MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2465–2471
0270-7306/88/062465-07$02.00/0

Vol. 8, No. 6

Investigation of Factors That Influence Phosphorylation of pp60$^{c\text{-}src}$ on Tyrosine 527

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Received 14 December 1987/Accepted 21 March 1988

Phosphorylation at tyrosine 527 of the proto-oncogene product, pp60$^{c\text{-}src}$, has been proposed to decrease the tyrosine kinase activity of the enzyme. We have investigated potential factors that might influence phosphorylation at this site by making mutant variants of the pp60$^{c\text{-}src}$ protein. By effectively eliminating the site of N-terminal myristylation, we demonstrated that stable membrane association is not necessary for tyrosine 527 phosphorylation. Furthermore, mutational elimination of the enzymatic activity of this mutant pp60$^{c\text{-}src}$ protein did not alter the efficiency of phosphorylation at tyrosine 527. These data are consistent with the proposal that pp60$^{c\text{-}src}$ may be phosphorylated at tyrosine 527 by a cellular tyrosine kinase distinct from pp60$^{c\text{-}src}$. In addition, using detergent-permeabilized cells, we established conditions that allow efficient phosphorylation of tyrosine 527 in vitro.

The proto-oncogene product, pp60$^{c\text{-}src}$, is the normal cellular homolog of the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (1). Although both of these proteins possess tyrosine-specific protein kinase activity, pp60$^{v\text{-}src}$ is a potent transforming protein, while pp60$^{c\text{-}src}$ does not induce oncogenic transformation even when expressed at levels comparable to those of pp60$^{v\text{-}src}$ (20, 21, 28). Investigations of the fundamental properties that may be responsible for the difference in the oncogenic potential of these proteins have revealed several criteria that distinguish these proteins. (i) pp60$^{v\text{-}src}$ represents a mutated version of pp60$^{c\text{-}src}$ which contains several single-amino-acid differences throughout the protein and a major digression located in the extreme carboxy terminus, where the last 12 amino acids of pp60$^{v\text{-}src}$ are unique and distinct from the last 19 amino acids of pp60$^{c\text{-}src}$ (35). This carboxy-terminal substitution (as well as some of the single-amino-acid changes) has been shown to be sufficient for activation of the oncogenic potential of pp60$^{c\text{-}src}$ (20, 33). (ii) The tyrosine-specific protein kinase activity of pp60$^{v\text{-}src}$ is 20- to 100-fold higher than the specific activity of pp60$^{c\text{-}src}$ (13, 19). (iii) pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ both undergo autophosphorylation in vitro at tyrosine 416 (29, 34); however, the in vivo site of tyrosine phosphorylation of pp60$^{v\text{-}src}$ is tyrosine 416, whereas pp60$^{c\text{-}src}$ is phosphorylated in vivo at tyrosine 527 (8). Tyrosine residue 527 is not found in pp60$^{v\text{-}src}$, and there is no tyrosine residue in the unique 12-amino-acid carboxy tail.

Several lines of evidence suggest that phosphorylation at tyrosine 527 may regulate the tyrosine kinase activity of pp60$^{c\text{-}src}$. (i) pp60$^{c\text{-}src}$ molecules isolated in the presence of sodium orthovanadate (a potent inhibitor of phosphotyrosine-specific phosphatases) possess a lower specific activity than pp60$^{c\text{-}src}$ molecules isolated under conditions that allow hydrolysis of phosphate at this site (11). (ii) Enzymatic dephosphorylation of tyrosine 527 causes an activation of pp60$^{c\text{-}src}$ tyrosine-specific protein kinase activity in vitro (10). (iii) pp60$^{c\text{-}src}$ molecules that are bound to the middle T antigen of polyomavirus and possess an elevated tyrosine-protein kinase activity are phosphorylated on tyrosine residue 416 instead of on 527 (6). (iv) All variant forms of pp60$^{c\text{-}src}$ which are oncogenic and display elevated levels of tyrosine-protein kinase activity are phosphorylated on tyrosine residue 416 and not on tyrosine 527 (16, 19). (v) Mutational substitution of phenylalanine for tyrosine 527 causes an activation of the kinase activity and oncogenic potential of pp60$^{c\text{-}src}$ (5, 24, 30, 32). Taken together, these results indicate that phosphorylation of tyrosine residue 527 suppresses the kinase activity and the oncogenic potential of pp60$^{c\text{-}src}$ and that this posttranslational modification plays a role in regulating the functional activity of this molecule.

These studies have raised the question of whether phosphorylation of pp60$^{c\text{-}src}$ tyrosine 527 is mediated by autophosphorylation or by another cellular protein kinase. The possibility that intramolecular autophosphorylation is responsible for tyrosine 527 phosphorylation was addressed in a recent analysis of the sites of phosphorylation of an enzymatically inactive mutant form of pp60$^{c\text{-}src}$ (22). This mutant protein, although incapable of autophosphorylation, is phosphorylated in vivo on tyrosine 527 at levels comparable to those for wild-type (wt) pp60$^{c\text{-}src}$. This result clearly demonstrates that tyrosine 527 can be phosphorylated by some mechanism other than intramolecular autophosphorylation. Further investigation of the mechanism of tyrosine 527 phosphorylation has been limited by the inability to obtain efficient phosphorylation of tyrosine residue 527 in vitro. When either crude cell extracts or purified pp60$^{c\text{-}src}$ are subjected to in vitro kinase assays, tyrosine phosphorylation of pp60$^{c\text{-}src}$ takes place almost exclusively at residue 416 (10, 34). A very low level of in vitro phosphorylation at tyrosine 527 was detected in pp60$^{c\text{-}src}$ immunoprecipitates that were pretreated with phosphatase; however, the phosphorylation at this site was only 10% of the level of tyrosine 416 phosphorylation (10). This result suggests that conditions within the cellular environment are essential to allow efficient phosphorylation of tyrosine 527. It is possible that phosphorylation of tyrosine 527 requires proper association of pp60$^{c\text{-}src}$ with the membrane in vivo, due either to changes in the conformation of pp60$^{c\text{-}src}$ upon association with the lipid bilayer or to interactions with other cellular proteins which either phosphorylate tyrosine 527 or indirectly affect the ability of pp60$^{c\text{-}src}$ to undergo intermolecular autophosphorylation at this site. In this report, we investigate whether stable association with the membrane is required for

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phosphorylation of tyrosine 527 in vivo, and we establish conditions that allow phosphorylation at this site in vitro.

MATERIALS AND METHODS

Cells, plasmids, and viruses. Chicken embryo fibroblasts (CEF) were prepared from 11-day-old gs$^-$ embryos (SPAFAS, Inc., Norwich, Conn.). The pc-*src* plasmid and pRSV plasmid vector system were obtained from S. Hughes (17) and modified by L. Fox (unpublished results) by substituting the c-*src* gene obtained from H. Hanafusa for the v-*src* gene and by eliminating the permutation in the envelope gene. Expression of plasmid DNA was accomplished by calcium phosphate transfection (15). NIH(pMc*src*/cos)A cells, which overexpress avian pp60$^{c\text{-}src}$ (21), were obtained from D. Shalloway.

Mutant constructions. All enzymes and the *Sac*II linker were obtained from New England BioLabs. DNA (pMc-src295) encoding the arginine 295 c-*src* mutant was obtained from T. Kmiecik and D. Shalloway and transferred into the pRSV c-*src* and pRSV LN c-*src* plasmids by substitution of the analogous *Mlu*I-to-*Bgl*I fragment.

In vivo labeling of cells. [^{35}S]methionine (Amersham, 50 μCi/ml, 900 μCi/mmol) and carrier-free $^{32}P_i$ (ICN, 1 mCi/ml) labeling was performed in methionine-minus or phosphate-minus Dulbecco modified Eagle medium supplemented with 10% complete Dulbecco modified Eagle medium (growth medium) for 18 h. ^{3}H-labeled myristic acid (New England Nuclear Corp.; 375 μCi) was dried under nitrogen, dissolved in 7 μl of dimethyl sulfoxide, and added to 750 μl of growth medium. Cells were labeled for 5 h.

Immunoprecipitation and in vitro kinase assay. pp60$^{c\text{-}src}$ was immunoprecipitated from RIPA (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris [pH 7.2], 5 mM EDTA)-solubilized lysates with 0.01 μl of monoclonal antibody 327 (obtained from ascites fluid), using Formalin-fixed *Staphylococcus aureus* prebound with anti-mouse antibody as the immunoabsorbent as described (27). In vitro kinase reactions were performed after immunoprecipitation of pp60$^{c\text{-}src}$ by the addition of 2 μg of acid-treated enolase (9), 5 μCi of [γ-^{32}P]ATP (ICN, 7,000 Ci/mmol), 10 mM Tris hydrochloride (pH 7.2), and 5 mM $MnCl_2$ for 15 min at 4°C.

Cellular fractionation. Cells were fractionated into soluble and particulate fractions by homogenization in hypotonic lysis buffer and sedimentation as described previously (2).

Cleavage of pp60$^{c\text{-}src}$ proteins. After immunoprecipitation and excision from the gel, pp60$^{c\text{-}src}$ was subjected to additional electrophoresis on a 12.5% SDS-polyacrylamide gel in the presence of 100 ng of V-8 protease. For CNBr cleavage, immunoprecipitated pp60$^{c\text{-}src}$ was eluted from the gel piece in 50 mM ammonium carbonate (pH 8.5)–0.1% SDS–1% β-mercaptoethanol. After filtration over glass wool, proteins were precipitated (along with 100 μg of Pentex bovine serum albumin as a carrier) with one-fourth volume of 100% trichloroacetic acid. Pellets were washed twice with cold ethanol-ether, vacuum dried, and cleaved for 1 h at room temperature in 30 μl of 50-mg/ml CNBr in 70% formic acid. Products were lyophilized four or five times with 1 ml of distilled H_2O. Dried pellets were suspended in borate sample buffer (75 mM Tris sulfate [pH 8.3], 2% SDS, 1% β-mercaptoethanol) and subjected to electrophoresis on a 27.5% acrylamide–0.18% bisacrylamide borate gel (separation gel buffer, 375 mM Tris sulfate [pH 8.3]; stacking gel, 5% acrylamide–0.0125% bisacrylamide; stacking gel buffer, 75 mM Tris sulfate [pH 8.3]; running buffer, 65 mM Tris borate [pH 8.3]–0.1% SDS). Gels were dried and exposed to film as described below.

In situ phosphorylation. Approximately 10^6 NIH(pMc*src*/cos)A cells were scraped into phosphate-buffered saline and pelleted for 5 s in a microcentrifuge (13,000 × *g*). The pellet was gently suspended in a small volume (25 μl) of in situ phosphorylation buffer (1% Triton X-100, 2.5 mM $MnCl_2$, 10 mM Tris [pH 7.4], 150 mM NaCl, 100 μM leupeptin, 1% aprotinin). After a 30-min incubation on ice, 5 μl of a kinase mix (25 μCi of [γ-^{32}P]ATP per reaction, 600 μM sodium orthovanadate, 6 μM unlabeled ATP) was added and gently mixed. After the kinase reaction was allowed to proceed for 15 min at 4°C, 250 μl of RIPA was added to further solubilize the cells, and pp60$^{c\text{-}src}$ was immunoprecipitated from the clarified lysate as usual.

Electrophoresis and autoradiography. Immunoprecipitates were suspended in SDS sample buffer and subjected to electrophoresis on 7.5% SDS-polyacrylamide gels as described by Laemmli (26). Gels containing ^{35}S or ^{3}H were fluorographed with En^{3}Hance (New England Nuclear Corp.) before drying. Dried gels were exposed to XAR-5 film (Kodak) and enhanced with DuPont Cronex Lightning-Plus intensifying screens (Fotodyne).

RESULTS

Construction of a myristic acid-minus pp60$^{c\text{-}src}$ molecule. The evidence that tyrosine 527 of pp60$^{c\text{-}src}$ is not efficiently phosphorylated under any condition that removes pp60$^{c\text{-}src}$ from the cellular environment raised the possibility that association with the membrane is required for phosphorylation at this site. To address this possibility, a mutant form of pp60$^{c\text{-}src}$ was constructed that was unable to associate stably with the membrane. The strategy utilized the fact that attachment of the 14-carbon saturated fatty acid, myristic acid, to the viral oncogene product, pp60$^{v\text{-}src}$, is necessary for membrane localization (14, 23). Myristic acid attachment to the N terminus of pp60$^{v\text{-}src}$ occurs either during or shortly after translation and shows a strict specificity for an N-terminal glycine (3, 36). Since pp60$^{c\text{-}src}$ also contains a myristyl modification (4, 19), a mutant variant of pp60$^{c\text{-}src}$ was created that disrupted myristic acid attachment to pp60$^{c\text{-}src}$. This mutant, referred to as LN c-*src* for "linker at *Nco*I," was made by inserting an 8-base-pair *Sac*II linker into the unique *Nco*I site located at the initiator ATG site of the c-*src* gene (Fig. 1). This linker insertion strategy was first utilized by Cross et al. (14) in constructing a myristic acid-deficient pp60$^{v\text{-}src}$. pp60$^{c\text{-}src}$ encoded by this mutant contained four additional amino acids N terminal to authentic pp60$^{c\text{-}src}$: an N-terminal methionine and three alanine residues. This insertion moved the original myristic acid acceptor glycine to amino acid position 5. The mutated c-*src* gene was transferred to the pRSV plasmid, which contains the entire Rous sarcoma virus genome (minus v-*src*) (Fig. 2).

Effect of the LN c-*src* mutation on the cellular localization of pp60$^{c\text{-}src}$. Previous studies have demonstrated that the majority of wt pp60$^{c\text{-}src}$ fractionates as a membrane-associated protein (12, 31), with only a small percentage (8 to 15%) of pp60$^{c\text{-}src}$ associated with the soluble cell fraction. To examine how disruption of myristylation affects the localization of pp60$^{c\text{-}src}$, we examined the fractionation of the mutant LN c-*src* gene product. CEF cultures were transfected with the pRSV plasmid containing either the LN or the wt c-*src* gene. One week later, cells labeled with [^{35}S]methionine or [^{3}H]myristic acid were broken by hypotonic lysis and were separated into soluble and particulate fractions. The c-*src*

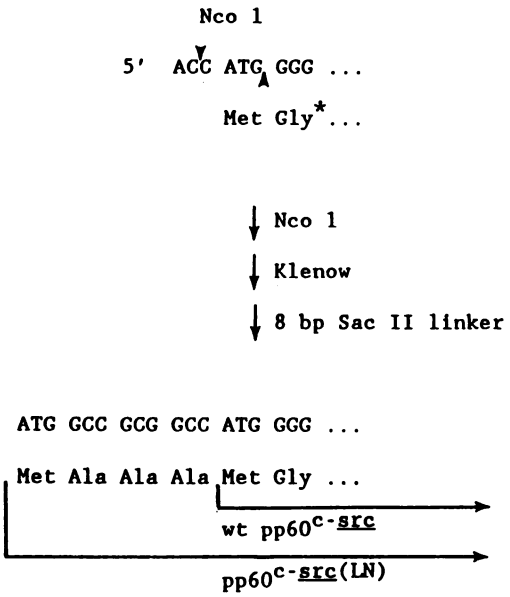

FIG. 1. Strategy for LN c-*src* construction. Asterisk indicates myristylation site.

gene products immunoprecipitated from these lysates are shown in Fig. 3. Unlike wt pp60$^{c\text{-}src}$, which fractionates predominantly as a particulate protein (Fig. 3A, lane 2), the majority of the LN c-*src* mutant gene product was found in the soluble fraction (Fig. 3A, lane 3). As expected, the soluble pp60$^{c\text{-}src(LN)}$ molecules were not labeled with [^{3}H]myristic acid (Fig. 3B, lane 3). The pp60$^{c\text{-}src}$ molecules detected in lanes 4 of Fig. 3 represent endogenous membrane-bound pp60$^{c\text{-}src}$ as well as exogenous myristylated pp60$^{c\text{-}src}$ produced by internal initiation at the second AUG within the LN c-*src* gene (see Fig. 1 and discussion of Fig. 5). [By comparing the levels of [^{3}H]myristate and [^{35}S]methionine incorporation into endogenous pp60$^{c\text{-}src}$ from uninfected CEF and the levels detected in CEF expressing pp60$^{c\text{-}src(LN)}$, we estimate that authentic pp60$^{c\text{-}src}$ generated by internal initiation of LN c-*src* mRNA represents approximately 5 to 10% of the total exogenous pp60$^{c\text{-}src}$ in LN c-*src*-transfected cells (data not shown).] Since the LN c-*src* gene can allow translation of a small amount of myristylated wt pp60$^{c\text{-}src}$, we have analyzed pp60$^{c\text{-}src}$ from the soluble cell fraction in all further experiments to examine exclusively the mutant pp60$^{c\text{-}src(LN)}$ molecules. The 52-kilodalton protein found in lane 1 of Fig. 3A is a degradation product of pp60$^{c\text{-}src}$ that is generated during

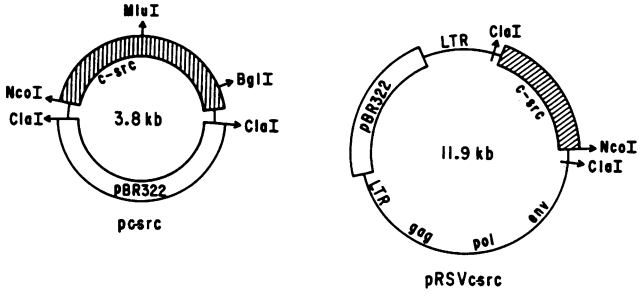

FIG. 2. Plasmids used in the construction and transfection of mutant c-*src* genes (as described in Materials and Methods). Hatched region represents the c-*src* gene.

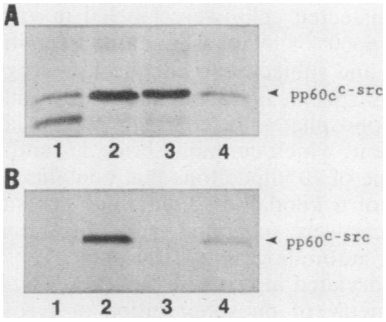

FIG. 3. Cellular fractionation of pp60$^{c\text{-}src(LN)}$. Labeled cell lysates were fractionated by hypotonic lysis into soluble (lanes 1 and 3) and particulate (lanes 2 and 4) fractions. pp60$^{c\text{-}src}$ was immunoprecipitated with monoclonal antibody 327 as described in Materials and Methods. (A) [^{35}S]methionine-labeled lysates from cells transfected with pRSV wt c-*src* (lanes 1 and 2) and pRSV LN c-*src* (lanes 3 and 4). (B) Identical to panel A except that cells were labeled with [^{3}H]myristic acid.

fractionation of the cells (12). The absence of this degradation product in lane 3 of Fig. 3A suggests that the protease-sensitive site is inaccessible to cleavage in the LN c-*src* gene product.

To confirm the identity of the immunoprecipitated protein from mutant-transfected cells, V-8 protease digestion was performed on in vivo ^{32}P-labeled pp60$^{c\text{-}src}$. The characteristic amino-terminal V_3 and V_4 fragments (7) from the LN c-*src* mutant protein migrated slightly more slowly than the analogous fragments from the wt protein (Fig. 4A, cf. lanes 2 and 1). This slower migration was presumably due to the four additional amino acids that were present in the N terminus of the pp60$^{c\text{-}src(LN)}$ molecules.

Phosphorylation sites of the pp60$^{c\text{-}src(LN)}$ molecule. To examine the sites of phosphorylation of a pp60$^{c\text{-}src}$ molecule that was not stably associated with the membrane, pRSV

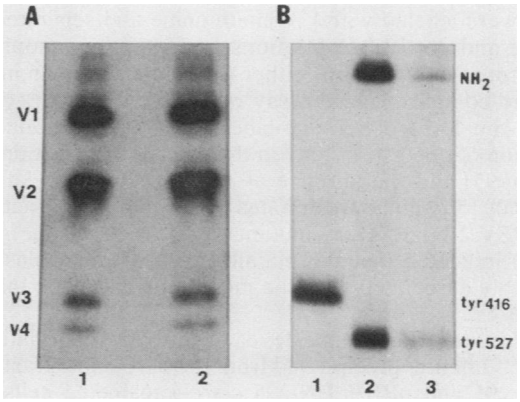

FIG. 4. Phosphopeptide analysis of pp60$^{c\text{-}src(LN)}$. pp60$^{c\text{-}src}$ was immunoprecipitated from total cell lysates of ^{32}P-labeled pRSV wt c-*src*-transfected CEF and from the soluble fraction of ^{32}P-labeled pRSV LN c-*src*-transfected CEF and subjected to electrophoresis on SDS-polyacrylamide gels. pp60$^{c\text{-}src}$ was excised from the gel and either subjected to limited proteolysis with V-8 protease (A) or cleaved to completion with CNBr (B) as described in Materials and Methods. (A) Lanes: 1, in vivo-labeled wt pp60$^{c\text{-}src}$; 2, in vivo-labeled soluble pp60$^{c\text{-}src(LN)}$. (B) Lanes: 1, pp60$^{c\text{-}src}$ phosphorylated in an immune complex kinase assay, used as a marker for the phosphopeptide containing tyrosine 416; 2, in vivo-labeled soluble pp60$^{c\text{-}src(LN)}$; and 3, in vivo-labeled wt pp60$^{c\text{-}src}$.

LN c-*src*-transfected cells were labeled in vivo with $^{32}P_i$. The soluble pp60$^{c\text{-}src(LN)}$ was immunoprecipitated, eluted from the gel, and subjected to chemical cleavage by CNBr. CNBr cleaves specifically at methionine residues and produces three phosphate-labeled fragments: a large 32-kilodalton fragment, which contains serine 17, and two smaller fragments, one of 10 kilodaltons that contains tyrosine 416, and another of 6 kilodaltons containing tyrosine 527 (22). These products were separated on a 27.5% borate gel and visualized by autoradiography. The pp60$^{c\text{-}src(LN)}$ molecules were phosphorylated at tyrosine 527 (Fig. 4B, lane 2), and the stoichiometry of phosphorylation at tyrosine 527 (as indicated by ^{32}P incorporation in the V-8 protease fragments shown in Fig. 4A) was indistinguishable from that of wt pp60$^{c\text{-}src}$. This result indicated that stable membrane association is not necessary for phosphorylation of pp60$^{c\text{-}src}$ at tyrosine 527. However, in these mutant-transfected cells, which contain high levels of soluble, active pp60$^{c\text{-}src}$, it is possible that the phosphorylation of tyrosine 527 is mediated by the tyrosine kinase activity of the mutant molecules themselves. To address the possibility that the mutant pp60$^{c\text{-}src(LN)}$ molecules were phosphorylated at tyrosine 527 by an intermolecular autophosphorylation event, an enzymatically inactive variant of the LN c-*src* mutant was made.

Analysis of an enzymatically inactive pp60$^{c\text{-}src(LN)}$ mutant. A double mutant of pp60$^{c\text{-}src}$ was constructed that contained the LN mutation combined with a mutation that encoded a single-amino-acid change from lysine to arginine at the ATP-binding site of pp60$^{c\text{-}src}$. This construction was accomplished by exchanging the *Mlu*I-to-*Bgl*I fragment (see Fig. 2) of the pRSV LN c-*src* mutant with the analogous fragment from pMc*src* 295, a mutant c-*src* gene that encoded the change at the ATP-binding site. This double-mutant c-*src* gene (LN-295 c-*src*) should encode a pp60$^{c\text{-}src}$ variant that is inactive as a kinase (since lysine 295 is required for binding ATP) and that cannot stably associate with the membrane (as demonstrated above). Chick cells transfected with the double-mutant plasmid pRSV LN-295 c-*src* or control constructions (pRSV wt c-*src*, pRSV LN c-*src*, and pRSV 295 c-*src*) were labeled with [^{35}S]methionine and separated into soluble and particulate fractions. pp60$^{c\text{-}src}$ immunoprecipitated from these fractions either was subjected to an in vitro immune complex kinase assay or was analyzed directly to detect the level of in vivo-labeled pp60$^{c\text{-}src}$ protein. The detection of pp60$^{c\text{-}src(LN\text{-}295)}$ in the soluble cell fraction (Fig. 5A, lane 7) and the absence of enolase phosphorylation in the pp60$^{c\text{-}src}$ immunoprecipitates from the soluble fraction of the pRSV LN-295 c-*src* mutant-transfected cells (Fig. 5B, lane 7) indicated that the mutant pp60$^{c\text{-}src}$ molecules were not stably associated with the membrane and were inactive as kinases.

In this experiment, the level of autophosphorylation of pp60$^{c\text{-}src}$ immunoprecipitated from the particulate fraction of pRSV 295 and pRSV LN-295 c-*src*-transfected cells (Fig. 5B, lanes 6 and 8) was higher than the observed autophosphorylation seen in uninfected CEF (Fig. 5B, lane 2). It is likely that this phosphorylation results from transphosphorylation of the mutant inactive pp60$^{c\text{-}src}$ molecules by the endogenous pp60$^{c\text{-}src}$ present in those immunoprecipitates. Consistent with this possibility is the evidence that, while the level of autophosphorylation of pp60$^{c\text{-}src}$ immunoprecipitated from the mutant transfected cells is higher than that of pp60$^{c\text{-}src}$ from uninfected CEF, the level of enolase phosphorylation is not higher than the levels detected in uninfected CEF (Fig. 5B, compare lanes 2 with 6 and 8). Although pp60$^{c\text{-}src(LN\text{-}295)}$ should not be present in the particulate cell fraction, the evidence that pp60$^{c\text{-}src}$ detected in this fraction migrates slightly faster than the retarded mobility of soluble pp60$^{c\text{-}src(LN\text{-}295)}$ (Fig. 5A, compare lanes 7 and 8) suggests that the membrane-bound pp60$^{c\text{-}src(LN\text{-}295)}$ protein is generated by internal initiation at the AUG at codon position 5 (see Fig. 1). Therefore, this protein would contain the authentic myristylation signal in the correct position, be subject to myristic acid addition, and be associated with the membrane.

Mutant pp60$^{c\text{-}src}$ from the soluble fraction of pRSV LN-295 c-*src*-transfected cell lysates was examined to determine the extent and the sites of in vivo phosphorylation. A direct comparison of the ratio of incorporation of $^{32}P_i$ to that of [^{35}S]methionine in the LN c-*src* mutant and wt pp60$^{c\text{-}src}$ molecules indicated no difference in the stoichiometry of phosphorylation in vivo (data not shown). Figure 6 shows an analysis of the sites of phosphorylation of this mutant variant of pp60$^{c\text{-}src}$. The profile of CNBr-cleaved fragments (Fig. 6B) demonstrated that pp60$^{c\text{-}src(LN\text{-}295)}$ was phosphorylated on tyrosine 527 (lane 4). The extent of phosphorylation at tyrosine 527 was determined by partial proteolytic digestion with V-8 protease (Fig. 6A). In this assay, the amount of phosphate incorporated at tyrosine 527 (V_2 fragment) could be directly compared with the amount incorporated at serine 17 (V_1 fragment). Using this comparison, it was evident that pp60$^{c\text{-}src(LN\text{-}295)}$ (Fig. 6A, lane 3) was phosphorylated at tyrosine 527 to the same extent as wt pp60$^{c\text{-}src}$ (lane 1) and pp60$^{c\text{-}src(295)}$ (lane 2). These results indicated that enzymatically inactive pp60$^{c\text{-}src}$ molecules that are not stably associated with the membrane are able to be phosphorylated at tyrosine 527.

In situ phosphorylation of pp60$^{c\text{-}src}$ at tyrosine 527. The

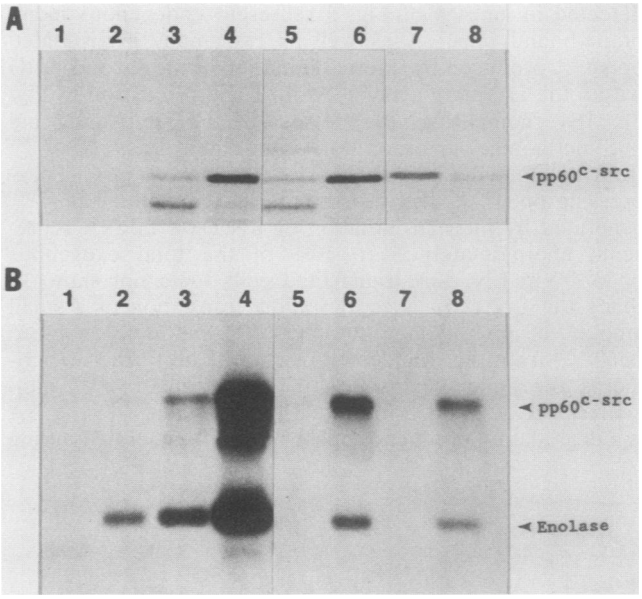

FIG. 5. Cellular fractionation of mutant pp60$^{c\text{-}src}$ and determination of in vitro kinase activity. [^{35}S]methionine-labeled cell lysates were fractionated by hypotonic lysis into soluble (lanes 1, 3, 5, and 7) and particulate (lanes 2, 4, 6, and 8) fractions. pp60$^{c\text{-}src}$ was immunoprecipitated with monoclonal antibody 327 as described in Materials and Methods. Immunoprecipitates were split; half were analyzed directly on a 7.5% SDS-polyacrylamide gel (A), and the other half were subjected to an in vitro kinase assay using enolase as an exogenous substrate (B). Lanes: 1 and 2, uninfected CEF; 3 and 4, pRSV wt c-*src*-transfected CEF; 5 and 6, pRSV 295 c-*src*-transfected CEF; and 7 and 8, pRSV LN-295 c-*src*-transfected CEF.

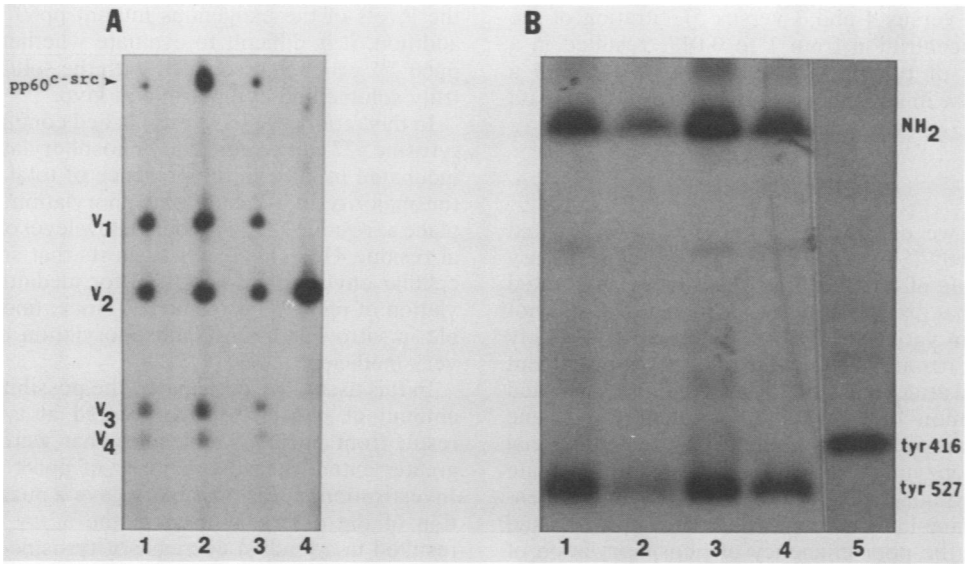

FIG. 6. Mutant pp60$^{c\text{-}src}$ phosphopeptide analysis. pp60$^{c\text{-}src}$ was immunoprecipitated from lysates of ^{32}P-labeled mutant-transfected cells and subjected to electrophoresis on SDS-polyacrylamide gels. pp60$^{c\text{-}src}$ was excised from the gel and either subjected to limited proteolysis with V-8 protease (A) or cleaved to completion with CNBr (B) as described in Materials and Methods. (A) Lanes: 1, in vivo-labeled wt pp60$^{c\text{-}src}$; 2, in vivo-labeled pp60$^{c\text{-}src(295)}$; 3, in vivo-labeled soluble pp60$^{c\text{-}src(LN\text{-}295)}$; and 4, wt pp60$^{c\text{-}src}$ phosphorylated in vitro in an immune complex kinase assay, used here as a marker. (B) Lanes: 1, in vivo-labeled wt pp60$^{c\text{-}src}$; 2, in vivo-labeled soluble pp60$^{c\text{-}src(LN)}$; 3, in vivo-labeled soluble pp60$^{c\text{-}src(295)}$; 4, in vivo-labeled soluble pp60$^{c\text{-}src(LN\text{-}295)}$; and 5, included as a marker, wt pp60$^{c\text{-}src}$ phosphorylated in vitro in an immune complex kinase assay.

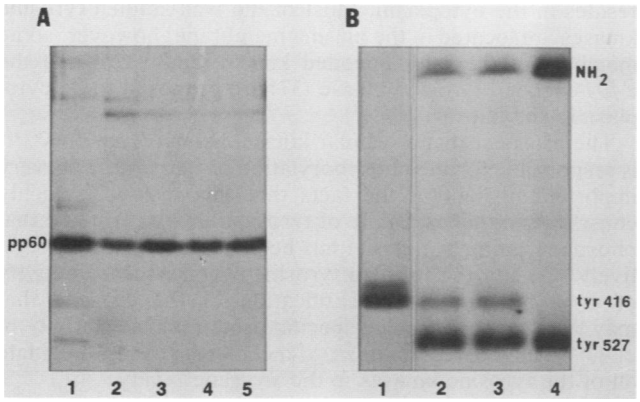

FIG. 7. Analysis of pp60$^{c\text{-}src}$ phosphorylated in situ. Cell pellets were incubated in in situ buffer in the presence of [γ-^{32}P]ATP as described in Materials and Methods. (A) RIPA-solubilized pellets were then immunoprecipitated with monoclonal antibody 327 and subjected to electrophoresis. Lanes: 1, pp60$^{c\text{-}src}$ from approximately 10^5 NIH(pMc*src*/cos)A cells phosphorylated in vitro in an immune complex kinase assay; 2 through 5, pp60$^{c\text{-}src}$ from approximately 10^6 NIH(pMc*src*/cos)A cells phosphorylated in the in situ assay in the presence of 0.5% Triton X-100 (lanes 2 and 3) or 1% Triton X-100 (lanes 4 and 5) and either 5 mM $MgCl_2$ (lanes 2 and 4) or 2.5 mM $MnCl_2$ (lanes 3 and 5). (B) pp60$^{c\text{-}src}$ was excised from the gel in panel A and cleaved to completion with CNBr. Lanes: 1, pp60$^{c\text{-}src}$ phosphorylated in vitro in an immune complex kinase assay; 2 and 3, pp60$^{c\text{-}src}$ phosphorylated in situ in the presence of 1% Triton X-100 and either 5 mM $MgCl_2$ (lane 2) or 2.5 mM $MnCl_2$ (lane 3); and 4, included as a marker, in vivo-phosphorylated pp60$^{c\text{-}src}$.

above data, combined with a previous study (22), indicate that mutations which either inactivate the kinase activity of pp60$^{c\text{-}src}$ or prevent stable membrane association do not interfere with phosphorylation at tyrosine 527. While these experiments did not eliminate the possibility that endogenous membrane-bound pp60$^{c\text{-}src}$ molecules may be responsible for tyrosine 527 phosphorylation by an intermolecular transautophosphorylation event, this event seems unlikely since the pp60$^{c\text{-}src(LN\text{-}295)}$ mutant was phosphorylated to the same extent as its membrane-bound counterpart, pp60$^{c\text{-}src(295)}$ (Fig. 6A, lanes 2 and 3). The results above, together with the evidence that pp60$^{c\text{-}src}$ is not efficiently phosphorylated on tyrosine 527 in vitro, support the possibility that another tyrosine kinase may be responsible for tyrosine 527 phosphorylation. To examine this possibility further, conditions were sought that would promote efficient pp60$^{c\text{-}src}$ tyrosine 527 phosphorylation in vitro. Since efficient tyrosine 527 phosphorylation had previously been demonstrated to occur only in vivo, conditions that mimicked the cellular environment were used. A concentrated slurry of permeabilized cells was allowed to undergo phosphorylation in the presence of [γ-^{32}P]ATP. After completion of the phosphorylation reaction the lysates were solubilized in RIPA buffer and were immunoprecipitated with antibody against pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ labeled by this method (Fig. 7A, lanes 2 through 5) was subjected to CNBr cleavage and was analyzed as described previously. The major site of tyrosine phosphorylation on the pp60$^{c\text{-}src}$ molecules labeled in this in situ assay was tyrosine 527, with a minor amount at tyrosine 416 (Fig. 7B, lanes 2 and 3). $MnCl_2$ used in this assay was only marginally better than $MgCl_2$ in promoting pp60$^{c\text{-}src}$ phosphorylation (Fig. 7A, compare lanes 2 versus 3 and 4 versus 5) and resulted in no difference in the sites of phosphorylation (Fig. 7B, lanes 2 and 3). Although there was no significant difference between the use of 0.5 or 1% Triton X-100 in the assay in promoting pp60$^{c\text{-}src}$ phosphorylation

(Fig. 7A, lanes 2 versus 4 and 3 versus 5), titration of the Triton X-100 concentration from 1 to 0.01% resulted in a gradual decrease of tyrosine 527 phosphorylation and a concurrent increase in phosphorylation at tyrosine 416 (data not shown).

DISCUSSION

In this report, we establish that myristic acid-mediated association of pp60$^{c\text{-}src}$ with the membrane is not necessary for phosphorylation of tyrosine 527. This was demonstrated by the evidence that phosphorylation at tyrosine 527 was not altered in a mutant variant of pp60$^{c\text{-}src}$ that could not stably associate with the membrane due to mutational displacement of its myristic acid attachment site. We further demonstrated that phosphorylation of this soluble, mutant c-*src* gene product on tyrosine 527 was not affected by the mutational loss of pp60$^{c\text{-}src}$ enzymatic activity. These results indicate that efficient phosphorylation of tyrosine 527 is not dependent on a membrane-induced conformation of pp60$^{c\text{-}src}$ and also suggest that the poor efficiency of phosphorylation of tyrosine 527 under in vitro phosphorylation conditions is not due to disruption of the interactions of pp60$^{c\text{-}src}$ with the lipid bilayer.

These results also suggest that the phosphorylation of tyrosine 527 is not strongly influenced by other membrane-bound proteins. These proteins could affect phosphorylation of tyrosine 527 either by directly phosphorylating this tyrosine residue or by inducing a conformational change in pp60$^{c\text{-}src}$ which would allow autophosphorylation to occur at tyrosine 527. If elimination of myristic acid-mediated attachment to the membrane has effectively removed pp60$^{c\text{-}src}$ from the direct influence of intrinsic membrane proteins, then the evidence presented here would suggest that neither membrane-associated endogenous pp60$^{c\text{-}src}$, nor another membrane-bound tyrosine kinase, is responsible for tyrosine 527 phosphorylation. However, it is difficult to evaluate the extent to which the interaction of pp60$^{c\text{-}src}$ with membrane proteins was affected by the additional N-terminal amino acids or by the disruption of myristic acid-mediated association of pp60$^{c\text{-}src}$ with the membrane. While myristic acid attachment is essential for stable association with the membrane (as defined by biochemical fractionation procedures), it is likely that other factors influence the association of pp60$^{c\text{-}src}$ with the membrane. The myristic acid-deficient mutant may be able to establish protein-protein interactions critical for mediating phosphorylation of tyrosine 527 in the absence of stable association with the membrane. However, since these mutant pp60$^{c\text{-}src}$ molecules are not stably associated with the membrane, it seems likely that any residual interaction that the mutant proteins may have established with membrane proteins would be weakened by the absence of myristic acid and that this weakened interaction would reduce the efficiency of phosphorylation at tyrosine 527 (if these interactions are crucial for mediating tyrosine 527 phosphorylation). Indeed, we demonstrate that these mutant pp60$^{c\text{-}src}$ molecules are phosphorylated on tyrosine 527 as efficiently as wt pp60$^{c\text{-}src}$. The data presented here are consistent with the proposal that another cellular tyrosine kinase may be responsible for the phosphorylation of pp 60$^{c\text{-}src}$ tyrosine 527. However, we cannot exclude the possibility that the small fraction of pp60$^{c\text{-}src}$ that has been shown to fractionate as a soluble cytoplasmic protein (12, 31) is responsible for the phosphorylation of pp60$^{c\text{-}src(LN\text{-}295)}$. This possibility seems unlikely since the levels of soluble endogenous pp60$^{c\text{-}src}$ would be approximately 200-fold lower than the levels of the exogenous mutant pp60$^{c\text{-}src}$ molecules. In addition, it is difficult to evaluate whether the 8 to 15% of pp60$^{c\text{-}src}$ which is associated with the soluble cell fraction is truly soluble and cytoplasmic in vivo.

In this report, we have established conditions under which tyrosine 527 can be efficiently phosphorylated in vitro. When incubated in vitro in the presence of total cellular proteins, the majority of tyrosine phosphorylation of pp60$^{c\text{-}src}$ took place at residue 527, with only a low level of phosphorylation at residue 416. This result suggests that some aspect of the cellular environment is crucial for mediating the phosphorylation of pp60$^{c\text{-}src}$ tyrosine 527 since, under the best possible in vitro conditions, phosphorylation of tyrosine 527 is very inefficient (10).

In this assay, we anticipated the possibility that the minor amount of phosphate incorporated at tyrosine 416 could result from pp60$^{c\text{-}src}$ molecules that were solubilized to a greater extent than the majority of pp60$^{c\text{-}src}$ in the extract. Investigation of this possibility gave a puzzling result. Titration of the detergent used in the assay from 1 to 0.01% resulted in a gradual decrease of tyrosine 527 phosphorylation and a concurrent increase in phosphorylation at tyrosine 416, with no change in the overall efficiency of phosphorylation of pp60$^{c\text{-}src}$. This result was unexpected since one might predict that, with increased concentrations of detergent, pp60$^{c\text{-}src}$ would be more likely to undergo autophosphorylation at tyrosine 416. One possible explanation of these results is that the tyrosine 527 phosphatase is detergent sensitive and that the detergent inhibits turnover of phosphate at this site.

Taken together, the results presented here are consistent with the hypothesis suggested previously (10, 22) that phosphorylation of pp60$^{c\text{-}src}$ on tyrosine 527 may be mediated by another cellular tyrosine kinase. Our data suggest that this kinase would have access to both a soluble and a membrane-bound substrate. This raises the possibility that this kinase resides in the cytoplasm. Most of the well-studied tyrosine kinases are located in the plasma membrane; however, some members of the c-*abl*-encoded kinase family (25) and the c-*fps*-encoded tyrosine kinase (37) are proposed to be cytoplasmic enzymes.

The prospect that a cellular kinase distinct from pp60$^{c\text{-}src}$ is responsible for the phosphorylation of tyrosine 527 is very important in light of the facts that this residue is highly conserved in the *src* family of tyrosine kinases (18) and that phosphorylation at this site has been demonstrated to negatively regulate the in vitro tyrosine-specific kinase activity of pp60$^{c\text{-}src}$ (10, 11). Identification of the enzyme that may regulate the tyrosine-specific protein kinase activity of pp60$^{c\text{-}src}$ may reveal a universal mechanism used to regulate all of the tyrosine kinases in the *src* gene family.

ACKNOWLEDGMENTS

We thank H. Hanafusa for providing the c-*src* gene, D. Shalloway and T. Kmiecik for the use of their pMc*src* 295 mutant and NIH(pMc*src*/cos)A cells, S. Hughes for use of his Rous sarcoma virus vector system, L. Fox for modification of plasmids used in this work, M. DeMarco for technical assistance, and K. Donnelly for superb secretarial assistance.

This work was supported by Public Health Service grant CA27951 from the National Institutes of Health, and J.S.B. receives partial salary support from an American Cancer Society Faculty Research Award.

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