1 h, and then the samples were lyophilized to dryness. Residual formate was removed by several cycles of washing with water and lyophilization. The samples were resuspended in Laemmli sample buffer (24) and run on a 32-cm 15 to 24% gradient polyacrylamide gel.

RESULTS

Deletions and insertions at residue 518 activate $pp60^{c\text{-}src}$ into a transforming protein. To test whether the spacing between the kinase domain and Tyr-527 is important for the regulation of $pp60^{c\text{-}src}$, we constructed a series of mutations which encoded *src* proteins with deletions and insertions of residues beginning at amino acid 518 (Fig. 1). Deletions of two, four, and seven amino acids were designed, as were insertions of two and four amino acids. The sequences of the insertions were chosen on the basis of secondary structure predictions (13) in order to minimize alterations in the predicted secondary structure of the adjacent regions. The mutations were constructed by oligonucleotide-directed mutagenesis, using an M13 vector containing the c-*src* cDNA. Following mutagenesis, the genes were cloned into pRLc, which is an RSV expression vector that contains a c-*src* cDNA in place of v-*src* (40).

Biological activity of the mutant c-*src* genes was tested by transfecting the pRLc vectors into CE cells. Untransfected cells as well as those transfected with the wild-type c-*src* remained morphologically normal (Fig. 2A and B). Cultures transfected with c-*src* 527F or any of the c-*src* genes containing the spacing mutations developed foci in 6 to 8 days. Transformation of the entire population followed within 2 days (Fig. 2C to I). When cells expressing the variant c-*src* genes were plated in soft agar, they formed macroscopic colonies within 14 days (data not shown).

Cells expressing the mutated c-*src* proteins were further characterized by analyzing the expression level of the c-*src* proteins and the phosphotyrosine content of cellular proteins following Western immunoblotting of total cell extracts. $pp60^{src}$ was detected by probing with the *src*-specific MAb 327 (26) (Fig. 3A). Expression of the genes containing the spacing mutations was comparable to the expression of wild-type c-*src* or the mutated gene, c-*src* 527F. The deletion and insertion of amino acids was evident from the shift in the mobility of the variant *src* proteins. Proteins phosphorylated on tyrosine were detected by probing a parallel immunoblot with antibodies to phosphotyrosine (39) (Fig. 3B). At this exposure, uninfected cells (lane 1) contained a single detectable tyrosine-phosphorylated protein with a molecular size of approximately 125 kDa. In cells expressing wild type c-*src* (lane 2), the only additional protein containing phosphotyrosine was $pp60^{c\text{-}src}$ itself. As expected, in cells expressing the mutated gene, c-*src* 527F (lane 3), numerous tyrosine phosphorylated proteins were detected by using the phos-

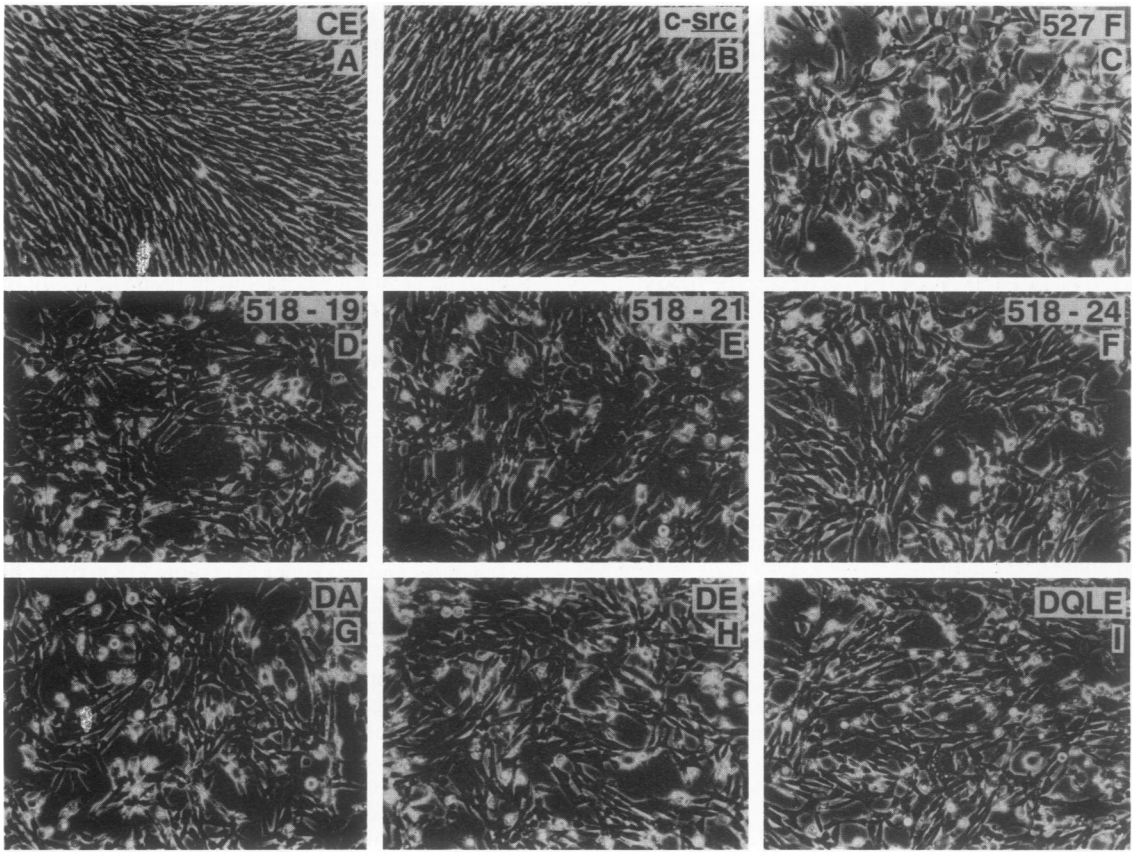

FIG. 2. Morphology of CE cells expressing the variant *src* genes. (a) Uninfected cells; (b) c-*src*; (c) c-*src* 527F; (d) c-*src* dl518-519; (e) c-*src* dl518-521; (f) c-*src* dl518-524; (g) c-*src* is518 DA; H, c-*src* is518 DE; I, c-*src* is518 DQLE.

photyrosine antibodies. Expression of c-*src* which contained any of the spacing mutations (lanes 4 to 9) produced a pattern of tyrosine-phosphorylated proteins similar if not identical to that seen in cells expressing c-*src* 527F. The increased tyrosine phosphorylation of cellular proteins indicated an increased in vivo kinase activity of the Src variants with the alterations at the C terminus.

Activated forms of $pp60^{src}$ form a stable complex with two tyrosine-phosphorylated proteins designated by their apparent molecular weights, pp130 and pp110 (38). To determine whether *src* proteins activated by deletions or insertions at residue 518 formed stable complexes with pp130 and pp110, *src* proteins were immunoprecipitated with a *src*-specific MAb, EC10 (33). The immunoprecipitated proteins were resolved via SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (Fig. 4). Neither pp130 nor pp110 was observed in immune complexes of wild type $pp60^{c\text{-}src}$ (lane 1). In contrast, immune complexes of $pp60^{527F}$ (lane 2) or the *src* proteins with the altered spacing between the kinase domain and Tyr-527 (lanes 3 to 8) contained both pp130 and pp110.

Alteration of Tyr-416 (the autophosphorylation site) (34, 42) to Phe abolishes the transforming potential of $pp60^{527F}$ (22, 36), whereas it does not inhibit the cellular transformation ability of $pp60^{v\text{-}src}$ (12, 44). To determine whether the activation caused by the spacing mutations required a tyrosine at residue 416, we constructed doubly mutated genes that encoded the deletions or insertions at the C terminus in addition to a phenylalanine at residue 416. Expression of these doubly mutated genes did not result in transformation. The cells were morphologically indistinguishable from cells expressing wild-type c-*src* or c-*src* 416F/527F (data not shown), and the *src* protein levels were comparable to the levels of $pp60^{c\text{-}src}$ or $pp60^{527F}$ (see Fig. 6). Thus, the presence of tyrosine at residue 416 was required for the activation of $pp60^{c\text{-}src}$ caused by alterations at the C terminus.

Altered *src* proteins have a reduced extent of phosphorylation at Tyr-527. Because phosphorylation of Tyr-527 may have been affected by the alterations, we examined the phosphorylation state of the variant *src* proteins. CNBr cleavage was performed on *src* proteins metabolically labeled with $^{32}P_i$ to assess the phosphorylation of specific residues. Three phosphorylated peptides are derived from this cleavage (Fig. 5B). The 32-kDa peptide is derived from the amino-terminal half of the protein, and it is phosphorylated predominantly on serines 12 and 17 (7, 15, 35, 37). The 10-kDa peptide contains Tyr-416, and the 5-kDa peptide contains Tyr-527 (20, 41). The *src* proteins were immunoprecipitated from parallel sets of unlabeled and labeled cells. To determine the relative amounts of the variant *src* proteins, immune complexes from unlabeled cell extracts were resolved by SDS-PAGE and Western blotted by using *src* MAb 327 (Fig. 5A). CNBr analysis was carried out on labeled *src* proteins (Fig. 5B). Phosphorylation of endogenous $pp60^{c\text{-}src}$ was barely detectable (lane 1, untransfected cells); therefore, the increased signals observed in lanes 2 to 9 were due to the phosphopeptides derived from the over-

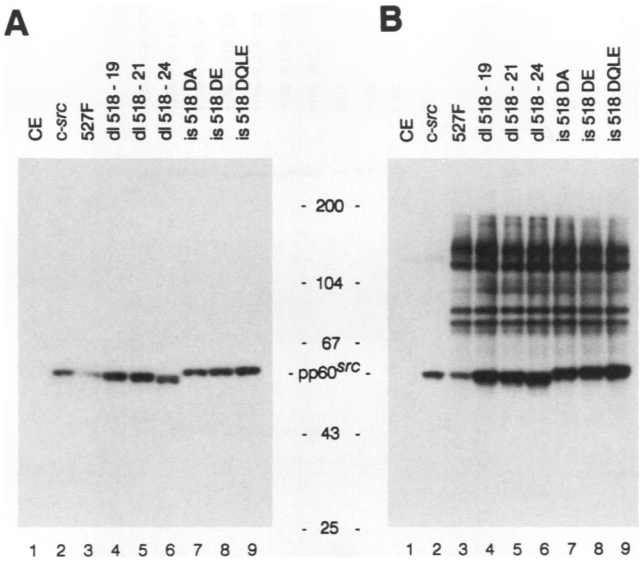

FIG. 3. Expression level of *src* proteins (A) and phosphotyrosine content of total cell proteins (B). CE cells expressing the indicated *src* variants were lysed in Laemmli sample buffer (24). Aliquots containing equal amounts of total cell protein were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting. (A) *src* proteins were visualized by immunoblotting with the *src*-specific MAb 327 as described in Materials and Methods. (B) Proteins containing phosphotyrosine were visualized by immunoblotting with antibodies to phosphotyrosine. Molecular weight markers (in kilodaltons) are indicated in the center.

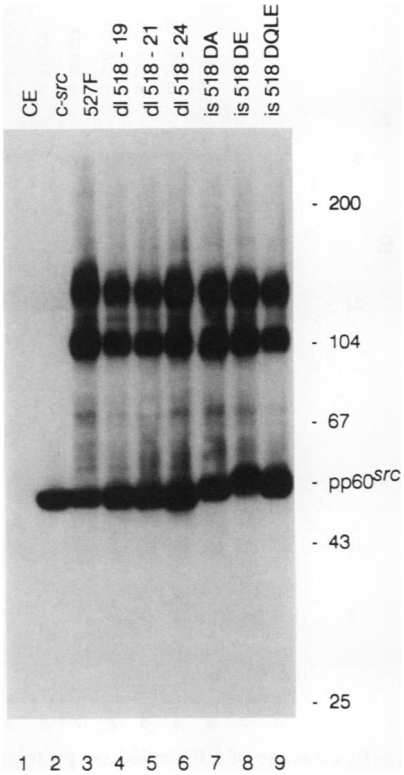

FIG. 4. Coimmunoprecipitation of pp130 and pp110 with activated c-*src* proteins. CE cells expressing the indicated *src* variants were lysed in modified RIPA buffer (9) and subjected to immunoprecipitation using the *src*-specific MAb EC10. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to phosphotyrosine. Molecular weight markers (in kilodaltons) are indicated at the right.

expressed *src* proteins. All of these *src* proteins appeared to be phosphorylated to the same relative extent within the 32-kDa peptide. As expected, overexpressed $pp60^{c\text{-}src}$ (lane 2) was phosphorylated on the Tyr-527-containing peptide, and $pp60^{527F}$ (lane 3) was phosphorylated on the Tyr-416-containing peptide (the autophosphorylation site). The proteins with the deletions or insertions (lanes 4 to 9) were all phosphorylated on both the Tyr-416-containing peptide and the Tyr-527-containing peptide; however, the extent of phosphorylation of the Tyr-527-containing peptide was significantly reduced compared with that of wild-type $pp60^{c\text{-}src}$. Densitometric analysis of the autoradiographs revealed that the stoichiometry of phosphorylation of the Tyr-527-containing peptides in the *src* proteins with the alterations at 518 ranged from 5 to 25% of that observed for $pp60^{c\text{-}src}$.

Reduced phosphorylation at Tyr-527 is independent of phosphorylation at Tyr-416. Autophosphorylation at Tyr-416 is thought to induce a conformational state that is required for transformation (22, 23, 36). It is possible that this alteration could affect the conformation around Tyr-527, which could lead to its lower phosphorylation state. To determine whether phosphorylation at Tyr-416 influenced the extent of phosphorylation at Tyr-527, we analyzed the phosphorylation state of the *src* proteins containing Phe-416 as described above. Figure 6 shows quantitation of the relative *src* protein levels (Fig. 6A) and analysis of the CNBr cleavage products (Fig. 6B). Changing the site of autophosphorylation to phenylalanine in the wild-type c-*src* protein ($pp60^{416F}$) did not affect the phosphorylation of Tyr-527 (lane 3). Altering both sites of tyrosine phosphorylation ($pp60^{416F/527F}$) resulted in no new detectable sites of phosphorylation in either the 10- or 5-kDa peptide (lane 5). The *src* protein variants containing both Phe-416 and the alteration in the region between the kinase domain and Tyr-527 still exhibited a lower level of phosphorylation on the Tyr-527-containing peptide than did $pp60^{c\text{-}src}$ or $pp60^{416F}$. Thus, in the context of these *src* proteins, autophosphorylation at Tyr-416 does not appear to affect the phosphorylation state of Tyr-527.

The results obtained with ^{32}P labeling were confirmed by steady-state phosphorylation analysis of intact proteins via Western immunoblotting. The *src* proteins containing Phe-416 were used to eliminate the contribution from the autophosphorylation site. Analysis was achieved by blotting one half of an MAb EC10 immune complex preparation with an antibody to phosphotyrosine and the other half with the *src* MAb 327 to normalize for the amount of *src* protein present. The *src* proteins containing the altered spacing at the C terminus in addition to Phe-416 had only 20 to 40% of the level of phosphotyrosine found in $pp60^{c\text{-}src}$ or $pp60^{416F}$ (data not shown).

DISCUSSION

The phosphorylation state of Tyr-527 in $pp60^{c\text{-}src}$ is an important component in the regulation of its oncogenicity (8, 32). Little is understood about the mechanism by which phosphorylation facilitates regulation. In this report, we have shown that the structural integrity of the region just downstream from the kinase domain is important for regulation. Harvey et al. (17) demonstrated that a deletion of

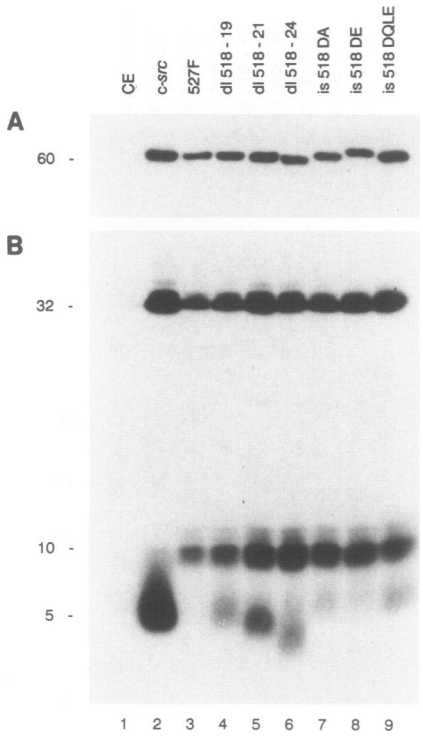

FIG. 5. CNBr cleavage of ^{32}P-labeled *src* proteins. (A) Relative expression levels of the *src* proteins. CE cells expressing the indicated *src* variants were lysed in modified RIPA buffer (9). The lysates were adjusted to equal protein concentration and volume, and the *src* proteins were immunoprecipitated with the *src*-specific MAb EC10. Immune complexes were resolved by SDS-PAGE and immunoblotted with the *src*-specific MAb 327. To examine the phosphorylation state (B), parallel plates of cells from those used in panel A were labeled with $^{32}P_i$. The *src* proteins were immunoprecipitated as in panel A and resolved by SDS-PAGE. Labeled *src* proteins were extracted from the gel and treated with CNBr. The resultant peptides were resolved on 15 to 24% SDS-polyacrylamide gels. Molecular sizes (in kilodaltons) of the cleavage products are shown at the left. The 32-kDa peptide contains the amino-terminal half of the *src* protein, and it is phosphorylated on Ser-12 and -17 (7, 15, 35, 37). The 10-kDa peptide contains the autophosphorylation site, Tyr-416, and the 5-kDa peptide contains the regulatory site of phosphorylation, Tyr-527 (20, 41).

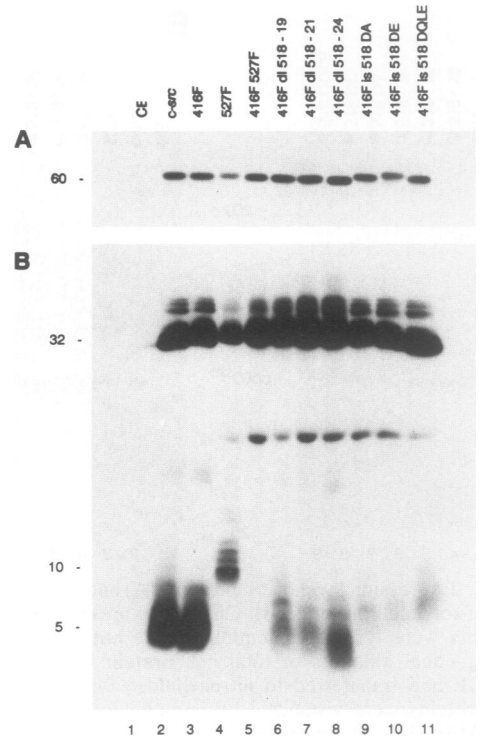

FIG. 6. Phosphorylation states of the variant *src* proteins containing the second alteration (416F). Relative protein level determination (A) and CNBr cleavage (B) were performed as described for Fig. 5. Molecular sizes (in kilodaltons) of the cleavage products are shown at the left.

residues 519 through 524 activated the oncogenic potential of $pp60^{c\text{-}src}$. Our results are in agreement, and they add to this view by showing that either a deletion or insertion of as few as two amino acids at residue 518 was sufficient to activate the oncogenic potential $pp60^{c\text{-}src}$. On the basis of these data, two basic mechanisms can be envisioned for activation.

In the first mechanism, the addition or deletion of amino acids may change the positioning of Tyr-527, which in turn would prevent specific interactions with other residues of the enzyme that normally cause a repressed conformation. Dephosphorylation of Tyr-527 might occur as a secondary event because of a more exposed environment, which might make it a better substrate for a phosphatase or a poorer substrate for the putative regulatory kinase. One possible site of interaction for phosphorylated Tyr-527 is within the active site. Regulation would be derived from the C terminus sterically inhibiting access to substrates, a mechanism similar to the steric inhibition model proposed for certain Ser/Thr kinases (43). These enzymes have negative regulatory sequences that appear to behave as pseudosubstrates. By analogy, the phosphorylated C terminus of $pp60^{c\text{-}src}$ might function as a pseudo-end product, and its interactions with the active site would produce the same net result of facilitating steric inhibition of enzymatic activity. An alternative site of interaction for phosphorylated Tyr-527 could be with an amino-terminal region called the SH2 domain (3, 28) which encompasses residues 137 to 241. The integrity of this region is important for enzymatically activated *src* proteins to cause transformation, and it is thought to be involved in targeting $pp60^{src}$ to certain relevant cellular substrates (18, 38, 46, 48). SH2 domains are found in several proteins, and evidence is mounting that this domain structure is involved in binding to regions of proteins containing phosphotyrosine (28, 29). Thus, the C terminus of $pp60^{c\text{-}src}$ containing Tyr-527 could bind to specific residues within the SH2 domain and therefore induce a repressed conformation. If phosphorylated Tyr-527 does interact with distal residues of the protein, then alteration of such residues should prevent interaction and cause activation of the kinase. There are examples of activating mutations in both the SH2 region (31, 47) and the kinase domain (21, 25). Therefore, interactions with either the kinase domain or the SH2 region are equally plausible on the basis of available evidence.

An alternative mechanism to explain the activation could be that the region between the kinase domain and Tyr-527 is required for the putative regulatory kinase to efficiently phosphorylate Tyr-527. The residues surrounding Tyr-527 appear not to be involved in Tyr-527 phosphorylation (6); therefore, the residues near 518 (the site of the alterations) may be important. Support for this hypothesis comes from

analysis of the binding of the polyomavirus middle T antigen to pp60$^{c\text{-}src}$. Middle T antigen transforms cells via its ability to bind pp60$^{c\text{-}src}$, prevent phosphorylation of Tyr-527, and activate pp60$^{c\text{-}src}$ kinase activity (1, 5, 10, 11). This may be a result of middle T antigen mimicking the binding of the regulatory Tyr-527 kinase. A deletion in pp60$^{c\text{-}src}$ of residues 519 to 524 prevents stable binding (17); thus, in the case of the deletion variants (deletion of residues 518 to 519, 521, and 524, respectively), decreased ability to serve as substrates for the regulatory Tyr-527 kinase may very well explain the decreased phosphorylation state of Tyr-527. However, this explanation seems much less likely for the three insertion variants, in which the actual sequence of the C-terminal 16, 17, or 18 amino acids, respectively, is unchanged from that in wild-type pp60$^{c\text{-}src}$. In fact, for the latter variant with the four-amino-acid insertion, the conservation of 18 residues is equivalent to conservation of the entire C terminus, including Leu-516. Of course, we cannot exclude the possibility that recognition by the regulatory kinase also involves residues within the kinase domain preceding Leu-516. If the residues near 518 are simply required for maintaining the phosphorylation state of Tyr-527, then regulation could be derived from a global conformational change in the kinase domain induced by phosphorylation of Tyr-527. This type of regulation is supported by mutational studies which have shown that the structure of the C-terminal region of the kinase domain is important for enzymatic activity (32). Additionally, an antibody made against a peptide containing residues 498 to 512 inhibits kinase activity (14).

We have shown that the spacing between the kinase domain and Tyr-527 is important for regulation. Whether this spacing is important for the proper positioning of Tyr-527 or for the simple maintenance of its phosphorylation state will require the examination of the population of *src* proteins which contain phosphorylated Tyr-527. If these molecules are fully activated, then the alterations would be directly responsible for preventing the conformational changes that lead to negative regulation, and the reduced phosphorylation of Tyr-527 would be only a trivial consequence. The answer awaits the ability to separate the two populations of *src* proteins and test their activities.

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