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A Mutation at the ATP-Binding Site of pp60$^{v\text{-}src}$ Abolishes Kinase Activity, Transformation, and Tumorigenicity

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We constructed a mutant, called RSV-SF2, at the ATP-binding site of pp60$^{v\text{-}src}$. In this mutant, lysine-295 is replaced with methionine. SF2 pp60$^{v\text{-}src}$ was found to have a half-life similar to that of wild-type pp60$^{v\text{-}src}$ and was localized in the membranous fraction of the cell. Rat cells expressing SF2 pp60$^{v\text{-}src}$ were morphologically untransformed and do not form tumors. The SF2 pp60$^{v\text{-}src}$ isolated from these cells lacked kinase activity with either specific immunoglobulin or other substrates, and expression of SF2 pp60$^{v\text{-}src}$ failed to cause an increase of total phosphotyrosine in the proteins of infected cells. Wild-type pp60$^{v\text{-}src}$ was phosphorylated on serine and tyrosine in infected cells, and the analogous phosphorylations could also be carried out in vitro. Phosphorylation of serine was catalyzed by a cyclic AMP-dependent protein kinase, and phosphorylation of tyrosine was perhaps catalyzed by pp60$^{v\text{-}src}$ itself. By contrast, SF2 pp60$^{v\text{-}src}$ could not be phosphorylated on serine or tyrosine either in infected cells or in vitro. These findings strengthen the belief that the phosphotransferase activity of pp60$^{v\text{-}src}$ is required for neoplastic transformation by the protein and suggest that the binding of ATP to pp60$^{v\text{-}src}$ elicits an allosteric change required for phosphorylation of serine in the protein.

The tumorigenicity and in vitro transforming ability of Rous sarcoma virus (RSV) reside in pp60$^{v\text{-}src}$ (4), the protein encoded by the viral oncogene v-*src* (3, 33, 48). pp60$^{v\text{-}src}$ has tyrosine-specific protein kinase activity (10, 11, 21, 26, 27, 36) and is itself phosphorylated, both on serine-17 (and, to a lesser degree, on at least one other serine) in the amino-terminal half of the protein (9, 16) and on tyrosine-416 (32, 41). Recent evidence has also demonstrated the existence of phosphotyrosines in the amino-terminal half of the protein that may play a role in regulation of enzymatic activity (7, 12, 34).

Evidence from studies on mutants of v-*src* suggests that pp60$^{v\text{-}src}$ may have as-yet-undiscovered functions independent of the kinase activity which are necessary for full expression of transformation (5, 13, 45). To examine this possibility more closely, it would be desirable to identify residues involved in kinase activity and then mutate them to eliminate this activity while preserving other possible functions of pp60$^{v\text{-}src}$. Both indirect (2) and direct (23) evidence now exists that lysine-295 is intimately involved with the ATP binding of pp60$^{v\text{-}src}$. We wanted to obtain a mutation at this site that would eliminate tyrosine kinase activity without altering overall structure or other active functions. In this paper, we report on the construction and expression of such a mutant, which we call RSV-SF2, and the effect of this mutation on the structure and activity of pp60$^{v\text{-}src}$ and on the expression of some parameters of transformation.

MATERIALS AND METHODS

Growth and labeling of cells. The procedures for growth and labeling of mammalian cells have been described elsewhere (42, 43), except that the ^{35}S-labeling medium was composed of methionine-free Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 20 μM L-methionine. Cells were labeled in this medium for 18 to 24 h. ^{32}P-labeling was for 4 or 18 h, as indicated.

Site-specific mutagenesis. Procedures for site-specific mutagenesis on M13 and selection of mutated DNA have been described earlier (43). The 19-base oligonucleotide used for converting lysine-295 of v-*src* to methionine was TGGC-CATAATGACTCTGAA.

Plasmid construction and DNA transfection. Plasmid pSVEneo-src was constructed by inserting into plasmid pSVsrcLTR (43) the neomycin resistance gene under control of the simian virus 40 early promoter (a 540-base-pair *Hin*cII-*Hin*dIII restriction fragment [1]) and hepatitis B virus polyadenylation sequences (a 536-base-pair *Sac*II-*Bgl*II restriction fragment from the hepatitis B viral genome which specifies a major viral polyadenylation site [40]). Plasmid pSVEneo-src/met295 is identical except for methionine at codon 295 of the v-*src* gene.

Rat-1 cells were transfected and selected in medium containing G-418 as described previously (35).

Immunoprecipitation. The general procedures for immunoprecipitation with tumor-bearing rabbit serum 5, which recognizes viral but not endogenous rat pp60$^{v\text{-}src}$, have been described previously (26, 43). In some cases, pp60$^{v\text{-}src}$ was immunoprecipitated with monoclonal antibody 443 (the generous gift of Joan Brugge); in this case, adsorption of the antibody-antigen complex to protein A was effected by the addition of an equal volume of rabbit anti-mouse immunoglobulin G (IgG) (Cappel Laboratories).

Enzymatic assays. The assay for phosphorylation of immunoglobulin by pp60$^{v\text{-}src}$ in an immune complex has been previously described (10, 26, 31). The assay for phosphorylation of enolase was similar to the procedure described previously (13), except that the monoclonal antibody used here recognizes pp60$^{v\text{-}src}$, and cells expressing pp60$^{v\text{-}src}$ were used as a source for kinase.

Half-life of pp60$^{v\text{-}src}$. For determining the stability of wild-type and mutant pp60$^{v\text{-}src}$, 60-mm plates of 25% confluent cells were labeled for 24 h with 0.2 mCi of L-[^{35}S]methionine. The labeling medium was then removed and replaced with growth medium (containing ca. 0.2 mM L-methionine). At this time and various times thereafter, lysates were prepared

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and pp60$^{v\text{-}src}$ was immunoprecipitated and isolated on a 9% polyacrylamide gel. The pp60$^{v\text{-}src}$ bands were visualized by direct autoradiography, excised, and then solubilized in 1.0 ml of NCS (Amersham Corp.) for 2 h at 55°C. After the addition of 14 ml of LiquiScint (Amersham), radioactivity was determined by liquid scintillation spectrometry.

Subcellular localization of pp60$^{v\text{-}src}$. The procedure used was that of Snyder and Bishop (42).

Phosphoamino acid analysis. The protocol for analyzing whole-cell phosphoamino acids by two-dimensional thin-layer electrophoresis has been described previously (21, 43).

In vitro phosphorylation of pp60$^{v\text{-}src}$. Phosphorylation by catalytic subunit of cAMP-dependent protein kinase (the generous gift of Theodoor van Daalen Wetters) was performed as follows: 100-mm plates of half-confluent cells were immunoprecipitated with 2 μl of serum 5 on 5 μl (settled volume) of protein A-Sepharose (Sigma Chemical Co.) and washed three times in RIPA buffer (4) and once in assay buffer (50 mM sodium phosphate [pH 7.0], 10 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mg of bovine serum albumin per ml, 0.1 mM EGTA). To 2.5 μl of beads was then added 10 μl of assay buffer containing 5 μCi of [γ-^{32}P]ATP (0.13 μM; 3,000 Ci/mmol; Amersham) and 0.3 μl of the catalytic subunit of cAMP-dependent protein kinase (4.3 μg/μl; 14.3 U/μl). Samples were incubated at 37°C for 15 min. The reaction was terminated by dilution into 1 ml of Puck saline plus 1 mM EDTA, followed by centrifugation and addition of 50 μl of sample buffer (25) to the beads.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were separated on 9 and 14% sodium dodecyl sulfate-polyacrylamide gels according to the procedure of Laemmli (25).

Autoradiography. All polyacrylamide gels were autoradiographed by using Kodak X-Omat AR film.

RESULTS

Mutagenesis of lysine-295 (wild type) to methionine (SF2). Our choice for methionine as the replacement amino acid for lysine-295 was based on structural considerations. The side chains of methionine and lysine are similar in terms of bond lengths and angles (47). The major difference is the substitution of a hydrogen atom on methionine for the ε-amino group of lysine. This substitution, it was hoped, would eliminate a positive charge necessary for ATP binding and, hence, kinase activity. Although some 95% of all lysines are exposed to solvent (22), 35% of all methionines are so positioned (compared, for instance, to 20% of valines or 18% of cysteines); a methionine at position 295 would thus have a reasonable chance to remain on the surface of the protein, preserving the overall structure of the local domain.

Two types of mutations at lysine-295 were not chosen. First, conversion of the positively charged lysine to a negatively charged amino acid, such as glutamate or aspartate, was ruled out because of the possibility that lysine might be found in an ionic bond with a negatively charged amino acid. Conversion of the charge at position 295 from positive to negative might then result in charge-charge repulsion, which would disrupt the local structure. Replacement of lysine with methionine, on the other hand, would also eliminate the ionic bond without also repelling the remaining negatively charged half of the original ionic pair. Second, we did not convert the lysine to arginine because of the possibility that this change would not completely eliminate ATP binding.

In support of the choice of methionine, this amino acid has been found to be a reasonable substitution when present-day protein sequences are compared to those of common ancestors (17). Lysine-to-methionine changes occur at no less than the rate for random mutations. Indeed, by this measurement, only arginine (3-fold more frequently than random), asparagine (1.3-fold), and glutamine (1.3-fold) occur more often than methionine as a replacement for lysine.

Strategy for transfection of rat-1 cells and expression of wild-type and SF2 v-*src*. Expression of the v-*src* genes was accomplished by using the simian virus 40 early promoter to initiate transcription and the RSV long terminal repeat to terminate it. A similar vector was used previously to express pp60$^{v\text{-}src}$ in mouse 3T3 cells (43). Because SF2 was not expected to transform cells, selection by focus formation was not expected to be possible. The neomycin resistance gene (6, 44), expressed by means of a second simian virus 40 early promoter and the hepatitis B viral polyadenylation sequence, was included in the plasmids to provide for a selectable marker of transfection.

The expression vectors for mutant and wild-type *src* were introduced into rat-1 cells by the procedure of Graham and van der Eb (20). After transfection with 10 μg of expression vector, cells carrying the plasmids were selected by growth in 350 μg of G-418 per ml. Approximately 80 to 100 G-418-resistant colonies appeared in both transfections. 95% of those transfected with wild-type v-*src* displayed the highly rounded cellular morphology typical of transformed cells; a representative clone is shown in Fig. 1C. Cells that received the plasmid expressing SF2 v-*src*, however, were always found to be flat (Fig. 1B) and virtually indistinguishable from the parent rat-1 cell line (Fig. 1A), indicating that RSV-SF2 is unable to induce morphological transformation.

Expression of the wild type and RSV-SF2 in G-418-resistant cells. To confirm that the selected clones were expressing pp60$^{v\text{-}src}$, cells were labeled overnight with [^{35}S]methionine, and cell lysates were immunoprecipitated with antiserum directed against pp60$^{v\text{-}src}$. The results are shown in Fig. 2. A protein with a molecular weight of 60,000 (lane 2) was obtained from cells transfected with the expression vector encoding wild-type pp60$^{v\text{-}src}$ and whose morphology was representative of the transformed state. Similar levels of a 60,000-dalton protein were found in four of five G-418-resistant clones selected from the SF2 plasmid transfection, with the cells analyzed in lane 3 showing appreciably lower levels of expression. Immunoprecipitation from untransfected cells produced no labeled material at 60,000 daltons (lane 1). The experiments described below used the wild-type clone analyzed in lane 2 and the SF2 pp60$^{v\text{-}src}$-expressing clone analyzed in lane 5, both of which express similar amounts of pp60$^{v\text{-}src}$ protein.

The antiserum used in this immunoprecipitation was selected because of its extremely low affinity for rat pp60$^{c\text{-}src}$, the cellular homolog of pp60$^{v\text{-}src}$. The quantity of labeled protein immunoprecipitated from the RSV-SF2-transfected cells represented levels of transcription consistent with expression of v-*src*, but not of c-*src*. V8 proteolysis of the 60,000-dalton proteins from cells transfected with both wild-type and SF2 v-*src* (data not shown) yielded patterns identical to those observed earlier for pp60$^{v\text{-}src}$ (29).

Anchorage-independent growth of SF2-expressing cells. One hallmark of the transformed state is the ability of cells to exhibit anchorage-independent growth. To determine whether RSV-SF2 conferred this property on cells, 10^3 cells expressing either wild-type or SF2 pp60$^{v\text{-}src}$ were seeded into soft agar and monitored for colony formation (Fig. 3). Cells expressing wild-type pp60$^{v\text{-}src}$ grew well in soft agar, with virtually all seeded cells eventually forming colonies. How-

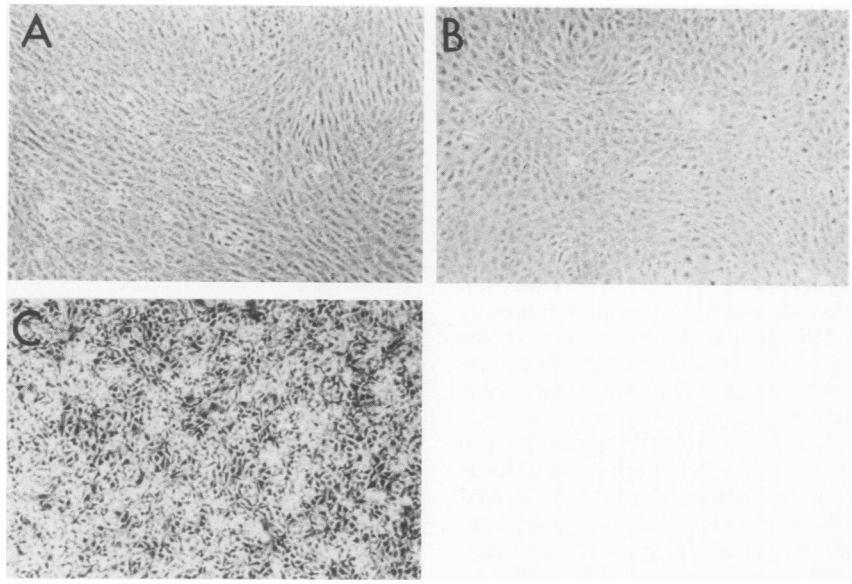

FIG. 1. Morphologies of normal cells and of G418-resistant cells expressing either wild-type or SF2 v-*src*. Monolayers were photographed at confluence. (A) Normal rat-1 cells. (B) G-418-resistant cells expressing SF2 pp60$^{v\text{-}src}$. (C) G-418-resistant cells expressing wild-type pp60$^{v\text{-}src}$.

ever, SF2 pp60$^{v\text{-}src}$-expressing cells were never observed to form colonies. Indeed, microscopic examination of the plates indicated that these cells, after 2 weeks, had failed to undergo even limited division. Thus, by two criteria, altered morphology and anchorage-independent growth, RSV-SF2 is unable to transform cells.

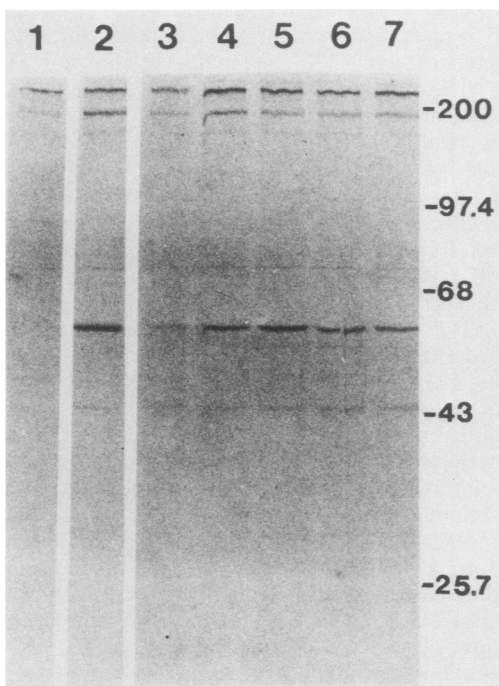

FIG. 2. Expression of pp60$^{v\text{-}src}$ in G-418-resistant transfectants. Cells were labeled for 18 h with L-[^{35}S]methionine before lysis and then analyzed by immunoprecipitation with TBR serum 5, which has low affinity for murine pp60$^{c\text{-}src}$. Lane 1, Normal cells; lane 2, visibly transformed cells from wild-type v-*src* transfection; lanes 3 through 7, clones from transfection with SF2 v-*src*. Numbers on the right refer to molecular weight markers ($\times 10^3$).

Tumorigenicity of cells expressing SF2 pp60$^{v\text{-}src}$. Because cells expressing RSV-SF2 displayed none of the tested properties associated with the transformed state, we expected that these cells would not be tumorigenic in nude mice. SF2-expressing cells failed to cause tumors when as many as 10^7 cells were injected, whereas cells transformed with wild-type v-*src* were tumorigenic when as few as 10^4 cells were given (Table 1). Thus, abolishing the kinase activity of pp60$^{v\text{-}src}$ by altering the ATP-binding site eliminates the tumorigenic potential of this protein.

Enzymatic activity of SF2 pp60$^{v\text{-}src}$. The lysine-to-methionine substitution was designed to eliminate the kinase activity of pp60$^{v\text{-}src}$ while at the same time preserving, as closely as possible, the overall wild-type structure of the protein. We tested SF2 pp60$^{v\text{-}src}$ for activity in two ways. First, the ability of the mutant to phosphorylate the heavy chain of IgG in an immune complex was measured. This assay was the one that was originally used to demonstrate the kinase activity of pp60$^{v\text{-}src}$ (10, 26). SF2 pp60$^{v\text{-}src}$ is devoid of kinase activity towards immunoglobulin (Fig. 4A).

The possibility existed that the mutant enzyme might still possess kinase activity but that this activity could no longer recognize the IgG heavy chain in the polyclonal immune complex. To exclude this possibility, mutant and wild-type pp60$^{v\text{-}src}$ were immunoprecipitated by using a monoclonal antibody, and the ability of pp60$^{v\text{-}src}$ to phosphorylate exogenously added enolase was measured as described previously (13). Enolase serves as a good substrate for wild-type pp60$^{v\text{-}src}$, as previously demonstrated (13) (Fig. 4B). The small amount of activity seen with the immunoprecipitate from untransformed cells may represent pp60$^{c\text{-}src}$. The immunoprecipitate from SF2-expressing cells shows the same amount of activity as in untransformed cells, providing a second indication that RSV-SF2 is devoid of kinase activity. The monoclonal antibody used in this experiment is not a substrate for pp60$^{v\text{-}src}$ and hence is not phosphorylated (28).

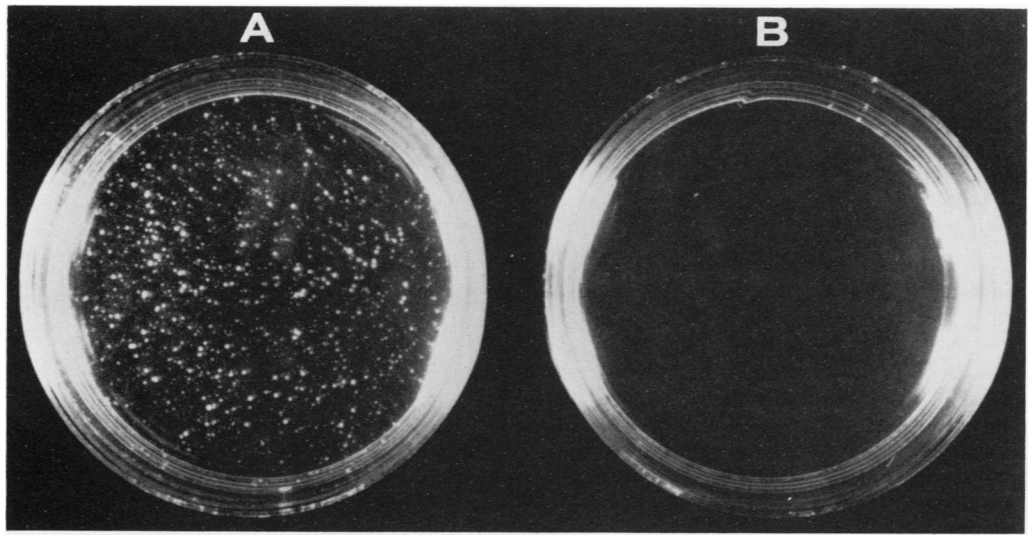

FIG. 3. Growth of transfected clones in soft agar. (A) Cells expressing wild-type pp60$^{v\text{-}src}$. (B) Cells expressing SF2 pp60$^{v\text{-}src}$. A total of 10^3 cells were seeded into growth medium containing 0.45% agar and photographed 2 weeks later.

Previous work (12, 19) indicated that phosphorylation of tyrosine-416 is due to autophosphorylation, although this finding is controversial (27). If the phosphorylation of tyrosine in pp60$^{v\text{-}src}$ is indeed autocatalytic, then SF2 pp60$^{v\text{-}src}$ should not be phosphorylated on tyrosine. The results shown in Fig. 4B, where wild-type pp60$^{v\text{-}src}$ (lane 3) but not SF2 pp60$^{v\text{-}src}$ (lane 2) becomes labeled in the presence of [γ-^{32}P]ATP, are consistent with this view. However, inasmuch as alteration of the tertiary structure around tyrosine-416 due to the SF2 mutation cannot be ruled out, the ability of pp60$^{v\text{-}src}$ to autophosphorylate is not resolved by this data.

To examine the possibility that SF2 pp60$^{v\text{-}src}$, although itself devoid of kinase activity, might somehow stimulate other tyrosine kinases in vivo, cells were labeled to equilibrium with $^{32}PO_4$, and the phosphoamino acids were separated and quantitated. The amount of phosphotyrosine in normal rat-1 cells is 0.09% of total phosphoamino acids, in agreement with levels observed for other normal cells (Table 2) (21, 38). After transformation by wild-type pp60$^{v\text{-}src}$, this level rises ninefold, to 0.84%. Expression of SF2 pp60$^{v\text{-}src}$, however, does not lead to an increase, indicating that the SF2 mutation neither directly nor indirectly causes phosphorylation of tyrosine residues in protein.

Half-life and subcellular location of SF2 pp60$^{v\text{-}src}$. We had hoped that the lysine-to-methionine mutation would eliminate the kinase and tumorigenic activities of pp60$^{v\text{-}src}$ without altering the overall protein structure. In an attempt to address this, two other properties of pp60$^{v\text{-}src}$ were measured, half-life and subcellular localization. The half-life of wild-type pp60$^{v\text{-}src}$ was found to be 8.6 h (Table 3), in agreement with the values reported previously (39). The half-life of SF2 pp60$^{v\text{-}src}$ was found to be 8.4 h, not significantly different from its wild-type counterpart.

Wild-type pp60$^{v\text{-}src}$ has been found to associate with the plasma membrane of transformed cells (15, 24, 49). Both wild-type pp60$^{v\text{-}src}$ and mutant pp60$^{v\text{-}src}$ were found largely in a membrane fraction, suggesting that the mutation does not alter the distribution of the protein within the cell (Table 3).

Effect of the SF2 mutation on phosphorylation of pp60$^{v\text{-}src}$. To determine whether the mutation in SF2 affected the phosphorylations of pp60$^{v\text{-}src}$ in vivo, cells were labeled with $^{32}PO_4$, and pp60$^{v\text{-}src}$ was isolated from cell lysates (Fig. 5). Whereas wild-type pp60$^{v\text{-}src}$ is phosphorylated as expected, SF2 pp60$^{v\text{-}src}$ lacked all phosphorylation, as evidenced by the absence of a band at 60,000 daltons. This experiment was repeated several times, and in no case was phosphorylation of SF2 pp60$^{v\text{-}src}$ ever observed. (The protein migrating in advance of pp60$^{v\text{-}src}$ arises from proteolysis of pp60$^{v\text{-}src}$; see reference 29.)

If, as has been suggested (19), pp60$^{v\text{-}src}$ autophosphorylates its tyrosines, then loss of this phosphorylation in vivo would be expected for SF2 pp60$^{v\text{-}src}$. However, phosphorylation of the amino-terminal serine(s) apparently results from the action of cellular cAMP-dependent protein kinase (8) rather than pp60$^{v\text{-}src}$ itself. Additionally, mutations at or near serine-17 do not appear to affect kinase activity (16). Given these observations, we were surprised by the absence of all phosphorylation in SF2 pp60$^{v\text{-}src}$.

The data suggest that the failure of SF2 pp60$^{v\text{-}src}$ to be phosphorylated in vivo is due to an intrinsic property of the protein. To test this, immunoprecipitated pp60$^{v\text{-}src}$ was incubated in the presence of [γ-^{32}P]ATP and the catalytic subunit from cAMP-dependent protein kinase. Wild-type pp60$^{v\text{-}src}$ is clearly a substrate for this enzyme, becoming heavily phosphorylated (Fig. 6). However, SF2 pp60$^{v\text{-}src}$ is not phosphorylated under these conditions. Although it was not explicitly determined that serine-17 was the residue

TABLE 1. Tumorigenicity of SF2 pp60$^{v\text{-}src}$-expressing cells[a]

Cell type	No. of cells injected	Tumor formation[b]	Time of tumor appearance (days after injection)
Rat-1	10^7	−	
Transformed by wild-type	10^4	+	15
	10^5	+	14
	10^6	+	12
Expressing SF2 pp60$^{v\text{-}src}$	10^7	−	

[a] Three-week-old *nu/nu* mice were injected intradermally and followed for tumor formation.

[b] +, Tumors were evident in three of three mice; −, no tumors were observed in three mice after 2 months.

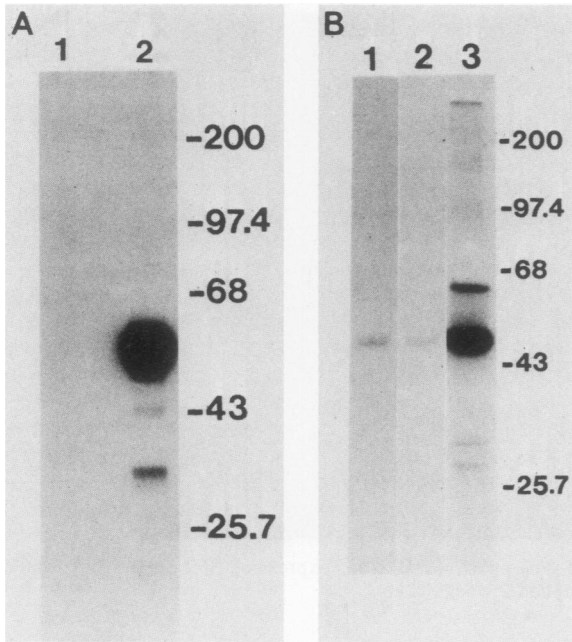

FIG. 4. Kinase activity of pp60$^{v\text{-}src}$ towards IgG and enolase. (A) One hundred-millimeter plates of cells expressing wild-type or SF2 v-*src* were immunoprecipitated with 2 μl of serum 5. The immune complexes were washed and incubated with [γ-^{32}P]ATP as described, and the phosphorylated products were separated on a 9% sodium dodecyl sulfate-polyacrylamide gel. Lane 1, SF2 pp60$^{v\text{-}src}$; lane 2, wild-type pp60$^{v\text{-}src}$. Numbers on the right refer to molecular weight markers (×10^3). (B) Enolase phosphorylations were carried out as described (13) by using monoclonal antibody 443. Each point represents immune precipitate from one-half of a half-confluent 100-mm plate. All reactions were for 15 min. Lane 1, Normal cells; lane 2, SF2-expressing cells; lane 3, cells transformed by wild-type pp60$^{v\text{-}src}$. Numbers to the right refer to molecular weight markers (×10^3).

phosphorylated in the wild type, V8 mapping (data not shown) indicated that the phosphorylation was in the amino-terminal half of the molecule.

It should be pointed out that in this experiment all components necessary for immune complex phosphorylation by wild-type pp60$^{v\text{-}src}$ were present, as reflected by the phosphorylation of the IgG heavy chain. A comparison of lanes 3 and 6 appears to show a decrease in IgG phosphorylation associated with the concomitant serine phosphorylation. This decrease is most probably due to the competition of pp60$^{v\text{-}src}$ with the catalytic subunit for ATP: under these conditions, the catalytic subunit and ATP were present in stoichiometric amounts and in great excess over pp60$^{v\text{-}src}$.

TABLE 2. Phosphoamino acid levels in normal cells and cells expressing pp60$^{v\text{-}src}$ [a]

Cell type	% Total phosphoamino acids in ^{32}P-labeled:		
	Serine	Threonine	Tyrosine
Normal	94.66	5.27	0.089
Transformed by wild-type pp60$^{v\text{-}src}$	94.93	4.46	0.84
SF2 pp60$^{v\text{-}src}$-expressing	95.81	4.10	0.087

[a] Procedures for the labeling of cells and the separation of phosphoamino acids are described in the text.

TABLE 3. Half-life and subcellular location of SF2 pp60$^{v\text{-}src}$ [a]

pp60$^{v\text{-}src}$ type	Half-life (h)	% pp60$^{v\text{-}src}$ in fraction:	
		Soluble	Membrane
Wild type	8.6	10	90
SF2	8.4	11	89

[a] Procedures for determining the half-life and subcellular location are discussed in the text.

DISCUSSION

Role of lysine-295 in catalysis by pp60$^{v\text{-}src}$. Lysine-295 has been implicated in the ATP binding of pp60$^{v\text{-}src}$ by homology with sequences of cAMP-dependent protein kinase (2) and by reaction with the ATP analog *p*-fluorosulfonylbenzoyl 5′-adenosine (23). As such, alteration of the properties of this residue would be expected to abolish kinase activity. We have converted lysine-295 to methionine by site-specific mutagenesis, using a change that removes the positive charge postulated to interact with the γ-phosphate group of ATP. We have shown that this change eliminates the ability of pp60$^{v\text{-}src}$ to phosphorylate substrates on tyrosine in vitro and to increase the amount of cellular phosphotyrosine in cells. These data are in accord with previous suggestions for the role of lysine-295 in the ATP-binding step of pp60$^{v\text{-}src}$ catalysis. We have not tested directly the ability of SF pp60$^{v\text{-}src}$ to bind ATP, as this will require the purification of large amounts of protein. Additionally, we have not examined SF2 pp60$^{v\text{-}src}$ for the loss of *p*-fluorosulfonylbenzoyl 5′-adenosine binding, since it is clear that the ←amino group

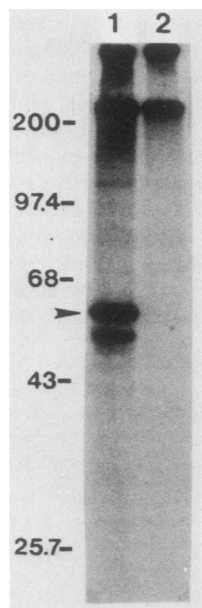

FIG. 5. In vivo phosphorylation of SF2 pp60$^{v\text{-}src}$. Half-confluent 100-mm plates of cells were labeled for 4 h with 1 mCi of $^{32}PO_4$ before lysis and immunoprecipitation with serum 5. Lane 1, Wild-type pp60$^{v\text{-}src}$; lane 2, SF2 pp60$^{v\text{-}src}$. The arrow points to the location to which pp60$^{v\text{-}src}$ migrates. Numbers to the left refer to molecular weight markers (×10^3).

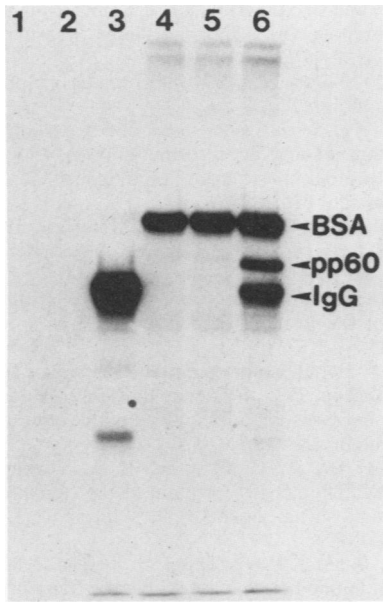

FIG. 6. Phosphorylation of pp60$^{v\text{-}src}$ by the catalytic subunit of cAMP-dependent protein kinase. Reactions were carried out as described in the text. Each point contained the immune precipitate from one-half of a half-confluent 100-mm dish. Lanes 1 and 4, Normal cells; lanes 2 and 5, cells expressing SF2 pp60$^{v\text{-}src}$; lanes 3 and 6, cells transformed by wild-type pp60$^{v\text{-}src}$. Incubations were done in the absence (lanes 1 through 3) or presence (lanes 4 through 6) of the catalytic subunit. BSA, Bovine serum albumin. IgG, heavy chain of IgG.

necessary for the reaction with *p*-fluorosulfonylbenzoyl 5'-adenosine is no longer present at position 295.

Effect of mutation at the ATP-binding site on phosphorylation of serine-17. Previous work (16) demonstrated that mutations at or near serine-17 had little effect on kinase activity. Indeed, a mutant of pp60$^{v\text{-}src}$ in which the first 25% of the protein is deleted still retains a modest amount of activity (30). Given these results, we were surprised to discover that a mutation at the ATP-binding site eliminated phosphorylation of serine-17, some 280 amino acids away from position 295.

Such a phenomenon is, however, not without precedent. The conversion of phosphorylase *a* to *b* requires serine-14 to be dephosphorylated (18). This reaction cannot take place if the ATP-binding site is occupied by ligands such as AMP. In this case, the serine residue is forced into a position inaccessibile to the phosphatase. Although this is an example of the dephosphorylation, rather than the phosphorylation, of serine being affected by changes at the ATP-binding site, the principle involved is the same. We therefore suggest that the binding of ATP to pp60$^{v\text{-}src}$ effects an allosteric change that is required to expose serine-17 for phosphorylation.

Effect of SF2 mutation on pp60$^{v\text{-}src}$ structure. The choice of methionine to replace lysine at position 295 was designed to minimize changes in tertiary structure. Such a requirement is necessary if other functions of pp60$^{v\text{-}src}$ are to be preserved. The side-chain backbone of methionine closely resembles that of lysine, even though sulfur has replaced the corresponding lysine carbon. The major change is in the replacement of the positively charged ε-amino group of lysine with a hydrogen atom. The backbone structure of the two residues is similar, and 35% of all methionines are found on the surface of proteins. Additionally, the choice of methionine for lysine does not seem to be particularly selected against during evolution. We thus had hoped that the change from lysine to methionine would be conservative. Two observations indicate that this hope has been borne out. First, the half-life of SF2 pp60$^{v\text{-}src}$ does not appear to be reduced relative to wild-type pp60$^{v\text{-}src}$. Second, SF2 pp60$^{v\text{-}src}$ associates with a membranous fraction of the cell. Although we have not rigorously shown this fraction to be the plasma membrane, previous results leave little doubt that it is the plasma membrane to which a major fraction of pp60$^{v\text{-}src}$ is bound. By these two measurements, the overall structure of pp60$^{v\text{-}src}$ appears not to be altered by the SF2 mutation.

Effect of loss of catalytic activity on transforming and tumorigenic activity of pp60$^{v\text{-}src}$. The loss of kinase activity was expected to eliminate the ability of pp60$^{v\text{-}src}$ to transform cells in culture and cause tumors. The in vitro studies described here on cells expressing SF2 pp60$^{v\text{-}src}$ indicate that kinase activity is an absolute requirement for the expression of elevated cellular phosphotyrosine levels, focus formation, growth in soft agar, and tumorigenicity.

In the past decade, a large number of mutants of v-*src* have been studied that cause only partial or altered transformation in a variety of cell types (reference 45 and references therein). These results have led to the hypothesis that pp60$^{v\text{-}src}$ may have activities other than strictly that of a protein kinase. Indeed, the description of pp60$^{v\text{-}src}$ has recently been expanded from merely tyrosine kinase to phospholipid kinase as well (46). The results presented here demonstrate that, upon elimination of protein tyrosine kinase activity, the most obvious and often-examined heralds of transformation disappear also. These findings do not distinguish between requirements for protein and phospholipid kinase activity in transformation, however, because there is apparently only one ATP-binding site in pp60$^{v\text{-}src}$ (23), which presumably services both forms of phosphotransfer. As a caveat to this conclusion, we should point out that, in spite of the similarities between wild-type and SF2 pp60$^{v\text{-}src}$ structure discussed above, we have not rigorously excluded the possibility that the SF2 mutation results in widespread denaturation of the protein.

A number of the above-mentioned studies on mutants based their hypotheses for other active functions of pp60$^{v\text{-}src}$ on the lack of kinase activity under conditions of partial transformation. However, as previously pointed out (37), lack of in vitro catalysis may be due only to the conditions used to measure catalysis and not to a true absence of kinase activity. By contrast, we have created a mutation that by its nature must fully inactivate the kinase activity of pp60$^{v\text{-}src}$. We therefore conclude that at least some of the classic transformation hallmarks are absolutely dependent upon tyrosine kinase activity. The partial or intermediate phenotypes observed previously may be due to a general reduction, but not elimination, of kinase activity or to loss of tyrosine phosphorylation of some, but not all, substrates.

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