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# Structural Elements That Regulate pp59$^{c\text{-}fyn}$ Catalytic Activity, Transforming Potential, and Ability To Associate with Polyomavirus Middle-T Antigen

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**Except for its unique amino-terminal region (residues 1 through 83), which possibly dictates substrate recognition, pp59$^{c\text{-}fyn}$ bears a high degree of homology with other members of the *src* family of tyrosine kinases. Here we show that the carboxy terminus of pp59$^{c\text{-}fyn}$ is necessary for stable middle-T-antigen association, that pp59$^{c\text{-}fyn}$is normally phosphorylated on both serine and tyrosine residues, and that Tyr-531 and Tyr-420 are phosphorylation sites in vivo and in vitro, respectively. Analysis of a spontaneously generated mutant encoding a truncated form of pp59$^{c\text{-}fyn}$ and of variants specifically mutated at the Tyr-531 and Tyr-420 phosphorylation sites indicates that pp59$^{c\text{-}fyn}$ has regulatory elements analogous to those that have already been identified for other *src*-like tyrosine kinases. However, further examination of the pp59$^{c\text{-}fyn}$ variants suggests the likelihood of additional means by which its activities might be regulated. Although alteration of Tyr-531 to phenylalanine (531F) in pp59$^{c\text{-}fyn}$ results in a protein which is more active enzymatically that the wild type, the enhancement is much less than that for the analogous variant of pp60$^{c\text{-}src}$. Furthermore, contrary to results of similar experiments on other *src*-like proto-oncogene products, 531F did not induce transformation of NIH 3T3 cells. Studies involving pp59$^{c\text{-}fyn}$-pp60$^{c\text{-}src}$ chimeras in which the unique amino-terminal sequences (residues 1 through 83) of the two kinases were precisely interchanged implied that the inability of 531F to induce transformation is probably not caused by the absence of substrates for pp59$^{c\text{-}fyn}$in NIH 3T3 cells but rather by the insufficient enhancement of pp59$^{c\text{-}fyn}$ kinase activity. It is therefore probable that the kinase and transforming activities of pp59$^{c\text{-}fyn}$ are repressed by additional regulatory elements possibly located in the amino-terminal half of the molecule.**

There is increasing evidence that members of the *src* family of protein tyrosine kinases participate either as mediators or amplifiers of cellular signal-transducing pathways (for reviews, see references 9 and 32). This concept was first formulated because of the ability of v-*src* family members to regulate cellular growth and was recently highlighted by the demonstration that p56$^{lck}$, a member of this family, is associated with the T-lymphocyte surface glycoproteins CD4 and CD8 (35, 39). The notion that these cellular enzymes have an ascribed role in cellular proliferation is further corroborated by the observation that mutants of pp60$^{c\text{-}src}$ (and its viral homolog), pp62$^{c\text{-}yes}$, pp59$^{c\text{-}fyn}$, and pp55$^{c\text{-}fgr}$ are capable of causing deregulated cellular proliferation and malignant transformation (for a review, see reference 9).

The *fyn* gene, a member of this family, encodes a 59-kDa *src*-like protein tyrosine kinase that is myristylated and phosphorylated (5, 18, 24, 28, 36). Originally identified in fibroblasts and endothelial cells, pp59$^{c\text{-}fyn}$ has since been detected in a variety of other cells and at especially high levels in the brain and hematopoietic cells (8, 25, 36). Two distinct *fyn*-encoded transcripts that result from alternative splicing of a single exon and that exhibit mutually exclusive patterns of expression have been reported (8). One of the isoforms has been shown to accumulate principally in the brain, while the other is located in thymocytes and certain hematolymphoid cell lines. Although the normal physiological functions of the *fyn* gene products have not been unequivocally established, a causal role between the pp59$^{c\text{-}fyn}$ kinase and T-cell development and differentiation has been demonstrated (20, 21).

Regulation of the *src* family of cellular kinases is achieved in part by control of phosphorylation at the tyrosines homologous to Tyr-527 and Tyr-416 of pp60$^{c\text{-}src}$. Preventing the phosphorylation of Tyr-527 of pp60$^{c\text{-}src}$ by mutation to phenylalanine (3, 26, 33), by association with middle-T antigen of polyomavirus (4, 12), or by phosphatase treatment (11) results in activation of the kinase and transforming activities of pp60$^{c\text{-}src}$. Similarly, the oncogenic potential of pp56$^{lck}$ (1, 30) and p59$^{hck}$ (40) is unmasked by mutation of the homologous residue corresponding to Tyr-527 of pp60$^{c\text{-}src}$. Concomitant with the activation observed with the 527F mutant of pp60$^{c\text{-}src}$ is phosphorylation at Tyr-416, the site of autophosphorylation. Phosphorylation of Tyr-416 was deemed important, since a mutant wherein both Tyr-416 and Tyr-527 were mutagenized to phenylalanine is nontransforming and exhibits a lower kinase activity than the 527F mutant (16, 26, 33).

The viral oncogene product of polyomavirus, middle-T antigen, is capable of forming stable complexes with pp60$^{c\text{-}src}$ (13), pp62$^{c\text{-}yes}$ (27), and pp59$^{c\text{-}fyn}$ (5, 18, 29). Complex formation with pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ results in activation of their catalytic activities to severalfold over those of their unassociated counterparts (2, 12, 27). However, in contrast to the c-*src* and c-*yes* gene products, association of pp59$^{c\text{-}fyn}$ with middle-T antigen does not result in similar activation (5, 18, 29), implying that there may be significant differences in

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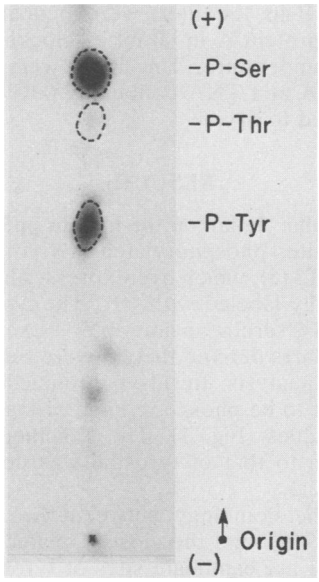


FIG. 1. Phosphoamino acid analysis of wild-type pp59$^{c\text{-}fyn}$. The 59-kDa protein obtained following immunoprecipitation of c-fyn/3T3 cells with the CFN-2 antibody was hydrolyzed in vacuo, applied to cellulose thin-layer chromatography plates, and then separated by electrophoresis at pH 3.5. The origin is marked with an X. Spots corresponding to ninhydrin-stained amino acid standards are circled. Autoradiography was for 3 weeks at −70°C.

the mechanisms by which the activities of these kinases are normally regulated. To investigate the cause of this anomaly, we sought to identify and characterize the regulatory sites of phosphorylation on pp59$^{c\text{-}fyn}$ and the sequences involved in mediating the interaction between pp59$^{c\text{-}fyn}$ and middle-T antigen.

## MATERIALS AND METHODS

**Mutagenesis of human c-*fyn* and plasmid constructions.** For mutagenesis experiments, a human c-*fyn* cDNA isolated from human embryo fibroblasts was used (36). All the mutations were introduced into the plasmid pSP65 c-*fyn* (5), which contains the entire cDNA for human pp59$^{c\text{-}fyn}$. Site-directed mutagenesis was performed by using the gapped heteroduplex approach essentially as described previously (5–7, 15, 16). The gapped heteroduplex used for making the 420F mutant was formed by annealing pSP65 c-*fyn* linearized at its *Bam*HI site with the 3.6-kb *Nde*I-*Bgl*I fragment of pSP65 c-*fyn*. For the 531F mutant, the gapped heteroduplex was made by annealing pSP65 c-*fyn* linearized with *Bam*HI with the 3.6-kb *Sca*I-*Sal*I fragment of pSP65 c-*fyn*. The synthetic oligonucleotide used to generate the 420F mutant was 5′-GACAATGAGTTCACAGCAAG-3′, and that used for the 531F mutant was 5′-GCCCCAGTTCCAACCTG GTG-3′. The combined 420F-531F mutant (420/531F) was assembled by replacing the 1.4-kb *Bam*HI-*Bst*EII fragment of 531F with that of 420F.

**Construction of chimeras HY F/S527 and HY S/F531.** To construct the chimeras HY F/S527 and HY S/F531, the codons for Gly-82, Val-83, and Thr-84 of pp60$^{c\text{-}src}$ and those for Gly-83, Val-84, and Thr-85 of pp59$^{c\text{-}fyn}$ were changed to 5′-GGGGTGACC-3′, thus creating a *Bst*EII recognition site in each cDNA without altering the coded amino acid sequence of either protein. The *Bst*EII restriction enzyme recognition site was engineered into pSP65 531F (36) and pSP65 527F (33) by using the oligonucleotides 5′-GAA CAGGGGTGACCCTCTTTG-3′ and 5′-GCTGGCGGGGT GACCACTTTC-3′, respectively. The resultant recombinant plasmids were referred to as 531F-*Bst*EII and 527F-*Bst*EII. HY F/S527 was assembled by replacing the 1.4-kb *Bst*EII-*Sal*I fragment of 531F-*Bst*EII with that of 527F-*Bst*EII. The chimera HY S/F531 was generated by annealing the 1.2-kb

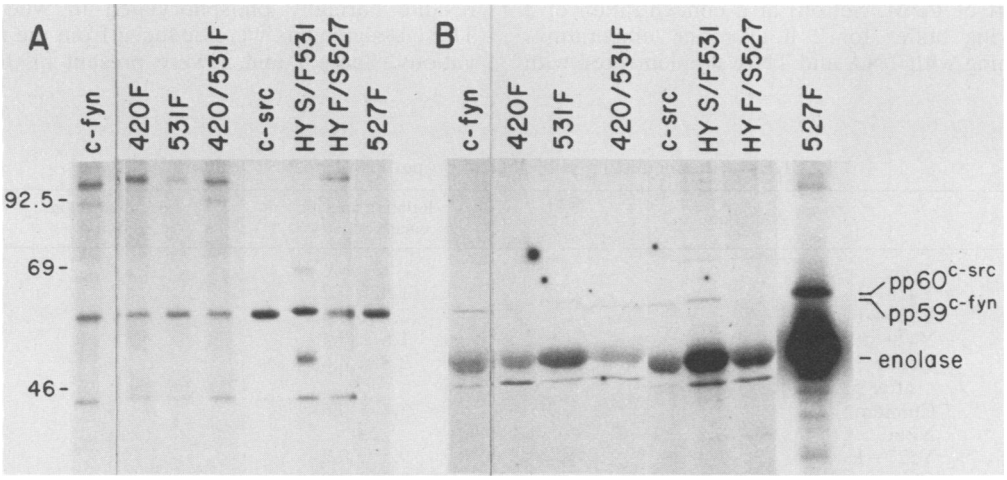


FIG. 2. Relative specific tyrosine kinase activities of the pp59$^{c\text{-}fyn}$ variants. Quantitative kinase assays were performed as described previously (7, 15, 16) and as indicated in Materials and Methods. Except for wild-type pp60$^{c\text{-}src}$, 527F, and HY S/F531, for which assays were performed on EC10-derived immunoprecipitates, the kinase activities of pp59$^{c\text{-}fyn}$ and its derivatives were assayed with the antibody CFN-2. The relative specific kinase activities were calculated by dividing the amount of $^{32}$P incorporated into enolase (B) by the amount of [$^{35}$S]methionine incorporated into the tyrosine kinases (A). Values for the mutant pp59$^{c\text{-}fyn}$ were normalized relative to those for wild-type pp59$^{c\text{-}fyn}$, and values for 527F and HY S/F531 were normalized to those for wild-type pp60$^{c\text{-}src}$. Average specific activities from three experiments of this type are given in Table 1. The [$^{35}$S]methionine gel was fluorographed and exposed for 4 days. Autoradiography time for the $^{32}$P gel was 2 h at −70°C. Molecular size markers are on the left in kilodaltons.

*Bst*EII-*Bst*EII and the 0.2-kb *Bst*EII-*Sal*I fragments of 531F-*Bst*EII to 527F-*Bst*EII digested with *Bst*EII and *Sal*I.

**Biological and biochemical assays.** The procedures for monitoring transforming activity by focus formation and by growth in soft agar were as described by Piwnica-Worms et al. (33) and Cheng et al. (7). All of the procedures for metabolic labeling of cells; preparation of cell lysates; immunoprecipitation of proteins using antibodies for pp59$^{c\text{-}fyn}$, pp60$^{c\text{-}src}$, and middle-T antigen; tyrosine kinase assays; complex formation assay; phosphoamino acid analysis; two-dimensional tryptic phosphopeptide analysis; one-dimensional peptide mapping using *Staphylococcus aureus* V8 protease; and in vitro transcription and translation of c-*fyn* and c-*src* cDNAs have been fully described elsewhere (5–7, 14–16, 33).

**Rescue of proviral fyn-T DNA from c-fyn/3T3 cells.** The fyn-T DNA was rescued from c-fyn/3T3 cells by the rescue-fusion protocol (5). Briefly, c-fyn/3T3 cells were trypsinized and plated at a ratio of 1:1 with cos-1 cells such that confluency was attained following approximately 48 h in culture. Cells were rinsed with serum-free medium and then fused by the addition of 50% polyethylene glycol for 1 min. The fused cells were allowed to grow for 3 days, after which Hirt DNA (17) was prepared and used to transform DH5α bacteria. The DNAs from kanamycin-resistant bacterial colonies were digested with *Bam*HI and *Sal*I and ligated to pUC18 predigested with the same enzymes. Two clones (pUCfyn-T1 and pUCfyn-T2) which exhibited anomalous restriction enzyme digestion patterns compared with those of wild-type c-*fyn* were selected for further analysis.

**Western blotting.** The method for preparing samples for Western blot (immunoblot) analyses and the conditions for transfer of proteins to nitrocellulose filters were as described by Kamps and Sefton (19). Typically, approximately 100 μg of protein from each lysate was loaded onto the gels. Following transfer to nitrocellulose, the filters were preblocked with blocking buffer (5% bovine serum albumin and 1% ovalbumin in TNA buffer [10 mM Tris, pH 7.2; 0.9% NaCl; 0.01% $NaN_3$]) for 18 h at room temperature. The filters were then probed with polyclonal antibodies to phosphotyrosine (gift of B. M. Sefton) at a concentration of 2 μg/ml in blocking buffer for 2 h at room temperature. Following washing with TNA and TNA supplemented with 0.05% Nonidet P-40, the filters were incubated with 10 μCi of $^{125}$I-labeled protein A in 40 ml of blocking buffer for 30 min at room temperature. The filters were washed extensively with TNA and TNA containing 0.05% Nonidet P-40 and then allowed to dry.

## RESULTS

**Sites of phosphorylation on the human pp59$^{c\text{-}fyn}$kinase.** To determine the sites phosphorylated in vivo, the engineered cell line c-fyn/3T3 (5), which overexpresses human pp59$^{c\text{-}fyn}$, was metabolically labeled with $^{32}P_i$. The lysates were incubated with CFN-2 serum, an anti-pp59$^{c\text{-}fyn}$ serum (5), and the immunoprecipitates derived therein were subjected to phosphoamino acid analysis. In unsynchronized cells, pp59$^{c\text{-}fyn}$ was determined to be phosphorylated primarily on tyrosine and serine residues (Fig. 1). The stoichiometry of serine phosphorylation to that of tyrosine was determined to be approximately 2:1.

Judged by the homology between the amino acid sequences of pp59$^{c\text{-}fyn}$ and the closely related pp60$^{c\text{-}src}$, Tyr-420 and Tyr-531 are candidate sites of tyrosine phosphorylation. To localize the sites of phosphorylation on pp59$^{c\text{-}fyn}$ and to ascertain the effect of these posttranslational modifications on the biochemical and biological activities of the cellular kinase, mutant variants of the human c-*fyn* gene product containing lesions at the presumptive phosphorylation sites were generated. Two point mutants (Tyr-420→Phe [420F] and Tyr-531→Phe [531F]) and a double mutant (420/531F) combining the lesions in 420F and 531F were generated. These mutant genes were engineered into a retroviral expression vector (33) which was then used to transfect the variant genes into NIH 3T3 cells. Stable NIH 3T3 cell lines expressing the mutant proteins were identified by immunoprecipitation using CFN-2 serum (Fig. 2A).

To localize the sites of phosphorylation on pp59$^{c\text{-}fyn}$, two-dimensional tryptic phosphopeptide analysis of $^{32}P$ metabolically labeled mutant proteins was performed (Fig. 3). Analysis of the resultant phosphotryptic maps suggested Tyr-420 as the site of autophosphorylation and Tyr-531 as a residue normally phosphorylated in wild-type pp59$^{c\text{-}fyn}$. These assignments were deduced from the following observations. Spots 1 and 2 were present in the tryptic phos-

TABLE 1. Biochemical and biological properties of pp59$^{c\text{-}fyn}$ mutants

| Mutant | Mutation | Relative specific kinase activity[a] | Focus-forming activity[b] | Growth in soft agar[c] |
|---|---|---|---|---|
| c-fyn | None | 1.0 | − | − |
| 420F | Y420→F | 1.0 ± 0.2 | − | − |
| 531F | Y531→F | 2.9 ± 0.4 | − | − |
| 420/531F | Y420 and Y531→F | 1.6 ± 0.4 | − | − |
| fyn-T | Delete 524–537 and 9-amino-acid insert after 523 | 2.8 ± 0.6 | − | − |
| HY F/S527 | Chimera | 3.4 ± 0.6 | + | − |
| c-src | None | 1.0 | − | − |
| 527F | Y527→F | 9.5 ± 0.2 | + | + |
| HY S/F531 | Chimera | 3.7 ± 0.8 | − | − |

[a] Determinations were performed with enolase as described in the legend to Fig. 2. The values for wild-type pp60$^{c\text{-}src}$ and for the 527F and HY S/F531 mutants were determined by using the monoclonal antibody EC10 (31) and were calculated in relation to the value for wild-type pp60$^{c\text{-}src}$, which was taken as 1.0. All other mutants were assayed with the anti-pp59$^{c\text{-}fyn}$ antibody CFN-2. The values for these represent fold activation relative to that of normal pp59$^{c\text{-}fyn}$, which was assigned a value of 1.0. The data are expressed as the arithmetic average from three separate trials ± the average deviation from the mean.

[b] Transforming activity was monitored by focus formation over a monolayer of NIH 3T3 cells following infection with the various recombinant retroviruses encoding the pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$ variants. +, Mutants demonstrating focus-forming activity; −, mutants demonstrating no focus-forming activity.

[c] Transforming activity was also assessed by monitoring the ability of NIH 3T3 cells expressing the various mutants to grow in a 0.3% agar suspension. Cells able to proliferate to a diameter of 1 mm after 3 weeks were counted. +, Cells capable of growth in suspension; −, cells which failed to proliferate.

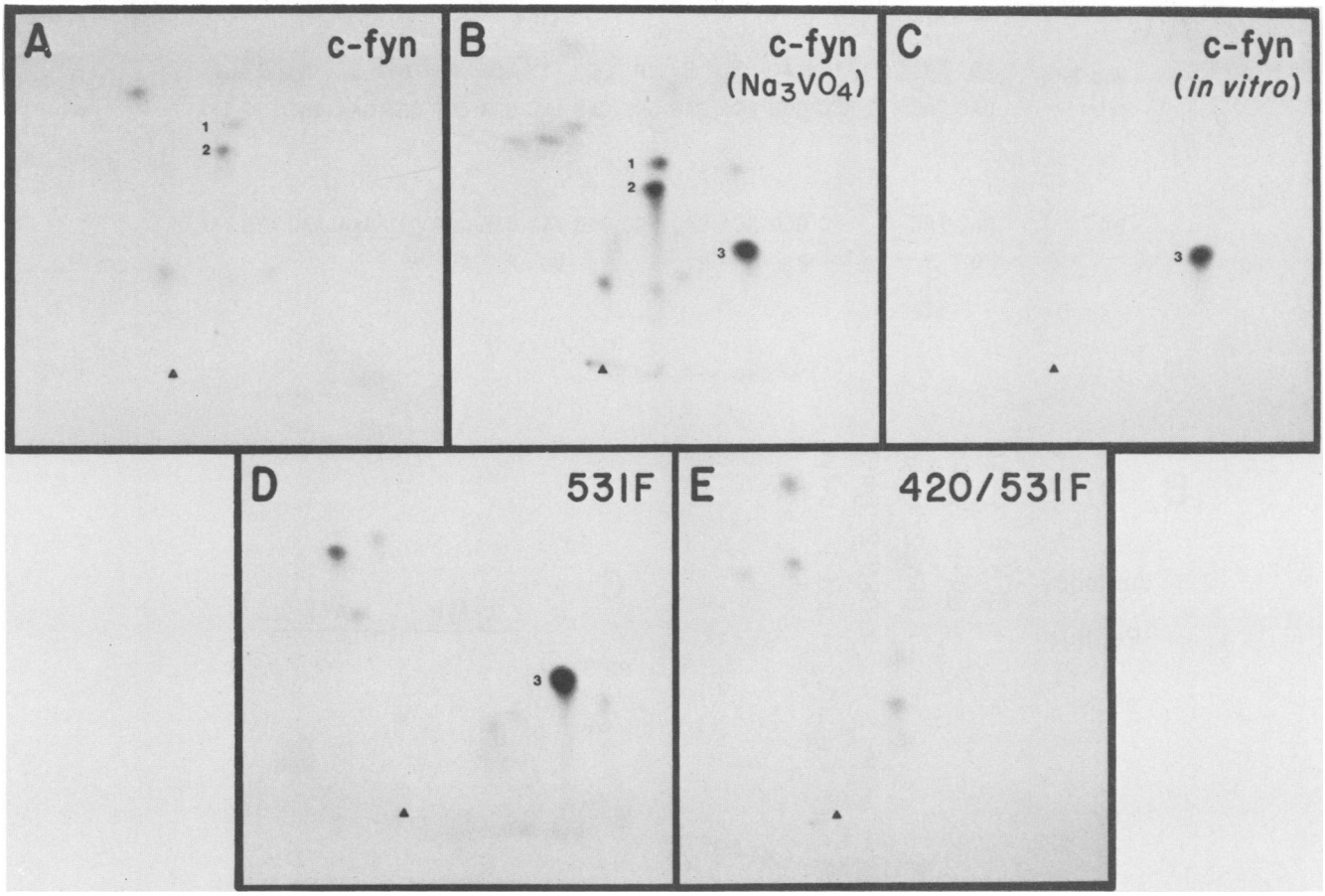


FIG. 3. Two-dimensional tryptic phosphopeptide maps of the pp59$^{c\text{-}fyn}$ mutant proteins. $^{32}$P-labeled pp59$^{c\text{-}fyn}$ proteins obtained by immunoprecipitation with CFN-2 antibody were gel purified, digested with trypsin, resolved by electrophoresis and chromatography in two dimensions, and then analyzed by autoradiography. Electrophoresis at pH 8.9 was first performed in the horizontal dimension with the anode on the right and was followed by ascending chromatography. The origins are marked with arrowheads. Spots 1 and 2, peptides containing Tyr-531; spot 3, peptide containing Tyr-420. Exposure time was 4 weeks at −70°C.

phopeptide map of wild-type pp59$^{c\text{-}fyn}$ (Fig. 3A) but absent in the maps of mutants 531F (Fig. 3D) and 420/531F (Fig. 3E) and were therefore deduced to correspond to peptides containing Tyr-531. Spot 3 was deduced to be the peptide harboring Tyr-420, since this phosphopeptide was observed in maps of in vitro-phosphorylated pp59$^{c\text{-}fyn}$ (Fig. 3C) and the 531F mutant (Fig. 3D) but absent in the double mutant 420/531F (Fig. 3E). The observation of increased phosphorylation on Tyr-420 with the 531F mutant and when wild-type pp59$^{c\text{-}fyn}$ was labeled in the presence of the phosphatase inhibitor sodium vanadate (Fig. 3B) is analogous to data obtained with pp60$^{c\text{-}src}$ (15).

**Biological activities of NIH 3T3 cells expressing wild-type and mutant pp59$^{c\text{-}fyn}$.** To investigate whether the phosphorylation sites identified above are involved in the regulation of the pp59$^{c\text{-}fyn}$ kinase, the mutants were tested for their abilities to transform cells in culture. Like wild-type pp59$^{c\text{-}fyn}$ (5), none of the pp59$^{c\text{-}fyn}$ point mutants were able to induce focus growth when assayed on NIH 3T3 cells or to sustain anchorage-independent growth in soft-agar suspension (Table 1).

During the course of this work, we also generated another mutant of c-*fyn* which we refer to as fyn-T. The mutant fyn-T was rescued from an NIH 3T3 cell line overexpressing wild-type human pp59$^{c\text{-}fyn}$ which had been continuously passaged for over 20 generations. Following repeated passaging, a proportion of the cells expressed a spontaneous truncated mutant of c-*fyn* whose gene product displayed aberrant enzymatic activity (see below). The cells expressing the mutant *fyn* protein were phenotypically normal. The mutant *fyn* DNA (fyn-T) was molecularly cloned from this cell line and upon DNA sequencing of the entire rescued cDNA clone was shown to harbor a deletion of nucleotides 2149 to 2153 (nucleotide numbering according to Semba et al. [36]). As a consequence of the frameshift mutation, fyn-T was predicted to encode a truncated protein of 532 residues (Fig. 4A). The carboxy-terminal truncated protein would contain residues 1 through 523 of pp59$^{c\text{-}fyn}$ and an additional nine unrelated residues resulting from translation of an alternative reading frame (Fig. 4A).

Analysis of [$^{35}$S]methionine-labeled, in vitro-translated fyn-T on polyacrylamide gels showed that the gene product migrated more rapidly than wild-type pp59$^{c\text{-}fyn}$, a fact consistent with the presence of the expected deletion (Fig. 4B; compare lanes 3 and 4). Immunoprecipitation analysis confirmed that the deletion resided at the carboxy terminus of fyn-T. In vitro-synthesized wild-type pp59$^{c\text{-}fyn}$ (Fig. 4B, lane 5) but not the fyn-T product (lane 2) was immunoprecipitated by cst-1, a peptide antibody raised against the carboxy-terminal seven residues common to both pp59$^{c\text{-}fyn}$ and

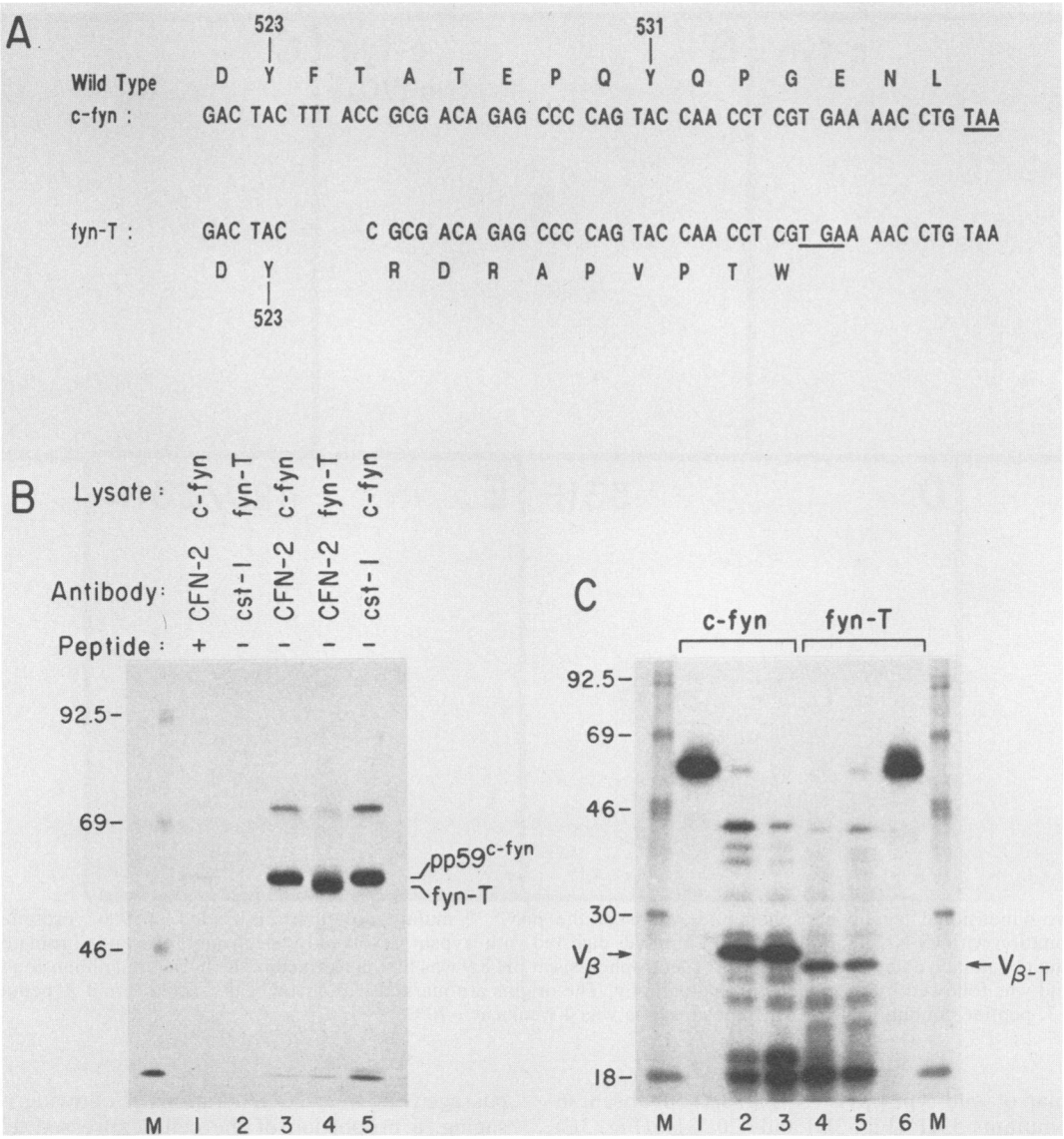


FIG. 4. Immunoprecipitation and one-dimensional peptide analysis of the fyn-T mutant. (A) Nucleotide sequence of the 3′ coding regions present in wild-type human c-*fyn* and fyn-T. Termination codons are underlined. Amino acid sequences predicted for human pp59$^{c\text{-}fyn}$ and fyn-T are also shown by one-letter designations. (B) pSP65 c-*fyn* and pSP65 fyn-T were transcribed with SP6 RNA polymerase. Following purification, the RNA transcripts were translated in vitro in a rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine. Samples were then immunoprecipitated with peptide antibodies raised against sequences present within the amino terminus of pp59$^{c\text{-}fyn}$ (CFN-2) or the carboxy terminus of pp59$^{c\text{-}fyn}$ (cst-1). Immunoprecipitation reactions were performed in the presence of the cognate peptide (+) or in the absence of added peptides (−). (C) Wild-type pp59$^{c\text{-}fyn}$ (lanes 1 to 3) and fyn-T mutant (lanes 4 to 6) were purified from $^{32}$P-labeled cells by immunoprecipitation with CFN-2 antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, digested with *S. aureus* V8 protease (0.0017 μg/μl [lanes 2 and 5] or 0.017 μg/μl [lanes 3 and 4]), and then electrophoresed on a 15% polyacrylamide gel. Lanes 1 and 6 were untreated controls. Lanes M contain molecular size markers in kilodaltons. The $V_\beta$ fragments corresponding to the carboxy-terminal portions of wild-type pp59$^{c\text{-}fyn}$ and fyn-T ($V_{\beta\text{-}T}$) are indicated by arrows. Exposure time was 1 week.

pp60$^{c\text{-}src}$ (14). Finally, the deletion within the fyn-T carboxy terminus was verified by one-dimensional peptide analysis using *S. aureus* V8 protease (Fig. 4C). The carboxy-terminal $V_\beta$ fragment of the fyn-T mutant protein ($V_{\beta\text{-}T}$) migrated with greater mobility on the gel, which is consistent with the expected deletion.

The fyn-T gene product which lacks Tyr-531, like the 531F mutant, was also judged to be nontransforming when examined by the focus-forming assay (Table 1). Hence, as is not the case with the other *src*-like tyrosine kinases, preventing phosphorylation at Tyr-531 does not result in activation of the transforming activity of pp59$^{c\text{-}fyn}$.

**Enzymatic activities of pp59$^{c\text{-}fyn}$ variants.** To determine the effect of the lesions on the protein tyrosine kinase activity of pp59$^{c\text{-}fyn}$, the relative specific kinase activities of the mutant proteins were assessed by monitoring their ability to phosphorylate enolase in vitro. Briefly, lysates prepared from [$^{35}$S]methionine-labeled cells were first normalized to contain approximately equivalent amounts of pp59$^{c\text{-}fyn}$. Immunoprecipitates derived by using CFN-2 serum were then

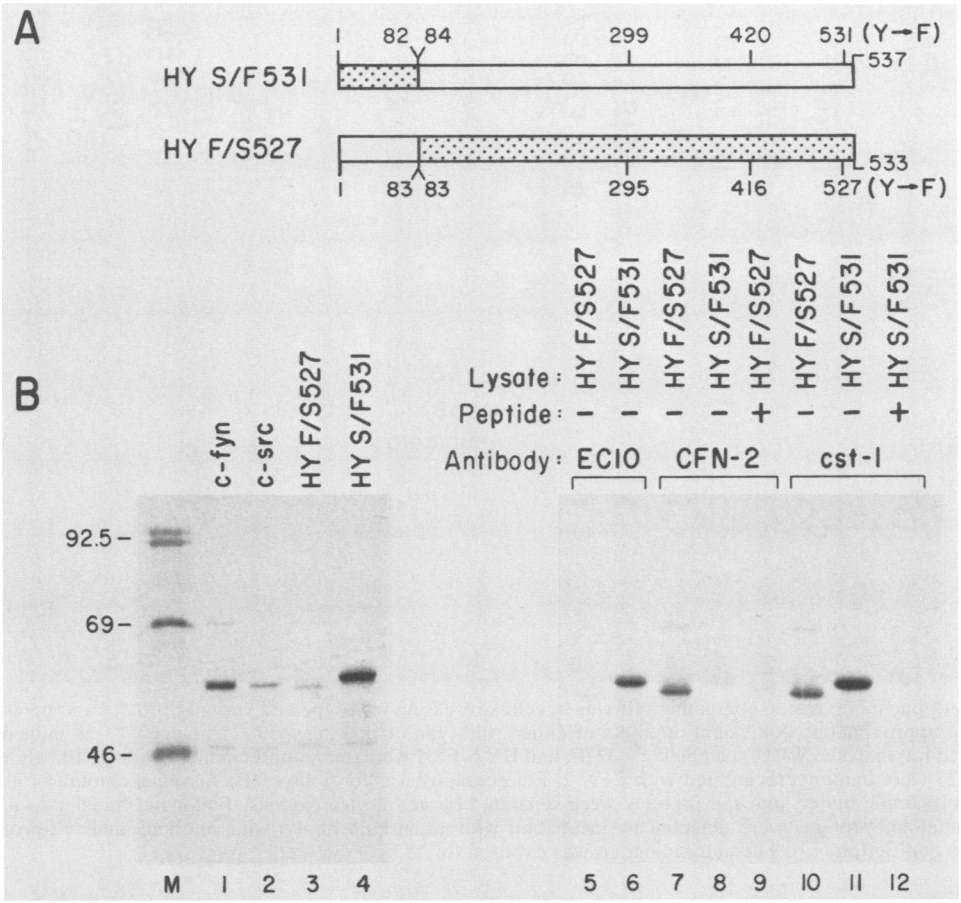


FIG. 5. Immunoprecipitation analysis of the pp59$^{c\text{-}fyn}$-pp60$^{c\text{-}src}$ chimeras. (A) Predicted amino acid sequences of the chimeras. The protein sequence coded for by c-*src* is shaded, while that for c-*fyn* is unshaded. The numbers correspond to amino acids in the wild-type proteins. (B) pSP65 HY S/F531 and pSP65 HY F/S527 were transcribed, and the products were translated in vitro in rabbit reticulocyte lysates. Samples were either analyzed directly on polyacrylamide gels (lanes 1 to 4) or immunoprecipitated with EC10 monoclonal antibody (lanes 5 and 6), CFN-2 antibody (lanes 7 to 9), or cst-1 peptide antibody (lanes 10 to 12). Immunoprecipitations were performed in the presence of the peptide immunogen (+) or in the absence of added peptide (−). The presence of an approximately 75-kDa band from in vitro translates of c-*fyn* has been described previously (5). Interestingly, the same 75-kDa band (which is *fyn* related) was also observed with the chimera HY F/S527 but not with HY S/F531. Fluorography was for 24 h at −70°C.

either resolved on gels (Fig. 2A) to confirm the amount of pp59$^{c\text{-}fyn}$ or incubated with [γ-$^{32}$P]ATP and enolase (Fig. 2B) to evaluate the abilities of the variant proteins to phosphorylate enolase.

Alteration of Tyr-531 to phenylalanine in the 531F mutant or deletion of Tyr-531 in the fyn-T mutant enhanced the ability of pp59$^{c\text{-}fyn}$ to phosphorylate enolase in vitro by approximately threefold (Table 1). Interestingly, although the modification of Tyr-420 to phenylalanine alone did not detectably alter the kinase activity of the mutant, combining the mutations at residues 420 and 531 (420/531F) resulted in activating the kinase activity, albeit to only approximately 50% of that observed with 531F. This observation is analogous to that obtained with pp60$^{c\text{-}src}$ (3, 16, 26, 33). Hence, preventing phosphorylation at Tyr-531 results in activation of the pp59$^{c\text{-}fyn}$ tyrosine kinase activity but to a level insufficient to transform NIH 3T3 cells in culture.

**Properties of chimeras HY F/S527 and HY S/F531.** It has been proposed that the divergence of sequences observed in the amino-terminal 80 or so residues of the various members of the *src* family of tyrosine kinases may confer different substrate specificity (for a review, see reference 9). The inability of the 531F mutant of pp59$^{c\text{-}fyn}$ to transform NIH 3T3 cells despite a detectable enhancement of its tyrosine kinase activity could therefore have been a consequence of a lack of appropriate substrates in the test cells. To examine this possibility, two chimeric constructs were generated by the reciprocal exchange of the amino-terminal sequences unique to the 531F mutant of pp59$^{c\text{-}fyn}$ and the transforming mutant (527F) of pp60$^{c\text{-}src}$ (7, 33). The resultant chimera, HY F/S527, encoded the amino-terminal 83 amino acids of pp59$^{c\text{-}fyn}$ and residues 83 to 533 of the 527F mutant of pp60$^{c\text{-}src}$, while the chimera HY S/F531 encoded the first 82 amino acids of pp60$^{c\text{-}src}$ and residues 84 to 537 of the 531F mutant of pp59$^{c\text{-}fyn}$ (Fig. 5A).

The structures of the recombinant chimeric plasmids were verified by restriction mapping, and those of their encoded products were verified by using domain-specific antibodies. Hence, both in vitro-synthesized chimeric proteins were immunoprecipitable with cst-1 peptide antibodies (14), whose epitopes are within the carboxy-terminal seven residues common to the two tyrosine kinases (Fig. 5B, lanes 10 and 11). The chimera HY S/F531 but not HY F/S527 was reactive with EC10 antibody (31), whose epitope is located

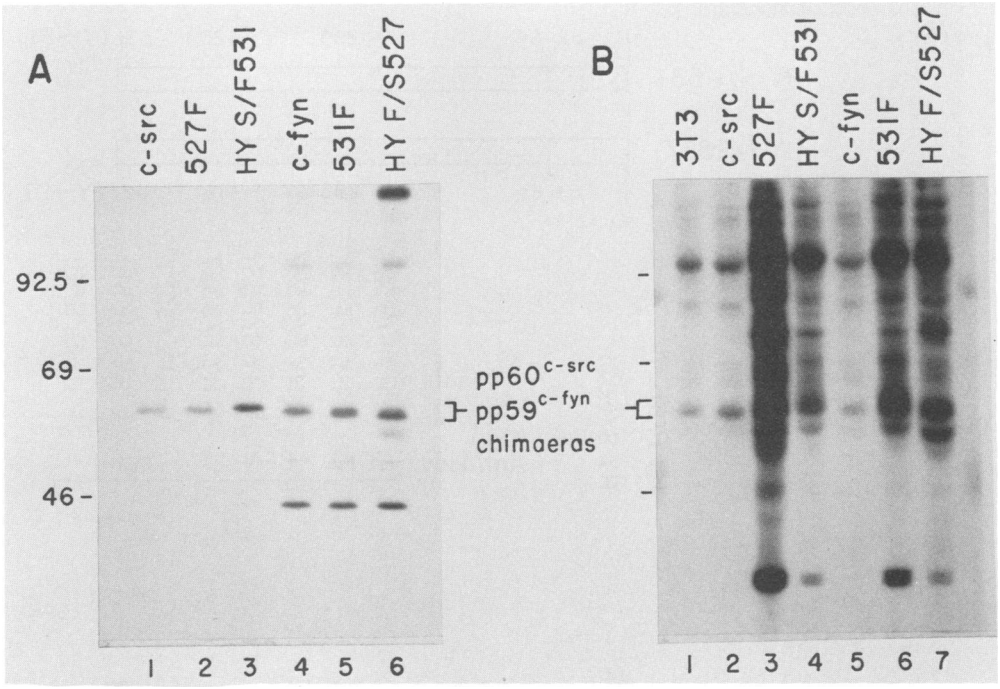


FIG. 6. Analysis of phosphotyrosine-containing proteins in cells expressing wild-type and variant pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$. (A) Clonal NIH 3T3 cells expressing approximately equivalent amounts of either wild-type or mutant pp59$^{c\text{-}fyn}$ or pp60$^{c\text{-}src}$ as judged by [$^{35}$S]methionine labeling were selected for analysis. Wild-type pp60$^{c\text{-}src}$, 527F, and HY S/F531 were immunoprecipitated with EC10, while wild-type pp59$^{c\text{-}fyn}$, 531F, and HY F/S527 were immunoprecipitated with CFN-2. Fluorography was for 4 days. (B) An equal amount of protein from each cell line was lysed in hot sample buffer, and the proteins were separated by gel electrophoresis. Following transfer to a nitrocellulose filter, phosphotyrosine-containing proteins were detected by incubation with an antiphosphotyrosine antibody and $^{125}$I-protein A essentially as described by Kamps and Sefton (19). The autoradiogram was exposed for 18 h.

within the amino terminus of pp60$^{c\text{-}src}$ (Fig. 5B, lanes 5 and 6), which is consistent with HY S/F531 having the amino-terminal portion of pp60$^{c\text{-}src}$. Conversely, HY F/S527 but not HY S/F531 was recognized by CFN-2 serum (5), an antibody raised against sequences located within the amino terminus of pp59$^{c\text{-}fyn}$, confirming that HY F/S527 contained *fyn*-like amino-terminal sequences (Fig. 5B, lanes 7 and 8).

Examination by the focus formation assay demonstrated that HY F/S527 but not HY S/F531 was transforming (Table 1). Foci appeared around 40 days postinfection compared with 14 days for 527F. Moreover, the number of foci induced was approximately 10% of the total number of G418-resistant colonies (average of 40 G418-resistant colonies per 90-mm dish when the transient viral supernatants were used) compared with 70% for 527F. Furthermore, unlike cells expressing 527F, stable NIH 3T3 cells expressing the HY F/S527 mutant were also unable to proliferate in soft-agar suspension (Table 1). The kinase activities of the chimeras were also determined by monitoring the phosphorylation of enolase in vitro (Fig. 2). Chimera HY F/S527 was judged to have a 3.4-fold-increased kinase activity relative to that of wild-type pp59$^{c\text{-}fyn}$ (Table 1), while HY S/F531 was determined to be activated 3.7-fold over wild-type pp60$^{c\text{-}src}$. The finding that HY S/F531 is nontransforming is consistent with the kinase activity measurements, since the fold elevation observed for this chimera is below the threshold we observed to be necessary for mutants of pp60$^{c\text{-}src}$ to transform NIH 3T3 cells (15, 16, 33). However, HY F/S527 was judged to be transforming, although its enolase-phosphorylating activity was shown to be less than that of HY S/F531. To test whether this result was misleading, for example, reflecting differences between the abilities of pp59$^{c\text{-}fyn}$ and pp60$^{c\text{-}src}$ to phosphorylate enolase rather than intrinsic enzymatic activity, the kinase activities of the variant proteins in vivo were compared by using antiphosphotyrosine antibodies.

**Tyrosine kinase activities of mutants assayed in vivo.** The in vivo kinase activities of the various mutants were assessed by monitoring the phosphorylation of cellular proteins in an immunoblot analysis using antiphosphotyrosine antibodies (19). Lysates from cell lines expressing approximately similar amounts of wild-type and mutant tyrosine kinases were used (Fig. 6A). The highest level of immunoreactivity was reproducibly observed with the transforming 527F variant of pp60$^{c\text{-}src}$ followed by HY F/S527, HY S/F531, and the 531F mutant pp59$^{c\text{-}fyn}$ (Fig. 6B). Furthermore, it is clear from this assay that HY F/S527 is more kinase active in cells than the HY S/F531 counterpart, with the former displaying both quantitatively and qualitatively increased immunoreactive bands.

**Carboxy terminus of pp59$^{c\text{-}fyn}$ is necessary for complex formation with middle-T antigen.** To address whether the carboxy-terminal region in pp59$^{c\text{-}fyn}$ was involved in binding middle-T antigen, the ability of the truncated fyn-T protein to associate with the viral antigen was assayed. Two additional cell lines, one coexpressing wild-type pp59$^{c\text{-}fyn}$ and middle-T antigen (c-fyn/mt) and the other coexpressing fyn-T and middle-T antigen (fyn-T/mt), were generated. Figure 7, lanes 2 and 3, shows the results of in vitro kinase assays of immunoprecipitates obtained by using HK3 serum, an antiserum specific for the early region of polyomavirus (6, 14). The signal observed represents the total kinase-active fraction of middle-T antigen present in these cells. To

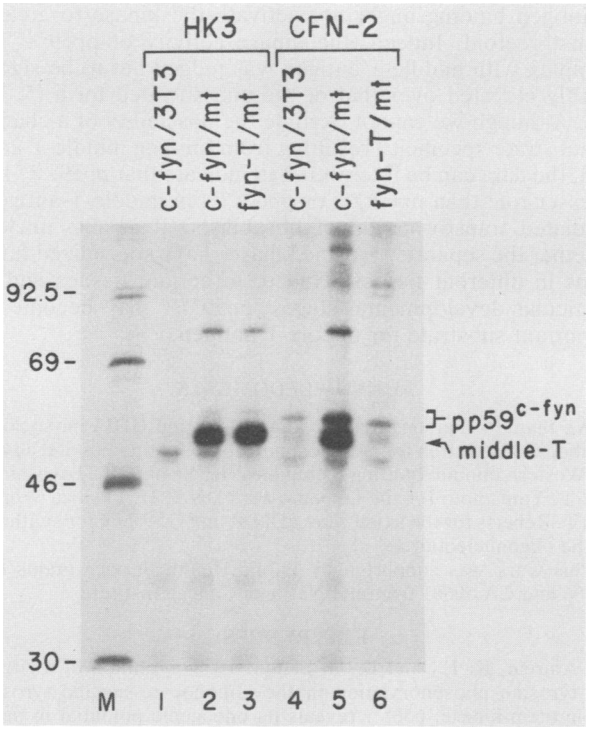


FIG. 7. The fyn-T mutant is unable to associate stably with middle-T antigen. Lysates were prepared from NIH 3T3 cells expressing wild-type pp59$^{c\text{-}fyn}$ (c-fyn/3T3), the fyn-T mutant (fyn-T/3T3), both wild-type pp59$^{c\text{-}fyn}$ and middle-T antigen (c-fyn/mt), and both fyn-T and middle-T antigen (fyn-T/mt). The lysates were normalized for protein content, immunoprecipitated with HK3 (anti-middle-T-antigen serum raised in hamsters) or CFN-2 (anti-pp59$^{c\text{-}fyn}$ serum) antibody, and phosphorylated in vitro by using [$\gamma$-$^{32}$P]ATP. The positions of pp59$^{c\text{-}fyn}$ and middle-T antigen are indicated on the right. Autoradiography was for 18 h.

determine whether fyn-T is capable of associating with middle-T antigen, in vitro kinase assays were repeated on CFN-2-derived immunoprecipitates. Phosphorylation of middle-T antigen in this assay is indicative of coimmunoprecipitation and hence of complex formation with the viral protein. Middle-T antigen was phosphorylated only in lysates containing wild-type pp59$^{c\text{-}fyn}$ but not detectably in those containing fyn-T (Fig. 7, lanes 5 and 6), indicating that fyn-T is unable to associate stably with middle-T antigen. We were also unable to detect coimmunoprecipitation of middle-T antigen from [$^{35}$S]methionine-labeled fyn-T/mt cells (data not shown).

## DISCUSSION

**pp59$^{c\text{-}fyn}$ kinase is regulated by phosphorylation at tyrosines 420 and 531.** Prevention of phosphorylation at Tyr-527 in the case of pp60$^{c\text{-}src}$ by mutagenesis to phenylalanine or by middle-T-antigen binding activates both the tyrosine kinase and transforming activities of the enzyme (3, 4, 12, 26, 33). Although middle-T-antigen binding also activates the kinase activity of complexed pp62$^{c\text{-}yes}$, in the case of complexed pp59$^{c\text{-}fyn}$ very little or no activation is observed (5, 18, 29). Therefore, either the pp59$^{c\text{-}fyn}$ kinase is regulated by mechanisms other than phosphorylation at Tyr-531 (the residue analogous to Tyr-527 of pp60$^{c\text{-}src}$) or middle-T-antigen binding does not directly affect the ability of cellular proteins to regulate phosphorylation at this site. When two-dimensional tryptic phosphopeptide mapping was used, the residue Tyr-531 was identified as a site normally phosphorylated in vivo and Tyr-420 was determined to be the site of autophosphorylation in vitro. These assignments are consistent with previous mapping data on the related pp60$^{c\text{-}src}$ (10, 38), pp56$^{lck}$ (1, 30), and p59$^{hck}$ (40) kinases. However, unlike the situation with pp60$^{c\text{-}src}$ (3, 26, 33), pp56$^{lck}$ (1, 30), or p59$^{hck}$ (40) mutated at the equivalent site, mutagenesis of the Tyr-531 of pp59$^{c\text{-}fyn}$ to phenylalanine as in the 531F mutant or its absence in the truncated fyn-T mutant did not result in oncogenic conversion of the pp59$^{c\text{-}fyn}$. The tyrosine kinase activities of the 531F and fyn-T mutants were, however, judged to be elevated approximately threefold above that of the normal counterpart. Hence the pp59$^{c\text{-}fyn}$ kinase, like the other members of the *src* family, is at least partially suppressed by tyrosine phosphorylation at Tyr-531. This finding is consistent with the report by Kypta et al. (28) indicating that pp59$^{c\text{-}fyn}$ is negatively regulated by tyrosine phosphorylation. Dephosphorylation results in activation of the catalytic activity, albeit to a low level previously determined to be insufficient for neoplastic transformation. Derepression of kinase activity in the 531F mutant is additionally associated with increased phosphorylation at Tyr-420, a site not normally phosphorylated in wild-type pp59$^{c\text{-}fyn}$. Stable phosphorylation of Tyr-420 is observed only in overexpressed pp59$^{c\text{-}fyn}$ in NIH 3T3 cells in the presence of the phosphatase inhibitor sodium orthovanadate. The phosphorylation of Tyr-420 in the 531F mutant is important, since the additional introduction of a Tyr-420→Phe mutation suppresses the kinase activity to approximately half the maximum observed with the Tyr-531→Phe mutation alone.

The finding that neither the 531F nor the fyn-T mutant is transforming in NIH 3T3 cells contrasts with the report by Kawakami et al. (23), who demonstrated that oncogenic forms of *fyn* selected for their abilities to transform NIH 3T3 cells lacked sequences including Tyr-531 at the carboxy terminus. We do not have a satisfactory explanation for this discrepancy. Differences in levels of expression or the choice of target cells may be responsible. However, interestingly, the site of mutation of the spontaneous transforming mutants described by Kawakami et al. (23) and our fyn-T mutant all impinge on codon 524.

The inability of 531F or fyn-T to transform NIH 3T3 cells suggests that pp59$^{c\text{-}fyn}$ may be additionally suppressed by mechanisms which include events other than or in addition to phosphorylation at the regulatory Tyr-531. The suggestion that different members of the *src* family have widely different kinase activities in NIH 3T3 cells is illustrated by the finding that p59$^{hck}$ mutated at the corresponding Tyr-501 is 100-fold more potent as a transforming agent than pp56$^{lck}$ or pp60$^{c\text{-}src}$ mutated at the homologous Tyr-505 and Tyr-527, respectively (40). Regulation of the transforming potential may be effected by restricting either the kinase activity or the substrate specificity of the cellular enzymes or both. This differential restriction may be imposed by the structural variability resulting from the unique amino terminus of each tyrosine kinase.

**Inability of 531F to transform NIH 3T3 cells is due to limited enhancement of its kinase activity.** To ascertain whether the inability of the 531F mutant to elicit neoplastic transformation resulted from the absence of pp59$^{c\text{-}fyn}$ substrates in NIH 3T3 cells or was related to insufficient activation of the pp59$^{c\text{-}fyn}$ tyrosine kinase activity, we constructed the chimeras HY S/F531 and HY F/S527. We rationalized that if the

lack of pp59$^{c\text{-}fyn}$-specific substrates was the cause, then the chimera HY S/F531, because it contains the amino-terminal domain of pp60$^{c\text{-}src}$ believed to confer substrate specificity, should be able to transform NIH 3T3 cells. However, HY F/S527 but not HY S/F531 was determined to be weakly transforming. Provided sufficient sequences were transferred to alter substrate specificity, this result suggests that the inability of 531F to transform was probably not due to lack of appropriate substrates in NIH 3T3 cells but more probably was a consequence of insufficient activation of its kinase activity. Consistent with the suggestion that 531F was insufficiently activated was the observation that the in vivo kinase activity of the 531F mutant was less than that of the transforming HY F/S527. Results from studies of other mutants of pp60$^{c\text{-}src}$ indicate that it is necessary for the kinase activity of the corresponding mutants to reach a threshold in order to transform (7, 15, 16, 26, 33).

**Elements within the amino terminus of pp59$^{c\text{-}fyn}$ downregulate its kinase activity.** The chimera HY F/S527 was only weakly transforming compared with the 527F mutant of pp60$^{c\text{-}src}$. HY F/S527, unlike 527F, induced focus growth only after an extended period, and cells expressing the HY F/S527 mutant were unable to proliferate in soft agar. Perhaps the structure of the chimera was altered deleteriously; alternatively, replacement of the unique portion of the amino terminus of 527F (residues 1 to 82) with that of pp59$^{c\text{-}fyn}$ could suppress the transforming activity of the 527F mutant. If this is the case, there may be elements within this unique portion of the amino-terminal region of pp59$^{c\text{-}fyn}$ which are absent in pp60$^{c\text{-}src}$ that are additionally involved in suppressing the tyrosine kinase activity of pp59$^{c\text{-}fyn}$. The involvement of the amino-terminal variable domain of these kinases in regulating kinase activity or interaction with other proteins has been documented earlier (15, 22, 29, 34, 37).

If the unique amino-terminal domain of pp59$^{c\text{-}fyn}$ is solely responsible for repressing the kinase activity of the enzyme, then the reverse chimera HY S/F531 should exhibit a kinase activity which is greater than that of the 531F mutant. However, we detected only a very modest increase compared with that detected with 531F, which we interpret to mean that there are other repressive elements within the amino-terminal half of the pp59$^{c\text{-}fyn}$ molecule. Such elements may be located within the conserved noncatalytic domain (residues 83 to 260) of the *src* family of kinases. This suggestion is supported by the demonstration that a fusion protein encoded by 5′ sequences for v-*fgr* (the oncogene present in Gardner-Rasheed feline sarcoma virus) and the 3′ two-thirds of the *fyn* gene (residues 220 to 537) is highly oncogenic (24). This fusion includes, in addition, the Tyr-531 residue of pp59$^{c\text{-}fyn}$. Taken together, these results suggest that the inability of 531F to transform may be a consequence of the presence of the aforementioned repressive elements within the amino terminus of pp59$^{c\text{-}fyn}$ which act to restrict its kinase activity.

**Complex formation with middle-T antigen requires the integrity of the carboxy-terminal sequences of pp59$^{c\text{-}fyn}$.** The fyn-T mutant which lacks carboxy-terminal sequences was unable to complex with middle-T antigen. This finding is consistent with previous mapping data with pp60$^{c\text{-}src}$. A domain influencing middle-T-antigen binding has been localized to sequences proximal to the regulatory Tyr-527 of the enzyme (3, 8). Presumably, as with pp60$^{c\text{-}src}$, middle-T-antigen binding disrupts the regulation of phosphorylation at Tyr-531 of pp59$^{c\text{-}fyn}$ and thereby activates its kinase activity. However, the data from the 531F mutant predict that middle-T-antigen binding might not activate the kinase to greater than threefold. Indeed, the kinase activity of pp59$^{c\text{-}fyn}$ in complex with middle-T antigen was judged not to be significantly elevated over that of the unassociated form (5, 19, 29). Although we cannot exclude the possibility of a change in substrate specificity resulting from binding middle-T antigen, the data can be interpreted to indicate that pp59$^{c\text{-}fyn}$ has a lesser role than pp60$^{c\text{-}src}$ or pp62$^{c\text{-}yes}$ in middle-T-antigen-mediated transformation of fibroblasts. It is also unclear whether the separate tyrosine kinases have specialized functions in different tissues. Hence, in certain tissues and at particular developmental stages, pp59$^{c\text{-}fyn}$ may become an important substrate for middle-T antigen.

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