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pp60$^{c\text{-}src}$ Variants Containing Lesions That Affect Phosphorylation at Tyrosines 416 and 527

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The biological and biochemical properties of pp60$^{c\text{-}src}$ are regulated, in part, by phosphorylation at Tyr-416 and Tyr-527. The tyrosine kinase and transforming activities of pp60$^{c\text{-}src}$ are suppressed by phosphorylation at Tyr-527, whereas full activation of pp60$^{c\text{-}src}$ requires phosphorylation at Tyr-416. To test specifically the significance of the negatively charged phosphate moieties on these tyrosine residues, we have substituted the codons for both residues with codons for either Glu or Gln. A negatively charged Glu at position 527 was unable to mimic a phosphorylated Tyr at this position, and, in consequence, the mutated pp60$^{c\text{-}src}$ was activated and transforming. Similarly, substitution of Tyr-416 with Glu was unable to stimulate the activities of the enzyme. However, mutagenesis of Tyr-416 to Gln (to form the mutant 416Q) activated the kinase activity approximately twofold over that observed for wild-type pp60$^{c\text{-}src}$. When introduced into the mutant 527F (containing Phe-527 instead of Tyr), the double mutant 416Q-527F exhibited weak transforming activity. This is in contrast to the other double mutants 416E-527F and 416F-527F, which were nontransforming. The biochemical basis by which 416Q activates pp60$^{c\text{-}src}$ is not understood but probably involves some local conformational perturbation. Deletion of residues 519 to 524 (RH5), a region previously shown to be necessary for association with middle-T antigen, led to loss of phosphorylation at Tyr-527 and activation of the enzymatic and focus-forming activities of pp60$^{c\text{-}src}$. Hence, the sequences necessary for complex formation with middle-T antigen may also be required by the kinase(s) which phosphorylates Tyr-527 in vivo. This suggests that normal cells contain cellular proteins which are analogous to middle-T antigen and whose action regulates the activity of pp60$^{c\text{-}src}$ by controlling phosphorylation or dephosphorylation at residue 527.

The phosphoprotein pp60$^{c\text{-}src}$ is a membrane-associated protein tyrosine kinase (2, 11, 21, 29). It is structurally and functionally related to pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus. However, unlike its viral homolog, the cellular pp60$^{c\text{-}src}$ protein normally does not elicit neoplastic transformation even when expressed at levels 10 times higher than that required by pp60$^{v\text{-}src}$ (23, 24, 34, 42). This disparity is due primarily to differences in their respective tyrosine kinase activities (18, 22). That pp60$^{c\text{-}src}$ does not normally promote cellular transformation appears to be due to suppression of its kinase activity.

Examination by site-directed mutagenesis has demonstrated that the kinase and transforming activities of pp60$^{c\text{-}src}$ are regulated, at least in part, by phosphorylation at multiple sites. The enzyme is negatively regulated by phosphorylation at Tyr-527, the major site of phosphorylation in vivo (3, 8, 13, 15, 26, 38, 40), and is positively regulated by phosphorylation at Tyr-416, the site of autophosphorylation in vitro (8, 26). Other potential regulatory sites include Ser-12, the site phosphorylated by protein kinase C (19), and Ser-17, the site phosphorylated by cyclic AMP-dependent kinase (36). Ser-17 is modified or dephosphorylated in mitotic cells, an event which correlates with increased kinase activity (5). Furthermore, pp60$^{c\text{-}src}$ from platelet-derived growth factor-treated cells (39) or from polyomavirus-infected cells (46) displays enhanced catalytic activity and contains additional sites of tyrosine phosphorylation within the amino terminus of the enzyme. However, it is unclear whether these phosphorylation events are a consequence of the observed activation of pp60$^{c\text{-}src}$ or indeed themselves contribute to the activation of pp60$^{c\text{-}src}$.

The role of Tyr residues (which are sites of phosphorylation in vivo) in modulating the activities of other protein tyrosine kinases of both the *src* family and the growth factor receptor family of kinases is well documented (for a review, see reference 45). Recently, we and others have shown that replacement of Tyr-527 with Ser, Cys, or Phe activates the kinase and transforming activities of pp60$^{c\text{-}src}$ (8, 40). This argues that the Tyr is normally phosphorylated by a tyrosine-specific kinase or that the Tyr must be conserved for reasons of structural integrity and cannot be replaced by other amino acids. In addition, phosphorylation of Tyr-416 is required for full activation of the 527F mutant (26, 38), since the 416F-527F mutant, containing both the Tyr-416 to Phe and the Tyr-527 to Phe mutations, exhibits reduced kinase activity and is nontransforming. In an attempt to understand in greater detail the role of the phosphorylated Tyr-527 in mediating its suppressive effect and that of phosphorylated Tyr-416 in augmenting the activities of pp60$^{c\text{-}src}$, the codons at positions 527 and 416 have been altered to encode Glu and Gln. The residue Glu was chosen because it is negatively charged and therefore might be able to mimic phosphorylated Tyr. An analogous study using the nuclear location signal of the simian virus 40 large T antigen has shown that a positively charged Arg is partially able to substitute for Lys-128 for nuclear compartmentalization (10).

Middle-T antigen, the transforming protein of polyomavirus, forms a complex with several cellular tyrosine kinases, including pp60$^{c\text{-}src}$ (16), pp62$^{c\text{-}yes}$ (27) and pp59$^{c\text{-}fyn}$ (6, 28). Complex formation of pp60$^{c\text{-}src}$ with the viral protein results in enhanced tyrosine kinase activity and reduced phosphorylation at Tyr-527 (1, 4, 15). Concomitant with this is phosphorylation at Tyr-416. Analysis of mutants of pp60$^{c\text{-}src}$ have implicated the carboxy terminus of pp60$^{c\text{-}src}$, specifi-

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cally the region between residues 518 and 525, as being necessary for stable association with middle-T antigen (3, 8). The close vicinity of this site to the regulatory Tyr-527 led us to speculate that the mechanism by which middle-T activates pp60$^{c\text{-}src}$ is by disrupting its ability to be regulated by kinase(s) or phosphatase(s) in vivo. Middle-T may mediate this by either masking or competing with the kinase(s) to affect phosphorylation at this site, or it may alter the local conformation such that Tyr-527 is more susceptible to dephosphorylation. Furthermore, since this region is highly conserved among the other members of the *src* family of tyrosine kinases (6; reviewed by J. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press), these protein kinases may normally be regulated by a common cellular counterpart of middle-T antigen. If this is correct, such a cellular homolog may be expected to require the integrity of this region for regulating the phosphorylation of Tyr-527. To test the hypothesis that removal of these sequences would prevent phosphorylation of Tyr-527 and thereby activate pp60$^{c\text{-}src}$, we have engineered a deletion mutant which lacks residues 519 to 524.

MATERIALS AND METHODS

In vitro mutagenesis procedures and plasmid constructions. Plasmid pGC11(Cm) containing the complete avian c-*src* cDNA was subjected to site-directed mutagenesis essentially as described by Piwnica-Worms et al. (38) and Cheng et al. (7, 8). To alter the codon for Tyr-527, a gapped heteroduplex was formed by annealing in equimolar quantities pGC11(Cm), linearized at its unique *Nco*I site, and the 4.2-kilobase-pair fragment generated by digestion of pGC11(Cm) with *Bgl*I and *Sal*I. The following synthetic oligonucleotides, both 20 residues long, were used: 5′-GAGCCCCAGGAACAGCCTGG-3′ to alter Tyr-527 to Glu and 5′-GAGCCCCAGCAACAGCCTGG-3′ to alter Tyr-527 to Gln. Site-directed mutagenesis of the codon for Tyr-416 was accomplished by using a combination of the *Nco*I linear fragment and the 297-base-pair *Pvu*II and 3.9-kilobase-pair *Pvu*II-*Bgl*II restriction enzyme fragments derived from pGC11(Cm) to form the gapped heteroduplex. The mutagenic oligonucleotides used to convert Tyr-416 to Glu and to Gln were 5′-GACAACGAGGAAACAGCACG-3′ and 5′-GACAACGAGCAAACAGCACG-3′, respectively. Double mutants containing altered codons at both positions 416 and 527 were constructed by reciprocal exchange of the *Bgl*I-*Sal*I fragments of mutants containing altered codons at position 416 with those containing lesions at position 527. To generate the deletion mutant, RH5, we used the 30-base oligonucleotide 5′-GCCTTCCTGGAGGACCCCCAGTACCAGCCT-3′, as the mutagen. The resultant mutant encodes residues 1 to 518 and 525 to 533 of pp60$^{c\text{-}src}$.

After the mutagens had been annealed to the gapped heteroduplex molecules, the gaps were filled with the Klenow fragment and T4 DNA ligase. Colonies containing the desired mutations were initially identified by hybridization with the appropriate mutant oligonucleotides and subsequently confirmed by DNA sequencing. Routinely, the entire span of the gaps was sequenced to ensure that no other spurious mutations had occurred. In addition, mutants were cloned into the transcription vectors SP64 and SP65 (Promega Biotec), and the in vitro-synthesized transcripts were translated in rabbit reticulocyte lysates. The in vitro-translated proteins were then analyzed and compared with that of wild-type pp60$^{c\text{-}src}$. This analysis ensured that no spurious frameshift mutations had occurred. The method for transcription and translation of c-*src* in vitro has been described previously (8).

Generation of recombinant retroviruses and infection of NIH 3T3 cells. All the c-*src* variants were inserted into the retroviral vector pLJ essentially as described previously (6, 8, 37, 38). Helper-free recombinant retroviruses were generated by transfection onto Ψ-2 packaging cells (33). At 24 h posttransfection, the cell supernatants were harvested and then used to infect NIH 3T3 cells. Medium was also collected from stable Ψ-2 producer lines established following the addition of G418 to the medium. G418-resistant clones were either clonally expanded or selected in bulk. Normally, viral stocks from the transient supernatants and from two different Ψ-2 clones were used to infect NIH 3T3 cells. At 24 h postinfection, cells were trypsinized and replated onto two 100-mm tissue culture dishes. Following an additional 24 h of incubation, one set of culture dishes was placed under selection with G418, whereas the other was scored for transforming activity (6, 8). Transforming activity was scored by dividing the number of foci observed by the total number of G418-resistant colonies. An average from three separate experiments was used to determine the final values. Stable NIH 3T3 cells expressing the various pp60$^{c\text{-}src}$ mutants were also established by clonal expansion of the G418-resistant clones.

Isolation of cell line expressing middle-T antigen. Cells expressing middle-T were generated by transfection of pAS100, a construct capable of directing the synthesis of middle-T alone, onto NIH 3T3 cells (20). Foci which grew over the monolayer of cells (they appeared 2 weeks posttransfection) were picked and expanded. A clonal cell line was obtained by plating a suspension of the transformed cells at low density in soft agar (32). Colonies which grew to approximately 1 mm were isolated and expanded. Two cycles of growth and selection in soft agar were used to generate the final cell line, designated mt.

Phosphoprotein analyses. Cleveland digests with *Staphylococcus aureus* V8 protease were performed as described by Cleveland et al. (9) with modifications (8). Two-dimensional tryptic phosphopeptide mapping was performed as described by Cheng et al. (8). The conditions for electrophoresis and chromatography were those described by Hunter and Sefton (21). Cyanogen bromide cleavage analysis was based on the protocol described by Schuh and Brugge (41), except that electrophoresis was performed on an 11 to 23% polyacrylamide gradient gel (Amersham Corp.).

Immunoprecipitations and in vitro kinase assays. Our procedures for isotopic labeling of cells, preparation of lysates, immunoprecipitation of proteins, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis have all been described before (6–8, 16, 17). Immune-complex kinase assays and quantitative kinase assays with enolase were conducted as described previously (6–8, 15). The method for monitoring complex formation of mutant pp60$^{c\text{-}src}$ with middle-T antigen was essentially that outlined by Cheng et al. (8).

Cells and antibodies. Generation of cell lines expressing the pp60$^{c\text{-}src}$ mutants 527F and 416F-527F has already been described (38). The monoclonal antibody EC10, which recognizes specifically the avian but not the murine pp60$^{c\text{-}src}$, was obtained from S. Parsons (35). cst-1 is a polyclonal antiserum raised in rabbits against a peptide corresponding to the carboxy-terminal 7 amino acids of pp60$^{c\text{-}src}$ (17), and HK3 is a polyclonal antiserum from hamsters bearing tumors induced by polyomavirus (8).

RESULTS

Construction of c-*src* mutants and derivation of cell lines expressing the pp60$^{c\text{-}src}$ variants. To investigate specifically the role of the phosphate moiety, we altered the codons for both Tyr-416 and Tyr-527 to either Glu (to generate the mutants 416E and 527E) or Gln (416Q and 527Q) by using site-directed mutagenesis. The residue Glu was chosen because we rationalized that since it is negatively charged, it may be capable of simulating phosphorylated Tyr-527 and thereby maintaining pp60$^{c\text{-}src}$ in the inactive state. Similarly, we also replaced Tyr-416 with Glu to examine whether the negatively charged Glu is capable of activating pp60$^{c\text{-}src}$.

Previously, we and others also demonstrated that replacement of Tyr-416 with Phe in the 527F mutant (Tyr 527 to Phe) dramatically decreases the kinase activity and transforming potential of the 527F mutant (26, 38). To test the ability of Glu or Gln at residue 416 to down-regulate the activities of 527F, we also constructed double mutants containing Glu (416E-527F) or Gln (416Q-527F) at residue 416 and Phe at residue 527.

Analysis of carboxy-terminal truncated mutants of pp60$^{c\text{-}src}$ defined the region adjacent to the regulatory Tyr-527, specifically between residues 518 and 525, as being necessary for complex formation with polyomavirus middle-T antigen (8). pp60$^{c\text{-}src}$ in complex with middle-T antigen is not phosphorylated at Tyr-527 (4). Since previous studies were performed with truncated mutants, we were unable to assess the effect of lesions in this region on phosphorylation at this site. To investigate the role of these sequences in the phosphorylation of Tyr-527, we constructed a deletion mutant which lacks amino acids 519 to 524 (RH5). Mutant RH5 thus lacks part of the putative middle-T-binding domain but retains Tyr-527.

All the mutations were introduced into an avian c-*src* cDNA (37). The variant genes were cloned into the retroviral vector pLJ, which places their expression under the control of the viral promoter in the 5′ long terminal repeat of the Moloney murine leukemia virus. Recombinant retroviruses generated by transfection onto Ψ-2 packaging cells were then used to infect NIH 3T3 cells. Following selection with the antibiotic G418, resistant colonies were isolated and expanded.

In vivo phosphorylation of pp60$^{c\text{-}src}$ mutants. All the mutants generated (except for RH5) contain lesions at one or more of the primary sites of tyrosine phosphorylation. To verify the mutations and, more importantly, to assess the effect of these mutations on the overall state of phosphorylation of the pp60$^{c\text{-}src}$ variants in vivo, we analyzed the mutant proteins by two-dimensional tryptic phosphopeptide analysis (Fig. 1). Wild-type pp60$^{c\text{-}src}$ is normally phosphorylated predominantly on Ser-17 and Tyr-527 (Fig. 1A) (12, 15). As expected, because the 527E and 527Q mutants cannot be phosphorylated on Tyr-527, their fingerprints demonstrated that they were phosphorylated predominantly on Ser-17 and Tyr-416 (Fig. 1D and E). Phosphorylation at Tyr-416 is observed with activated mutants of pp60$^{c\text{-}src}$ (8, 30, 38), suggesting that the mutants 527E and 527Q are activated.

The double mutants 416E-527F and 416Q-527F were phosphorylated significantly only on Ser-17 (Fig. 1F and G), since they contained altered codons at both positions 416 and 527. Mutants 416E and 416Q displayed tryptic phosphorylation patterns which were indistinguishable from that of wild-type pp60$^{c\text{-}src}$ (Fig. 1B and C), as observed previously for mutant 416F (8). Hence, all the mutants displayed phosphorylation patterns consistent with their respective lesions.

Biological properties of NIH 3T3 cells expressing wild-type and mutant pp60$^{c\text{-}src}$. Table 1 summarizes the results obtained when the cells expressing pp60$^{c\text{-}src}$ were examined for focus-forming activity, specific kinase activity, and phosphorylation in vivo. The morphology of the cells expressing the 416E and 416Q mutants appeared unaltered and was indistinguishable from that of cells infected with the control virus pLJ or cells expressing the wild-type avian pp60$^{c\text{-}src}$. Cells expressing the 527E and 527Q mutants, however, showed substantial morphologic alterations when compared with cells expressing the wild-type protein. Both displayed a highly rounded morphology, similar to cells transformed by 527F (26, 38). The double mutants 416E-527F and 416Q-527F, like 416F-527F (38), also appeared altered to a more spherical morphology.

The alteration in fibroblast morphology induced by expression of the pp60$^{c\text{-}src}$ mutants was also associated with acquisition of other properties of the transformed phenotype. For example, when the transforming potential of the various pp60$^{c\text{-}src}$ variants was assayed by using focus formation, cells expressing the 527E or 527Q mutant but not the 416E or the 416Q mutant were able to induce growth of foci (Table 1). As has been shown for 416F-527F (38), the double mutant 416E-527F was nontransforming. However, unexpectedly, cells expressing the mutant 416Q-527F were able to induce growth of foci, albeit at a reduced frequency and following an extended incubation (approximately 28 days as compared with 14 days for 527F). Moreover, only 10 to 20% of the total G418-resistant colonies were capable of forming foci, as compared with 60 to 70% for 527F. In each case, the transformation data were obtained by using transient viral supernatants and supernatants of two independent clonal Ψ-2 producers. The results obtained were similar irrespective of the source of the virus.

Relative specific kinase activities of the pp60$^{c\text{-}src}$ variants. The specific activities of phosphorylation of enolase by the *src* proteins were compared following immunoprecipitation with the monoclonal antibody EC10 (35). Since the EC10 antibody is specific for an amino-terminal epitope in avian pp60$^{c\text{-}src}$ (34), the data obtained reflect the activity of the carboxy-terminal mutant avian pp60$^{c\text{-}src}$ species but not that of the endogenous murine counterpart. The cells were labeled with [^{35}S]methionine, and volumes of lysates were adjusted to contain approximately equivalent amounts of pp60$^{c\text{-}src}$. Immunoprecipitates obtained were then either analyzed directly on gels (Fig. 2A) or incubated in the presence of enolase and [γ-^{32}P]ATP (Fig. 2B). The levels of pp60$^{c\text{-}src}$ in each lysate (Fig. 2A) were normalized and quantitated by densitometry, and the ^{32}P measurements were determined directly by scintillation counting. Although the immunoprecipitates contained approximately equivalent amounts of pp60$^{c\text{-}src}$ protein, the mutants varied in their ability to phosphorylate enolase (Fig. 2). Average activities were calculated from the results of four separate experiments (Table 1), such as the one shown in Fig. 2, and with data from at least two different G418-resistant clones. In agreement with previous results, the transforming mutants 527E and 527Q, like 527F, exhibited kinase activities which were between 8- and 10-fold higher than that of normal pp60$^{c\text{-}src}$. Modification of Tyr-416 to Glu (416E) or Phe (416F) did not alter their kinase activities significantly from that obtained for the wild-type protein. By contrast, the catalytic activity of the 416Q mutant was enhanced approximately twofold when compared with that of normal

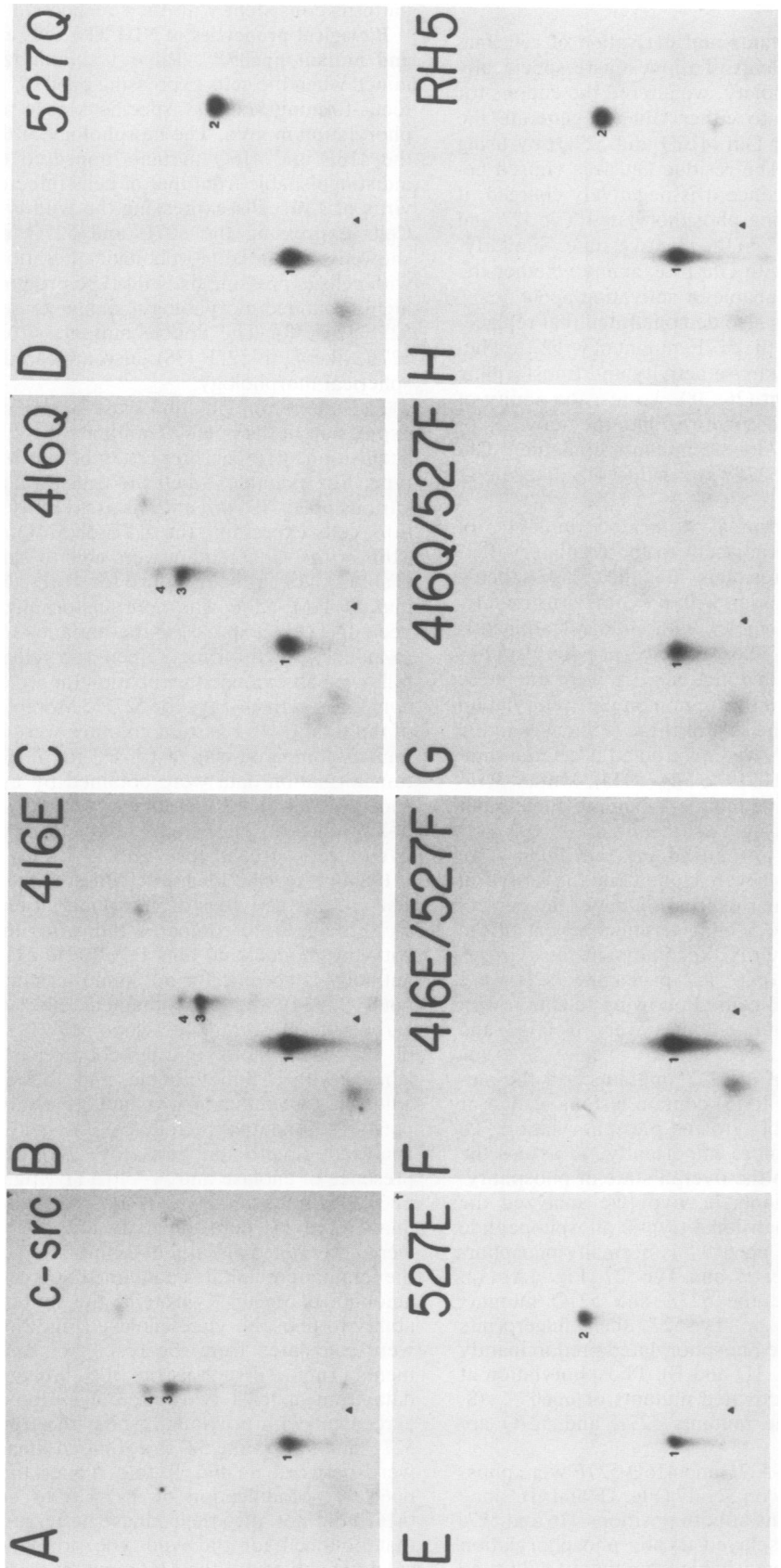

FIG. 1. Tryptic phosphopeptide analysis of the mutant $pp60^{c\text{-}src}$. ^{32}P-labeled $pp60^{c\text{-}src}$ proteins were immunoprecipitated with the monoclonal antibody EC10. The *src* proteins were extracted from the polyacrylamide gels, treated with trypsin, separated by electrophoresis and chromatography in two dimensions, and analyzed by autoradiography. The origins are marked with arrowheads. Electrophoresis at pH 8.9 was performed in the horizontal dimension with the anode on the right. Spot numbering: 1, peptides containing Ser-17; 2, peptides containing Tyr-416; 3 and 4, peptides containing Tyr-527. The correlation of spots and peptides is described by Cheng et al. (8). Exposure time was 2 days.

TABLE 1. Biochemical and biological properties of the carboxy-terminal pp60$^{c\text{-}src}$ mutants

Mutant	Mutation	Focus-forming activity[a]	Relative specific kinase activity[b]	Phosphorylation on residue[c]:		
				17	416	527
c-src		−	1.0	+	−	+
527F	Y527→F	+	8.9 (1.5)	+	+	−
416E	Y416→E	−	1.0 (0.3)	+	−	+
416Q	Y416→Q	−	1.8 (0.4)	+	−	+
527E	Y527→E	+	10.2 (1.9)	+	+	−
527Q	Y527→Q	+	8.3 (1.5)	+	+	−
RH5	del.519–524	+	4.7 (1.0)	+	+	−
416E-527F	Y416→E; Y527→F	−	3.0 (0.7)	+	−	−
416Q-527F	Y416→Q; Y527→F	+/−	5.9 (0.9)	+	−	−
416F-527F	Y416→F; Y527→F	−	3.4 (0.5)	+	−	−

[a] Transforming activity was monitored by focus formation over a monolayer of NIH 3T3 cells following infection with the various recombinant retroviruses encoding the pp60$^{c\text{-}src}$ variants. Symbols for focus-forming activity: −, pp60$^{c\text{-}src}$ mutants demonstrating no focus-forming activity; +, mutants demonstrating focus-forming activity; +/−, mutants demonstrating weak focus-forming activity.

[b] Kinase activity was measured by using enolase as described in the legend to Fig. 2. Enolase-specific kinase activities were determined from the amounts of ^{32}P incorporated into the enolase bands and the amounts of [^{35}S]pp60$^{c\text{-}src}$ in the immunoprecipitated bands. The values are averages from four experiments and represent the fold activation relative to wild-type pp60$^{c\text{-}src}$. Numbers in parentheses indicate the average deviation from the mean.

[c] In vivo phosphorylation was determined by two-dimensional tryptic phosphopeptide mapping (Fig. 1). Symbols for phosphorylation: +, phosphorylated; −, no detectable phosphorylation.

pp60$^{c\text{-}src}$. That the 416Q mutant activates the kinase activity of pp60$^{c\text{-}src}$ was not unexpected, since it is consistent with the above observation that the double mutant 416Q-527F (but not the 416E-527F or the 416F-527F mutant) displays transforming potential. The specific kinase activity of the 416Q-527F mutant is lower than that of 527F but higher than those of both the 416F-527F and 416E-527F mutants (Table 1). Hence, Gln at residue 416 is capable of activating the kinase activity of pp60$^{c\text{-}src}$.

Analysis of the deletion mutant RH5. To monitor the significance of the region between residues 518 and 525 in determining phosphorylation at Tyr-527, we constructed a deletion mutant, RH5, which lacks residues 519 to 524. To verify the presence of the deletion, cells expressing the mutant were labeled with ^{32}P, the lysates were immunoprecipitated by using the antibody EC10, and the recovered c-*src* protein was then subjected to partial digestion with *S. aureus* V8 protease (Fig. 3A). The amino-terminal fragments V1, V3, and V4 were indistinguishable from those generated from wild-type pp60$^{c\text{-}src}$. The carboxy-terminal V2 fragment of the mutant RH5 (indicated by the arrow in Fig. 3A) migrated with greater mobility on the gel, which is consistent with the expected deletion.

In addition, to demonstrate that the RH5 deletion mutant retained the carboxy-terminal residues 525 to 533 (and hence Tyr-527), the cells expressing the mutant were also subjected to immunoprecipitation with the peptide antibody cst-1, an antibody which was raised against the carboxy-terminal 7 residues of pp60$^{c\text{-}src}$ (17). RH5 which had either been transcribed and then translated in vitro in a rabbit reticulocyte lysate (Fig. 3B) or been expressed in NIH 3T3 cells (Fig. 3C) was immunoprecipitable with cst-1 antiserum. The antibody cst-1 recognizes both the avian and endogenous pp60$^{c\text{-}src}$. Hence, in Fig. 3C, the signal observed with cst-1 represents a combination of both the mutant avian and endogenous murine pp60$^{c\text{-}src}$. However, because the mutant RH5 is expressed at an approximately eightfold higher level than the endogenous murine pp60$^{c\text{-}src}$ (data not shown), the equal intensity of the bands observed with EC10 and cst-1 (Fig. 3C) argues that RH5 pp60$^{c\text{-}src}$ was also immunoprecipitated with the cst-1 antibody. Therefore, taken together with the information from DNA sequencing, the data here show that RH5 encodes a pp60$^{c\text{-}src}$ variant which is composed of residues 1 to 518 and 525 to 533.

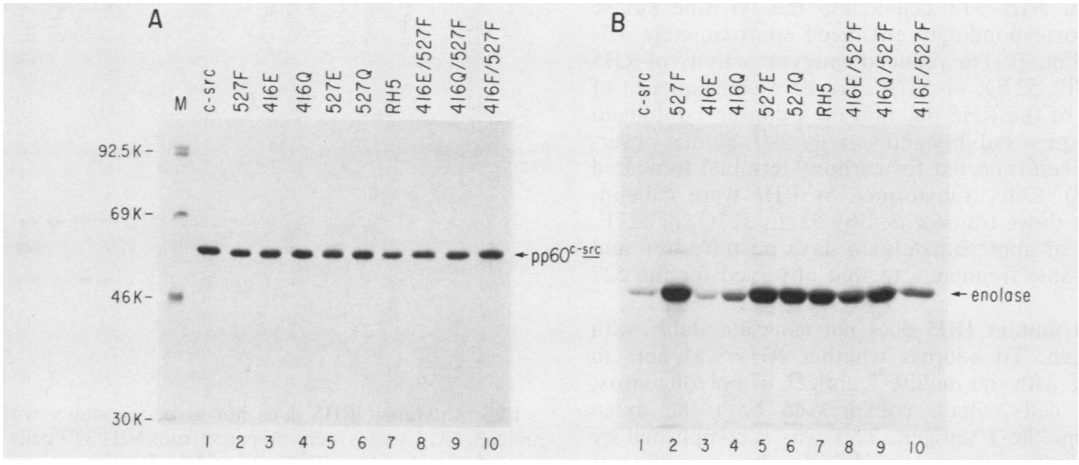

FIG. 2. Relative specific kinase activities of the pp60$^{c\text{-}src}$ mutants. The determination of specific activities for phosphorylation of enolase was essentially as described by Cheng et al. (8). Cells were labeled with [^{35}S]methionine, and the lysates were normalized to contain approximately equivalent amounts of pp60$^{c\text{-}src}$ (as determined by prior quantitation) and then immunoprecipitated with EC10 serum. The immunoprecipitates were either analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) or incubated with enolase and [γ-^{32}P]ATP for 3 min at 30°C (B). Control experiments indicated that incorporation of ^{32}P into enolase was linear for 5 min. The [^{35}S]methionine gel was fluorographed and exposed for 24 h. Autoradiography time for the enolase gel was 1 h at −70°C. The specific kinase activity was determined by dividing the amount of ^{32}P incorporated into enolase by the amount of [^{35}S]methionine incorporated into pp60$^{c\text{-}src}$. Values for the mutant pp60$^{c\text{-}src}$ were normalized relative to wild-type pp60$^{c\text{-}src}$. Average specific activities from several experiments of this type are given in Table 1. Lanes: 1, wild-type pp60$^{c\text{-}src}$; 2, 527F; 3, 416E; 4, 416Q; 5, 527E; 6, 527Q; 7, RH5; 8, 416E-527F; 9, 416Q-527F; 10, 416F-527F; M, molecular size markers; sizes are on the left in kilodaltons (K).

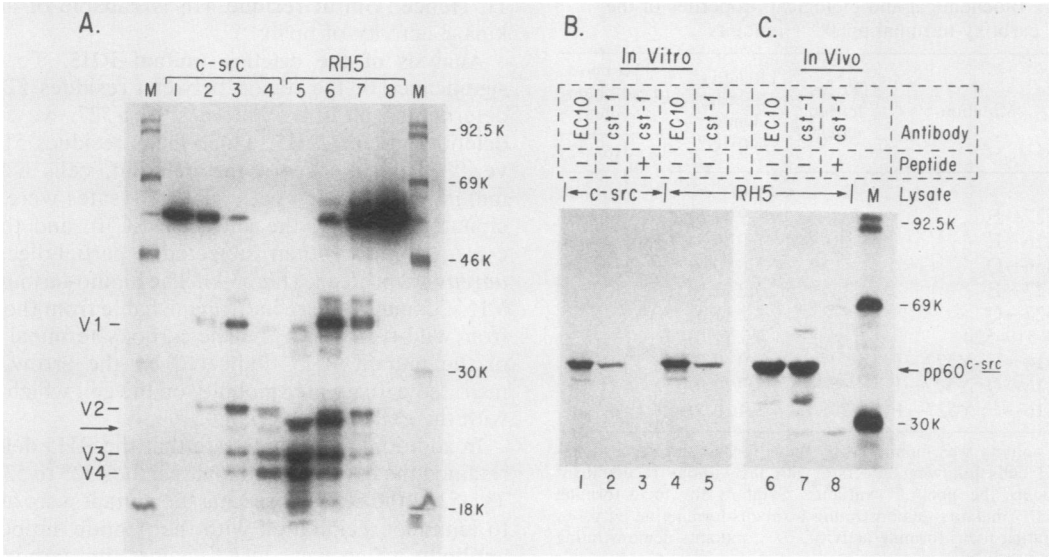

FIG. 3. Cleveland and immunoprecipitation analysis of the deletion mutant RH5. (A) ^{32}P-labeled wild-type and RH5 pp60$^{c\text{-}src}$ were purified by immunoprecipitation with EC10 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The excised bands were digested with *S. aureus* V8 protease (0.0017 μg/μl [lanes 2 and 7], 0.017 μg/μl [lanes 3 and 6], or 0.17 μg/μl [lanes 4 and 5]) and then electrophoresed on a 15% polyacrylamide gel. Lanes 1 and 8 show untreated samples. Lanes M contain molecular size markers; sizes are on the right in kilodaltons (K). The arrow indicates the V2 fragment of RH5. V1, 34-kDa amino-terminal fragment; V2, 26-kDa carboxy-terminal fragment; V3, 18-kDa amino-terminal subfragment of V1; V4, 16-kDa amino-terminal subfragment of V1. Autoradiography time was 3 days. (B) pSP65-c-*src* and pSP65-RH5 containing the wild type and RH5 c-*src* cDNA, respectively, were transcribed with SP6 RNA polymerase. Following purification, the RNA transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and then immunoprecipitated with either EC10 or cst-1 serum (a peptide antibody raised against the carboxy-terminal 7 amino acids of pp60$^{c\text{-}src}$). Lanes 1 to 3 contain immunoprecipitates from in vitro-synthesized wild-type pp60$^{c\text{-}src}$, and lanes 4 and 5 contain lysates with in vitro-synthesized RH5. The [^{35}S]methionine gels were fluorographed and exposed for 24 h. (C) Cells expressing the pp60$^{c\text{-}src}$ variant RH5 were metabolically labeled with [^{35}S]methionine, and the cell lysates were immunoprecipitated with either EC10 (lane 6) or cst-1 (lanes 7 and 8). When the peptide antiserum cst-1 was used, (+) indicates that the immunoprecipitation reactions were performed in the presence of the cognate peptide and (−) indicates that they were performed in the absence of added peptide. Exposure time was 2 days.

Mutant RH5 exhibits activated tyrosine kinase and transforming activities. Cells expressing the RH5 mutant exhibited a transformed phenotype (cells were highly rounded and formed foci on NIH 3T3 cells), and the tyrosine kinase activity was correspondingly enhanced approximately 4.7-fold (Table 1; Fig. 2). The reduced catalytic activity of RH5 relative to 527E, 527Q, or 527F may be a consequence of encroachment of the RH5 deletion on a conserved domain required for protein stability and activity (44). Similar observations have been reported for carboxy-terminal truncated mutants (8, 43). Cells transformed by RH5 were indistinguishable from those transformed by 527E, 527Q, or 527F. Foci appeared at approximately 14 days postinfection and with a comparable frequency to that observed for the 527 variants.

The deletion mutant RH5 does not associate stably with middle-T antigen. To address whether RH5 was able to complex stably with the middle-T antigen of polyomavirus, we generated cells which coexpressed both the avian pp60$^{c\text{-}src}$ and middle-T antigen. This was accomplished by infecting middle-T-expressing NIH 3T3 cells (mt cells; see Materials and Methods) with the recombinant retroviruses containing c-*src* coding sequences. Clonal cell lines were obtained following selection of the infected cultures with G418. Two such cell lines, one containing the wild-type pp60$^{c\text{-}src}$ (c-src/mt) and the other containing RH5 (RH5/mt), were generated. Examination of the lysates from cells coexpressing wild-type pp60$^{c\text{-}src}$ or RH5 and the viral protein revealed that the wild-type but not RH5 pp60$^{c\text{-}src}$ was capable of associating with middle-T (Fig. 4). Complex

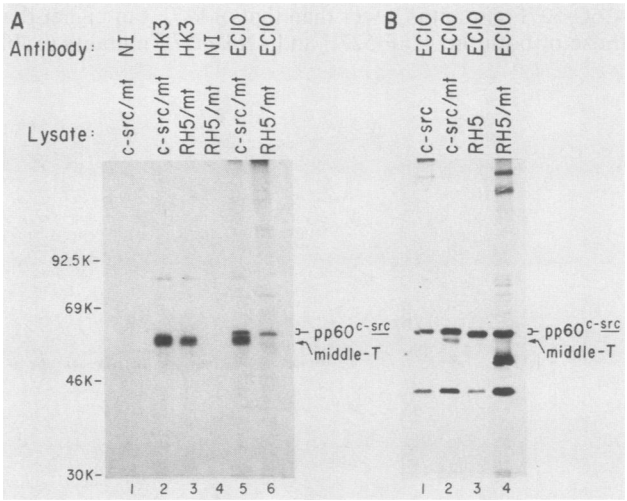

FIG. 4. Mutant RH5 does not associate stably with middle-T antigen. (A) Lysates were prepared from NIH 3T3 cells coexpressing either wild-type pp60$^{c\text{-}src}$ and middle-T antigen (c-src/mt) or RH5 and middle-T antigen (RH5/mt). The lysates were normalized for protein content, immunoprecipitated with EC10, HK3 (anti-middle-T serum raised in hamsters), or NI (nonimmune) serum and phosphorylated in vitro by using [γ-^{32}P]ATP. The positions of pp60$^{c\text{-}src}$ and middle-T are indicated on the right margin. Autoradiography time was 3 h. (B) Cell lines expressing wild-type pp60$^{c\text{-}src}$ (lane 1), pp60$^{c\text{-}src}$ and middle-T antigen (lane 2), RH5 (lane 3), or RH5 and middle-T antigen (lane 4) were labeled with [^{35}S]methionine, and the cell lysates were immunoprecipitated with EC10. The gel was fluorographed and exposed for 24 h.

formation was monitored by the ability of immunoprecipitates obtained with EC10 antibody to phosphorylate middle-T antigen in a kinase assay (6, 8). To determine whether both the c-src/mt and RH5/mt cells expressed middle-T antigen, we performed immune-complex kinase assays with HK3 serum (an antiserum against middle-T). Under these conditions, middle-T is phosphorylated (7). Both cell lines express approximately equivalent amounts of middle-T antigen (Fig. 4A, lanes 2 and 3). To determine whether RH5 is capable of associating with middle-T, we performed in vitro kinase assays on EC10-derived immunoprecipitates. Phosphorylation of middle-T in this assay is indicative of coprecipitation of the viral protein and hence complex formation with the avian pp60$^{c\text{-}src}$. Middle-T was phosphorylated only in lysates containing the wild type but not detectably in those containing RH5 (Fig. 4A, lanes 5 and 6). Using the antibody cst-1, we could demonstrate that the middle-T in RH5 is able to associate with the endogenous murine pp60$^{c\text{-}src}$ (data not shown). However, it is possible that RH5 bound middle-T antigen but was unable to phosphorylate it. To test this, the RH5/mt and c-src/mt cell lines were labeled with [^{35}S]methionine and the lysates were immunoprecipitated with EC10 serum. Middle-T was coimmunoprecipitated with wild-type pp60$^{c\text{-}src}$ (Fig. 4B, lane 2) but not with RH5 (Fig. 4B, lane 4). Hence, mutant RH5 is unable to associate stably with the middle-T antigen.

RH5 is not detectably phosphorylated at Tyr-527. Two-dimensional tryptic phosphopeptide analysis demonstrated that RH5 was phosphorylated predominantly at Ser-17 and Tyr-416 but not at Tyr-527, since no labeled peptide of the expected altered mobility was detected (Fig. 1H). However, it is possible that the RH5 Tyr-527-containing tryptic peptides were not resolved in this system. To further characterize the carboxy-terminal sites of phosphorylation, we subjected RH5 to cyanogen bromide cleavage analysis. Cleavage of wild-type pp60$^{c\text{-}src}$ with cyanogen bromide generated two phosphorylated peptides with approximate molecular masses of 32 kilodaltons (kDa), which corresponds to the amino-terminal fragment containing Ser-17, and 6 kDa, which corresponds to the fragment containing Tyr-527 (Fig. 5, lane 1). By contrast, analysis of RH5 revealed a 32-kDa phosphopeptide and a 10-kDa phosphopeptide, the latter corresponding to the internal peptide containing Tyr-416 (Fig. 5, lane 2). The absence of a phosphorylated Tyr-527 supports the fingerprint data indicating that RH5 was phosphorylated mainly at Ser-17 and Tyr-416 in vivo. The phosphorylation observed at Tyr-416 is consistent with the observation that RH5 is transforming and has been observed with other activated mutants of pp60$^{c\text{-}src}$ and also with pp60$^{c\text{-}src}$ in complex with middle-T. The inability to phosphorylate Tyr-527 suggests strongly that residues 519 to 524, in addition to being required for binding to middle-T antigen, are necessary for recognition by a cellular protein(s) which regulates the phosphorylation at Tyr-527 in vivo. Alternatively, deletion of the same residues enhances dephosphorylation of Tyr-527.

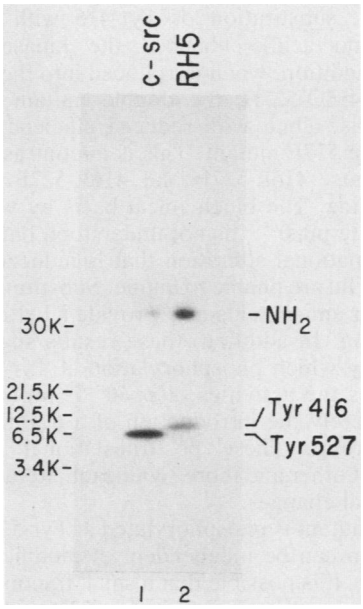

FIG. 5. Cyanogen bromide cleavage analysis of pp60$^{c\text{-}src}$ phosphoproteins. Cell lines expressing wild-type pp60$^{c\text{-}src}$ and the RH5 variant were metabolically labeled with $^{32}P_i$. Following purification by immunoprecipitation with EC10 serum and gel electrophoresis, the pp60$^{c\text{-}src}$ proteins were cleaved with cyanogen bromide. Cleavage products were resolved by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gradient gel (11 to 23% acrylamide) and visualized by autoradiography. The positions of the peptides containing Tyr-416 and Tyr-527 are denoted. The 32-kDa phosphopeptide corresponds to the amino-terminal peptide and contains Ser-17. Molecular size markers are on the left in kilodaltons (K). Autoradiography time was 24 h.

DISCUSSION

Since the observation that the kinase and transforming activities of pp60$^{c\text{-}src}$ are maintained in an inactivated state by phosphorylation at Tyr-527, several issues requiring further study have emerged. These include (i) identification of the events which lead to normal dephosphorylation and activation of pp60$^{c\text{-}src}$ during normal cellular proliferation, (ii) identification of the kinase(s) and phosphatase(s) which regulate the phosphorylation of Tyr-527 in vivo, and (iii) identification of the mechanism by which phosphorylation at Tyr-527 suppresses the activities of the proto-oncogene. In this paper we report studies which attempt to address the last two issues. The impetus to answer these questions is driven in part by the desire to understand the normal cellular function of pp60$^{c\text{-}src}$ in vivo and the processes governing normal cellular regulation.

It is apparent that the pp60$^{c\text{-}src}$ kinase is modulated by a balance of phosphorylation between Tyr-416 and Tyr-527. Removal of phosphate at Tyr-527 by enzymatic means (13, 15), by mutagenesis (3, 26, 38, 40), or by binding to middle-T (4) leads to activation of the catalytic and transforming activities. On the other hand, in vivo phosphorylation of Tyr-416 is characteristic of activated forms of pp60$^{c\text{-}src}$ (8, 22, 26, 30, 38). Prevention of phosphorylation on Tyr-416 in otherwise activated mutants such as 527F dramatically reduces the kinase activity (26, 38). Hence, phosphorylation at Tyr-416 or the presence of the hydroxyl group of Tyr is necessary to activate pp60$^{c\text{-}src}$ fully.

Effect of Glu and Gln at positions 416 and 527 on pp60$^{c\text{-}src}$. To investigate specifically the role of the phosphate moiety on these Tyr residues, we have attempted to simulate its negative charge by replacing Tyr with Glu. The results obtained demonstrate that the presence of a negatively charged Glu residue at position 527 is not sufficient to prevent activation of the transforming activity of pp60$^{c\text{-}src}$. Both the 527E and 527Q mutants were capable of inducing growth of foci on NIH 3T3 cells. Similarly, the introduction of a Glu residue at position 416 is not able to activate the biological and biochemical activities of pp60$^{c\text{-}src}$. However,

unexpectedly, substitution of Tyr-416 with Gln (an uncharged amino acid) enhanced the kinase activity of pp60$^{c\text{-}src}$. In addition, when introduced into the mutant 527F to generate 416Q-527F, the double mutant was able to transform cells, albeit with reduced efficiency when compared with the 527F mutant. This is in contrast to the other double mutants, 416F-527F and 416E-527F, which were nontransforming. The biochemical basis by which 416Q is able to activate pp60$^{c\text{-}src}$ is not understood but may involve some conformational alteration that is induced by Gln and that Phe and Glu are unable to mimic. Substitution of residue 416 with other amino acids may provide a better insight into the mechanism. In addition, these results suggest that the mechanism by which phosphorylation at Tyr-416 and Tyr-527 modulates the activities of pp60$^{c\text{-}src}$ probably involves more than merely the introduction of a negatively charged phosphate group. These posttranslational modifications probably elicit other and more significant alterations, such as conformational changes.

The 416Q mutant is phosphorylated at Tyr-527, suggesting that activation can be independent of modification at Tyr-527. However, it is possible that a small fraction of the 416Q mutant is dephosphorylated at Tyr-527 and that this is responsible for the observed activation. In this regard, 416Q more closely resembles the situation observed with overexpressed pp60$^{c\text{-}src}$. Under these conditions, pp60$^{c\text{-}src}$ is phosphorylated at Tyr-416 and Tyr-527 and exhibits enhanced kinase activity (8, 25). Studies by Kmiecik et al. (25) comparing activities of pp60$^{c\text{-}src}$ in overexpressing cells isolated in the absence or presence of a phosphatase inhibitor suggest that the observed activation is probably caused by stabilization of phosphorylation at Tyr-416. Hence, phosphorylation at Tyr-416 is able to activate the pp60$^{c\text{-}src}$ kinase. Our observation with 416Q further supports the notion that residue 416, independently of Tyr-527, is able to modulate the activity of pp60$^{c\text{-}src}$. Although Gln and phosphorylated Tyr at position 416 are both able to activate pp60$^{c\text{-}src}$, it is unclear whether activation is manifest via the same mechanism. Overexpressed pp60$^{c\text{-}src}$ containing stabilized phosphorylated Tyr-416 is activated threefold (25), whereas the 416Q mutant is activated only approximately twofold. This difference is consistent with the observation that 416Y-527F is more transforming than 416Q-527F.

All three mutants, 416Q-527F, 416E-527F and 416F-527F, exhibited enhanced tyrosine kinase activities when compared with wild-type pp60$^{c\text{-}src}$. The observation that 416Q-527F but not 416F-527F or 416E-527F was able to transform NIH 3T3 cells, however, was probably due to the fact that the kinase activity of 416Q-527F was higher than those of either of the other two mutants. This is consistent with results of previous studies indicating a correlation between the degree of activation of the kinase activity and the transforming activity (8, 26, 37). The data here suggest, perhaps, that a threshold for activation of the kinase activity is required prior to observing the transformed phenotype.

Mutant lacking the middle-T-binding region is not detectably phosphorylated at Tyr-527. As yet, very little is known about the cellular kinase(s) which phosphorylates Tyr-527. Recent studies suggest that pp60$^{c\text{-}src}$ itself is able to transautophosphorylate Tyr-527, albeit not to a level sufficient to account for all the pp60$^{c\text{-}src}$ molecules in fibroblast cells (14). In addition, other members of the *src* family of kinases contain a Tyr residue equivalent to that of Tyr-527 of pp60$^{c\text{-}src}$ (for a review, see Cooper, in press), but it is not established whether these kinases are regulated by the same or different kinases.

The results reported here show that deletion of residues 519 to 524, a region which is required for middle-T binding, abrogates the ability of pp60$^{c\text{-}src}$ to be phosphorylated at Tyr-527. Presumably, either the sequences deleted are required for recognition by the Tyr-527 kinase(s) or the deletion has predisposed Tyr-527 to enhanced dephosphorylation. This, in turn, probably causes activation of the kinase and transforming activities observed for the mutant RH5. The finding that the same sequence required for middle-T binding is also required for phosphorylation at Tyr-527 leads us to speculate that perhaps the mechanism by which middle-T activates pp60$^{c\text{-}src}$ is (i) by competing with the cellular kinase(s) which regulates phosphorylation at Tyr-527 for binding to pp60$^{c\text{-}src}$, (ii) by binding and thereby masking the ability of the cellular kinase(s) to phosphorylate pp60$^{c\text{-}src}$, or (iii) by binding and thereby altering the local conformation such that Tyr-527 becomes more accessible for dephosphorylation.

Irrespective of the mode, this finding also allows us to suggest that the cells may contain a cellular protein, a homolog of middle-T antigen, which modulates the normal regulation of pp60$^{c\text{-}src}$. Such a cellular protein may be the Tyr-527 kinase or a phosphatase inhibitor maintaining pp60$^{c\text{-}src}$ in the quiescent state. In this model, middle-T would represent an aberrant version of such a kinase or phosphatase inhibitor and would be without the activities necessary for maintaining Tyr-527 in the phosphorylated state. If this line of reasoning is correct, middle-T may be related to a tumor suppressor gene product. Alternatively, the homolog may function like middle-T but acting only transiently as a transforming protein. Such a cellular protein may be a phosphatase or a kinase inhibitor. These cellular proteins may bind pp60$^{c\text{-}src}$ and thereby activate the kinase activity of the enzyme, but, unlike middle-T, may do so only transiently and reversibly. Hence, unlike middle-T, the homolog would not lead to constitutive dephosphorylation of Tyr-527 and activation of pp60$^{c\text{-}src}$. Instead, it might activate pp60$^{c\text{-}src}$ only at specific times in the cell cycle. Since middle-T has been shown to be able to associate with pp60$^{c\text{-}src}$ (16), pp62$^{c\text{-}yes}$ (27), and pp59$^{c\text{-}fyn}$ (6, 28), and since the carboxy-terminal sequences of these enzymes are highly conserved, they, too, may be regulated by a similar transient transforming protein or suppressor gene product. These possibilities warrant further studies.

The requirement of the residues 519 to 524 of pp60$^{c\text{-}src}$ for stable association with middle-T and for phosphorylation of Tyr-527 by the cellular kinase(s) suggests either that the region is required for direct binding with middle-T and the cellular kinase(s) or that its presence is necessary for pp60$^{c\text{-}src}$ to adopt the appropriate conformation for recognition by middle-T and the kinase(s). Recently, it was shown that a chimeric p60$^{src/lck}$ (containing amino acids 1 to 516 of pp60$^{c\text{-}src}$ and 495-509 of p56^{lck}) but not p56^{lck} is able to associate with middle-T (31). This argued that sequences present within both the carboxy and amino termini of pp60$^{c\text{-}src}$ may be required to bind middle-T. However, it is possible that the p56^{lck} is non-middle-T binding because amino-terminal sequences unique to p56^{lck} block its association with the viral protein. Hence, it is presently unresolved whether more than one domain on pp60$^{c\text{-}src}$ is required for complex formation with middle-T and, if so, which domain is involved in direct binding with middle-T. Neither is it known whether middle-T and the cellular kinase(s) bind to the same or different domains on the pp60$^{c\text{-}src}$ protein. Future experiments will address these questions.

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