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Potential positive and negative autoregulation of p60$^{c\text{-}src}$ by intermolecular autophosphorylation

(phosphotyrosine/protein-tyrosine kinase/protooncogene/phosphorylation sites)

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ABSTRACT **The product of the protooncogene c-*src* is a protein-tyrosine kinase, p60$^{c\text{-}src}$, that is normally inhibited by phosphorylation at a tyrosine residue close to the C terminus (Tyr-527). If activated by dephosphorylation of Tyr-527, or by other means, p60$^{c\text{-}src}$ becomes phosphorylated at a tyrosine residue in the catalytic domain (Tyr-416). To test whether either or both of these tyrosines can be phosphorylated by p60$^{c\text{-}src}$ itself, we have created four mutations in c-*src*. One mutant product can receive but cannot donate phosphate, and other mutants are capable of catalysis but lack phosphorylation sites. The mutant genes were expressed singly or in combination in yeast. Analysis of the phosphorylation of mutant p60$^{c\text{-}src}$ in the yeast cells and in immunoprecipitates showed that p60$^{c\text{-}src}$ molecules can phosphorylate each other at Tyr-416 and -527. Prohibiting intramolecular phosphorylation had little effect on reaction rates and extents, suggesting that intermolecular phosphorylation predominates. If the same situation pertains in the milieu of the vertebrate fibroblast, phosphorylation of one p60$^{c\text{-}src}$ by another at Tyr-416 or -527 could permit positive or negative autoregulation.**

Many protein kinases are regulated by phosphorylation. The product of the c-*src* protooncogene, the protein-tyrosine kinase p60$^{c\text{-}src}$, has two known tyrosine phosphorylation sites that may regulate activity. Tyr-416, in the catalytic region, is the major *in vitro* phosphorylation site (1, 2). Tyr-527, close to the C terminus, is the major *in vivo* phosphorylation site (3) and a minor *in vitro* site (4).

Tyr-527 is extensively phosphorylated in p60$^{c\text{-}src}$ from normal fibroblasts (3), and this phosphorylation inhibits p60$^{c\text{-}src}$ activity. Enzymatic removal of phosphate from Tyr-527 *in vitro* stimulates p60$^{c\text{-}src}$ (4, 5). Many mutations that directly or indirectly reduce the phosphorylation state of the C terminus constitutively activate the p60$^{c\text{-}src}$ kinase (6–12). Such mutations include truncation at residue 518 or 523, replacement of Tyr-527 with phenylalanine or serine, and point mutations in the kinase domain. Association of p60$^{c\text{-}src}$ with middle-sized tumor antigen in cells transformed by polyoma virus reduces the level of phosphate at Tyr-527 and concomitantly activates the kinase (13, 14). Since the C-terminal 17 residues of p60$^{c\text{-}src}$ lie outside the minimal catalytic region, inhibition by phosphorylated Tyr-527 must involve a direct or indirect interaction between the C-terminal tail and the kinase domain, in which the tail may act as a product–analog inhibitor (4).

The effect of phosphorylation at Tyr-416 on the kinase activity of p60$^{c\text{-}src}$ is poorly understood. *In vivo*, phosphorylation of Tyr-416 is a characteristic of activated forms of p60$^{c\text{-}src}$ (1, 2, 6–9, 11, 13). It is difficult to evaluate whether the activity of p60$^{c\text{-}src}$ is stimulated by Tyr-416 phosphorylation because phosphorylation of this residue accompanies substrate phosphorylation during assay *in vitro* (1) and appears not to exceed a stoichiometry of ≈30% *in vivo* (15). *In vitro* kinase activity is not altered by a Phe-416 mutation in p60$^{c\text{-}src}$ (7, 9), and experiments with purified p60$^{v\text{-}src}$ have suggested that autophosphorylation may not alter activity (16). However, when a Phe-416 mutation is made in a p60$^{c\text{-}src}$ activated by a Phe-527 mutation, then activity is reduced 50% (7, 9). This suggests that either the Tyr-416 hydroxyl group or its phosphorylation is necessary for full activation of p60$^{c\text{-}src}$.

It is important to identify kinases and phosphatases that modulate the extent of phosphorylation of Tyr-416 and Tyr-527. Here we show that both residues can be phosphorylated by p60$^{c\text{-}src}$. Neither phosphorylation is decreased when intramolecular phosphorylation is blocked, suggesting that the reactions are predominantly intermolecular. Phosphorylation of one p60$^{c\text{-}src}$ molecule by another at Tyr-527 may provide a means to down-regulate kinase activity after stimulation by dephosphorylation.

MATERIALS AND METHODS

Plasmids. All c-*src* sequences were derived from p5H (12), which was modified by placing a *Bam*HI site at the 5′ end to create pJC21 (17). A modified Bluescribe M13+ cloning vector (Stratagene, San Diego, CA) containing the pJC21 c-*src* sequence flanked by *Bam*HI and *Bgl* II sites was introduced in *Escherichia coli* strain TG1 cells (a derivative of JM101; ref. 18). Single-stranded DNA, containing c-*src* sequences oriented in an antisense direction, was produced by infection with the helper phage M13K07 (19). Specific oligonucleotides were used for mutagenesis (20). Transformants were screened by high-stringency hybridization with the oligonucleotides used for mutagenesis, and single-stranded DNA from positive colonies was sequenced.

The c-*src* sequence was first modified to introduce two unique restriction sites close to the 3′ end: an *Xho* I site at codons 516/517 and a *Spe* I site at codon 521 [using the sense oligonucleotide CCTTCCTCGAGGACTACTTCACTAGTACAGAGCC (altered bases underlined)]. Neither of these mutations altered the coding potential of c-*src*. Further mutations were introduced into this wild type at codons 295 (oligonucleotide GGCCATAGAACTCTGAAG), 416 (CAACGAGTTTACAGCACG), and 527 (CCCCAGTTTCAGCCTGG). A plasmid that incorporates changes at both codons 416 and 527 was then constructed by using the *Xho* I site.

Both yeast shuttle vectors used contained a unique *Bam*HI expression site. pLGSD5(-ATG) (here called pU) contains the *URA3* gene, the *GAL1,10* upstream activating sequence, *CYC1* promoter, a portion of *lacZ*, pBR322 sequences, and an origin region from the yeast 2-μm circle (21). A related vector (pL) with a *LEU2* gene replacing the 5′ half of *URA3* was derived using a *LEU2* fragment from YEp13 (22).

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Wild-type and mutant c-*src* sequences were moved into pU and pL after insertion of the chloramphenicol acetyltransferase (CAT) gene adjacent to the 3′ end of c-*src* (17). This allowed excision of c-*src* and CAT on a common *Bam*HI/*Bgl* II restriction fragment that was inserted into *Bam*HI-cut vector DNA and recipients were selected on media containing chloramphenicol.

Yeast Strains. The *Saccharomyces cerevisiae* strain BJ2168 (*a, ura3-52, leu2, trp1, prb1-1122, pep4-3, prc1-407, gal2*; E. W. Jones, Carnegie Mellon University, Pittsburgh) was used throughout. Cultures were maintained on plates containing synthetic complete medium with 2% glucose or selected on similar plates lacking either uracil or leucine (24). Plasmids were introduced into the cells by the lithium acetate transformation procedure (25). Yeast containing two plasmids with different selectable markers were selected on plates lacking both uracil and leucine.

$p60^{c\text{-}src}$ Expression and Analysis. Yeast were grown in synthetic complete medium (24) lacking uracil and/or leucine. Cultures were induced in the same medium lacking glucose and containing 2% galactose for 22–24 hr. When labeling with $^{32}P_i$ was required, low-phosphate rich medium (17) containing galactose was used, with $^{32}P_i$ added for the final 2 hr. Procedures for lysis, immunoprecipitation with monoclonal antibody 327, phosphorylated amino acid analysis, and phosphopeptide analysis have been described (4, 17). Immunoblotting of immunoprecipitates obtained from 5×10^7 yeast cells was done as described (17). Protein kinase assays were carried out with immunoprecipitates containing $p60^{c\text{-}src}$ derived from 10^6 yeast cells, incubating with acid-treated enolase and 70–185 kBq of [γ-^{32}P]ATP (1.1×10^{17} Bq/mol) in a 10-μl volume for 5 min at 30°C (17). Reactions were linear.

RESULTS

The mechanism of $p60^{c\text{-}src}$ self-phosphorylation is best studied under conditions in which other protein-tyrosine kinases are absent. Therefore, we studied phosphorylation in $p60^{c\text{-}src}$ immunoprecipitates and in yeast in which avian $p60^{c\text{-}src}$ had been expressed artificially. In the former situation, $p60^{c\text{-}src}$ is phosphorylated predominantly at Tyr-416 (1, 2), whereas in yeast both Tyr-416 and Tyr-527 are phosphorylated at a low level (17, 26). We show here that a kinase-inactive mutant $p60^{c\text{-}src}$ is not phosphorylated in immunoprecipitates and has no phosphotyrosine in yeast cells, indicating that $p60^{c\text{-}src}$ is the catalyst in both situations. We then assayed for intermolecular phosphorylation by using $p60^{c\text{-}src}$ mutants that lacked phosphorylation sites to transfer phosphate to the inactive mutant.

Requirements for Phosphorylation of $p60^{c\text{-}src}$ *in Vitro*. We generated an inactive $p60^{c\text{-}src}$ ($p60^{R295}$) by mutating the codon for Lys-295 to specify arginine. Lys-295 interacts with the β–γ phosphate bond of ATP (27), and an Arg-295 mutation in $p60^{v\text{-}src}$ abolishes its kinase activity (28). We expressed $p60^{R295}$ in yeast cells from a galactose-inducible promoter with plasmid pU, which carries the *URA3* gene for selection in uracil auxotrophs, or plasmid pL, selectable by leucine prototrophy. Expression of $p60^{R295}$ and the wild-type ($p60^{WT}$) was detected by immunoblotting of immunoprecipitates (Fig. 1*A Upper*). Both proteins were expressed to similar levels. When immunoprecipitates of $p60^{R295}$ were incubated with [γ-^{32}P]ATP, no phosphorylation of either $p60^{R295}$ or an exogenous substrate protein, enolase, was detected (Fig. 1*A Lower*, lane 4). We conclude that the *in vitro* phosphorylation of $p60^{WT}$ and enolase (Fig. 1*A*, lane 2) is catalyzed by $p60^{WT}$ itself, and not by a contaminating kinase.

In fibroblasts, mutant $p60^{c\text{-}src}$ having phenylalanine at residue 527 ($p60^{F527}$) is more active than $p60^{WT}$, because the latter is inhibited by phosphate at Tyr-527 (4–10). In yeast, the majority of $p60^{WT}$ molecules lack phosphate at Tyr-527 (17, 26), so we expected that $p60^{F527}$ and $p60^{WT}$ would have similar specific activities. We also expected that a mutant lacking the major *in vitro* phosphorylation site ($p60^{F416}$) would be poorly phosphorylated *in vitro* but would phosphorylate substrates with at least 50% of wild-type activity (7, 9).

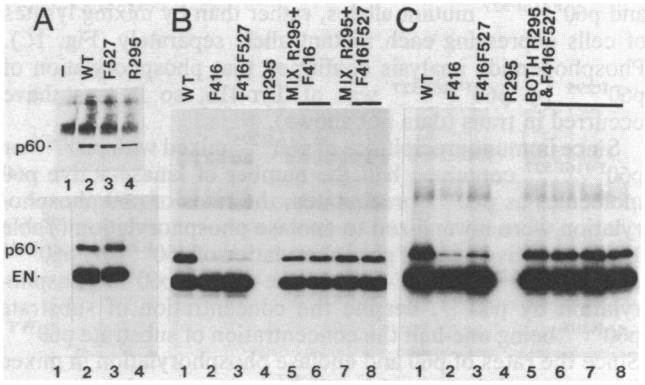

FIG. 1. Phosphorylation of $p60^{c\text{-}src}$ and enolase in immunoprecipitates. Yeast cells expressing various plasmids were induced and p60 was immunoprecipitated. p60 was detected in immunoprecipitates (from 5×10^7 cells) by immunoblotting after $NaDodSO_4$/PAGE (17). Kinase reactions were performed by incubating immunoprecipitates (from 10^6 cells) with [γ-^{32}P]ATP and enolase. Reaction products were separated by $NaDodSO_4$/PAGE and gels were autoradiographed. The positions of p60 and enolase (EN) are indicated. (*A Upper*) Immunoblot of immunoprecipitates of cells expressing plasmids pU (lane 1), pUWT (lane 2), pUF527 (lane 3), pUR295 (lane 4). The bands above p60 are immunoglobulin. (*A Lower*) Products of kinase assays performed with the same lysates. (*B*) Kinase assays performed with p60 immunoprecipitates from individual lysates (lanes 1–4) or mixed lysates (5×10^5 cells each, duplicate immunoprecipitations, lanes 5–8). Cells were expressing plasmids pUWT (lane 1), pUF416 (lane 2), pL:pUF416F527 (lane 3), pLR295:pU (lane 4), pUF416 mixed with pLR295:pU (lanes 5 and 6), pL:pUF416F527 mixed with pLR295:pU (lanes 7 and 8). (*C*) Kinase assays with p60 from cells expressing plasmids pUWT (lane 1), pUF416 (lane 2), pL:pUF416F527 (lane 3), pLR295:pU (lane 4), pLR295:pUF416F527 (lanes 5–8, four independent clones analyzed).

Mutant c-*src* alleles encoding $p60^{F527}$, $p60^{F416}$, and a double mutant, $p60^{F416F527}$, were constructed and expressed in yeast with the pU vector. Immunoblotting showed that all mutants were expressed to similar levels as $p60^{WT}$ (Fig. 1*A Upper*; data not shown). When approximately equal amounts of mutant and wild-type $p60^{c\text{-}src}$ were assayed with enolase as substrate in immunoprecipitates, similar phosphorylation rates were found, to within an estimated 0.7- to 1.5-fold experimental error (Fig. 1*A Lower*, lane 3; Fig. 1*B*, lanes 2 and 3). The rates of phosphorylation of $p60^{F527}$ and $p60^{WT}$ were similar (Fig. 1*A*, lane 3), but phosphorylation of $p60^{F416}$ and $p60^{F416F527}$ was reduced by 80–90% (Fig. 1*B*, lanes 2 and 3). This was expected, since $p60^{F416}$ and $p60^{F416F527}$ both lack the major *in vitro* phosphorylation site. Phosphorylation occurred instead at sites that were weakly phosphorylated in $p60^{WT}$ (data not shown).

Intermolecular Phosphorylation of $p60^{c\text{-}src}$ at Tyr-416 in an Immunoprecipitate. We tested whether intermolecular phosphorylation of $p60^{c\text{-}src}$ could occur in an immunoprecipitate with $p60^{R295}$ as a substrate for $p60^{F416F527}$ or $p60^{F416}$. Since the latter two mutants lack Tyr-416 as a target for possible intramolecular phosphorylation, any phosphorylation of Tyr-416 detected must be intermolecular.

Increased initial rates of p60 phosphorylation were observed in immunoprecipitates of mixtures of $p60^{R295}$ and $p60^{F416}$ or in mixtures of $p60^{R295}$ and $p60^{F416F527}$, relative to the labeling of any of the p60 mutants alone (Fig. 1*B*). This indicated that intermolecular phosphorylation of $p60^{R295}$ could occur. Very similar results were obtained if the experiment was done with a lysate of cell clones simultaneously expressing both $p60^{R295}$

and $p60^{F416F527}$ mutant alleles, rather than by mixing lysates of cells expressing each mutant allele separately (Fig. 1*C*). Phosphopeptide analysis confirmed that phosphorylation of $p60^{R295}$ by $p60^{F416F527}$ was at Tyr-416, so it must have occurred in trans (data not shown).

Since immunoprecipitates of $p60^{R295}$ mixed with $p60^{F416}$ or $p60^{F416F527}$ contained half the number of kinase-active p60 molecules as $p60^{WT}$ precipitates, the rates of p60 phosphorylation were normalized to enolase phosphorylation (Table 1). The relative rates of phosphorylation of $p60^{R295}$ by $p60^{F416}$ or $p60^{F416F527}$ were 57–94% of the rate of $p60^{WT}$ phosphorylation by $p60^{WT}$, despite the concentration of substrate $p60^{R295}$ being one-half the concentration of substrate $p60^{WT}$. Since the rates of p60 and enolase phosphorylation in mixed immunoprecipitates and in $p60^{WT}$ immunoprecipitates were directly proportional to p60 concentration, over a 30-fold concentration range (data not shown), we presume that the substrate p60 concentration exceeds the K_m for the intermolecular phosphorylation reaction. Significantly, the similarity of the rates of phosphorylation of $p60^{WT}$ and $p60^{R295}$ means that intermolecular phosphorylation can account for most of the phosphorylation in the $p60^{WT}$ immunoprecipitate, assuming that $p60^{WT}$ and $p60^{R295}$ are equally good substrates.

Phosphorylation of $p60^{c\text{-}src}$ Mutants in Yeast Cells. Tyr-527 phosphorylation is difficult to detect in immunoprecipitates but is readily observed by labeling yeast cells with $^{32}P_i$ (4, 17, 26). To determine the requirements for Tyr-527 phosphorylation, the phosphorylation states of $p60^{c\text{-}src}$ mutants in yeast cells were examined. Phosphorylated amino acid analysis of $p60^{WT}$ from $^{32}P_i$-labeled yeast showed phosphoserine and phosphotyrosine, but $p60^{R295}$ lacked phosphotyrosine (Fig. 2 *E* and *F Insets*). This showed that $p60^{c\text{-}src}$ is not phosphorylated by yeast protein-tyrosine kinases. The phosphorylation-site mutants contained reduced, but detectable, levels of phosphotyrosine (Fig. 2 *G* and *H Insets*). Sites of phosphorylation in p60 molecules from $^{32}P_i$-labeled yeast were identified by thin-layer electrophoresis and chromatography of peptides produced by digestion with trypsin or chymotrypsin. The former enzyme is useful for detecting phosphorylated Tyr-416, and the latter is useful for detecting Tyr-527 (3). $p60^{R295}$ was phosphorylated at the same serine residues as $p60^{WT}$ but lacked phosphate at Tyr-416 and -527 (Fig. 2 *A*, *B*, *E*, and *F*). The phenylalanine mutants were phosphorylated normally at serine and at the unmutated tyrosine sites (Fig. 2 *C*, *D*, *G*, and *H*).

Table 1. Relative phosphorylation of $p60^{c\text{-}src}$ in immunoprecipitates

p60 species present in immunoprecipitate	Ratio of p60 and enolase phosphorylation rates, % of ratio for $p60^{WT}$		
	Exp. 1	Exp. 2	Exp. 3
$p60^{WT}$	100	100	100
$p60^{R295}$	*	*	*
$p60^{F416}$	14	10	15
$p60^{F416F527}$	10	10	15
$p60^{R295}$,$p60^{F416}$†	57	82	ND
$p60^{R295}$,$p60^{F416F527}$†	69	94	ND
$p60^{R295}$,$p60^{F416}$	39	ND	ND
$p60^{R295}$,$p60^{F416F527}$‡	41	ND	57
$p60^{R295}$,$p60^{F416F527}$‡	46	ND	61
$p60^{R295}$,$p60^{F416F527}$‡	36	ND	62
$p60^{R295}$,$p60^{F416F527}$‡	56	ND	78

Immunoprecipitates were prepared from 10^6 induced yeast cells containing various plasmids and incubated with [γ-^{32}P]ATP and enolase. Radioactivity in p60 and enolase was quantified by scintillation counting of bands cut from a NaDodSO$_4$/polyacrylamide gel. Values were corrected for gel background (<1% of $p60^{WT}$ incorporation). Incorporation into p60 and enolase for $p60^{WT}$ immunoprecipitates was as follows: exp. 1, 6517/43,103 cpm (p60/enolase); exp. 2, 15,630/67,023 cpm; exp. 3, 18,047/103,331. Variation between experiments is probably due to various amounts of [γ-^{32}P]ATP. ND, not determined.

*No incorporation into either p60 or enolase.

†Two lysates (5×10^5 cells each) were mixed and immunoprecipitated in duplicate. The average ratio is presented.

‡Four independent cultures expressing both $p60^{R295}$ and $p60^{F416F527}$ were immunoprecipitated and assayed.

Unexpectedly, serine phosphorylation of $p60^{R295}$ seemed to be more extensive than for $p60^{WT}$ (see Fig. 2; similar quantities of protein were analyzed). Also, $p60^{R295}$ migrated as a single band on NaDodSO$_4$/PAGE, but $p60^{WT}$ was a doublet, with a second faster-migrating band (Fig. 1*A Upper*). These differences could reflect an altered conformation or subcellular localization of the mutant protein but seem unlikely to affect interpretation of the tyrosine phosphorylation results.

These data show that yeast cells contain protein-serine kinases, but not protein-tyrosine kinases, able to phosphorylate avian $p60^{c\text{-}src}$. Phosphorylation in yeast cells of $p60^{WT}$ at either Tyr-416 or -527 requires $p60^{c\text{-}src}$ kinase activity but does not require the other tyrosine residue.

Intermolecular Phosphorylation of $p60^{c\text{-}src}$ in Living Yeast Cells. To examine whether Tyr-527 was a substrate for intermolecular phosphorylation in yeast, we studied the phosphorylation of $p60^{R295}$ when expressed simultaneously with kinase-active p60 molecules. Yeast cell clones expressing both $p60^{R295}$ and $p60^{F527}$ were selected by making use of vectors with different genetic markers. Phosphopeptide maps of ^{32}P-labeled p60 molecules immunoprecipitated from these clones showed all the phosphopeptides detected in the maps of $p60^{F527}$ and $p60^{R295}$ expressed separately (Fig. 3 *B* and *C*), with the addition of the Tyr-527 phosphopeptide (Fig. 3*D*). Labeling at Tyr-527, relative to other sites, approached that in $p60^{WT}$ maps (Fig. 3*A*), suggesting that intramolecular phosphorylation does not contribute significantly to the phosphorylation state of $p60^{WT}$ in yeast cells.

Similar experiments were performed with cell clones expressing $p60^{R295}$ together with $p60^{F416}$ or $p60^{F416F527}$. As expected from *in vitro* results (Figs. 1 and 2), Tyr-416 was phosphorylated when $p60^{R295}$ and $p60^{F416}$ were coexpressed, and both Tyr-416 and -527 were phosphorylated when $p60^{R295}$ and $p60^{F416F527}$ were coexpressed (data not shown). Each of these peptide mapping experiments was repeated with four independent clones of coexpressing cells. These observations show that $p60^{R295}$ can be phosphorylated in yeast cells at Tyr-527 and/or Tyr-416 by kinase-active $p60^{c\text{-}src}$ mutant alleles.

The apparent trans phosphorylation between c-*src* mutant alleles could be explained trivially if recombination between plasmids had regenerated the wild-type allele. To explain our data, recombination would need to occur in each of the independent clones. However, no recombinant plasmids were detected when DNA was extracted from one yeast clone, recloned through bacteria, restriction mapped, and probed with oligonucleotides that distinguish the mutant alleles (data not shown).

DISCUSSION

Our experiments show that tyrosine phosphorylation of avian $p60^{c\text{-}src}$ in immunoprecipitates or yeast cells is catalyzed by $p60^{c\text{-}src}$ itself in a reaction that appears to be largely intermolecular. We have not directly addressed whether intramolecular phosphorylation is possible, but we suggest that it is quantitatively minor in either yeast or immunoprecipitates. Since the level of expression and location of $p60^{c\text{-}src}$ are similar in fibroblasts and yeast (ref. 17; data not shown), intermolecular phosphorylation may also occur in fibroblasts. Unfortunately, the ubiquity of $p60^{c\text{-}src}$ in fibroblasts

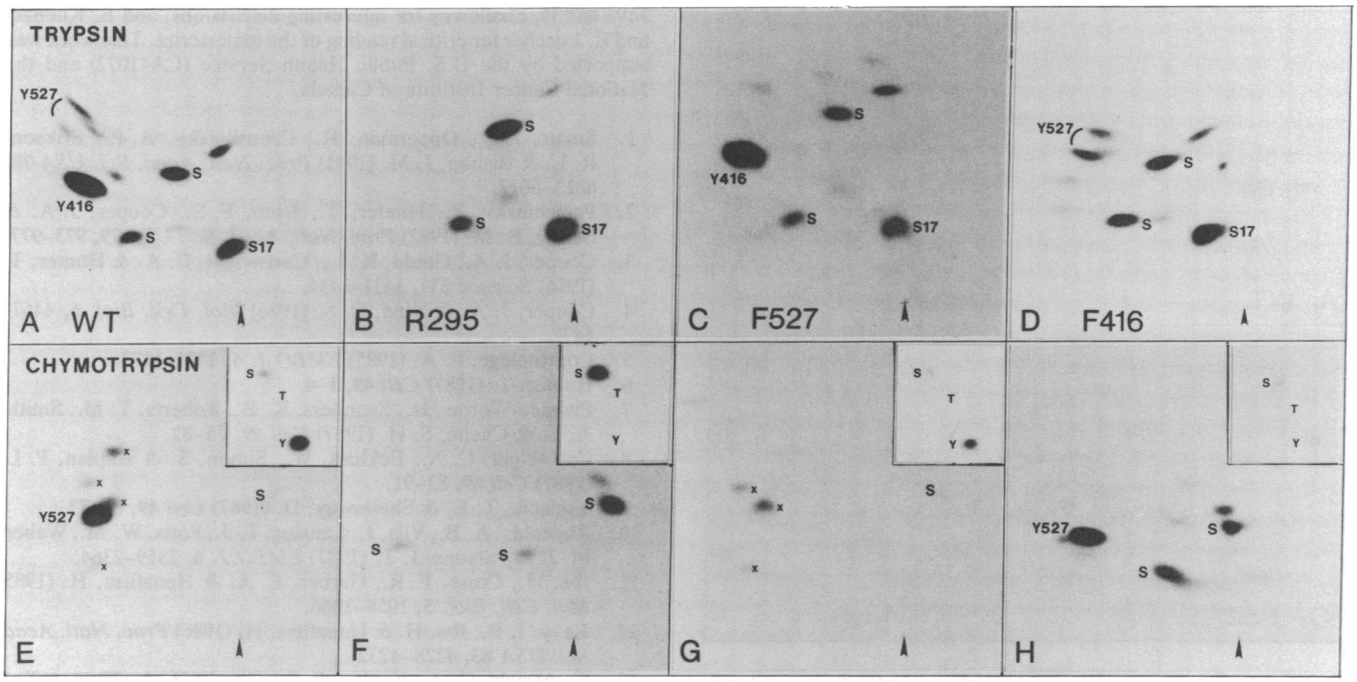

FIG. 2. Phosphorylated amino acids and phosphopeptides of wild-type and mutant $p60^{c\text{-}src}$ from $^{32}P_i$-labeled yeast cells. Yeast cells expressing various plasmids were induced in low-phosphate medium and labeled with $^{32}P_i$. $p60^{c\text{-}src}$ was immunoprecipitated from $\approx 10^8$ cells in each case, purified by $NaDodSO_4$/PAGE, and digested with trypsin (*A–D*) or chymotrypsin (*E–H*). Maps were loaded with samples derived from similar amounts of p60 protein. (*A* and *E*) $p60^{WT}$ from cells expressing pUWT. (*B* and *F*) $p60^{R295}$ from cells expressing pUR295. (*C* and *G*) $p60^{F527}$ from cells expressing pUF527. (*D* and *H*) $p60^{F416}$ from cells expressing pUF416. Phosphopeptides containing Tyr-416 (Y416), Tyr-527 (Y527), Ser-17 (S17), and other serines (S) are indicated. Chymotrypsin digestion in the vicinity of Tyr-416 is incomplete and releases several peptides, some of which are indicated (x). (*Insets*) Autoradiographs of $^{32}P_i$-labeled phosphorylated amino acids released from the $p60^{c\text{-}src}$ samples by partial acid hydrolysis. The positions of nonradioactive phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) markers are indicated.

precludes similar experiments with p60 mutants in their natural environment.

We had assumed that Tyr-416 phosphorylation was intramolecular, because of numerous observations that the level of Tyr-416 phosphorylation correlates with activity for $p60^{c\text{-}src}$ and its mutants (6–9, 11, 13). Also, autophosphorylation of purified, monomeric, $p60^{v\text{-}src}$ is independent of its concentration, suggesting an intramolecular reaction (16). However, since this purified $p60^{v\text{-}src}$ autophosphorylated at N-terminal tyrosines as well as Tyr-416, concentration dependence of Tyr-416 phosphorylation may have been obscured (16). It is also possible that conditions in yeast or immunoprecipitates promote intermolecular interactions that are rare with purified $p60^{v\text{-}src}$. Local concentrations of $p60^{c\text{-}src}$ could be high in immunoprecipitates, although it might be expected that the tethering of molecules to a solid support would limit diffusion. Weinmaster and Pawson (29) found that another protein-tyrosine kinase could undergo intermolecular phosphorylation at the Tyr-416 homolog in an immunoprecipitate. Perhaps immunoprecipitated molecules are sufficiently flexible for one kinase-active molecule to phosphorylate several kinase-inactive molecules, or perhaps the rate of dissociation of $p60^{c\text{-}src}$ from the antibody is sufficient for the reaction to occur in solution.

Intermolecular autophosphorylation appears to regulate many protein kinases. Among the protein-tyrosine kinases, the *fps* gene product and the insulin receptor are stimulated by phosphorylation at their Tyr-416 homologs. For the *fps* product, intermolecular phosphorylation stimulates *in vitro* kinase activity 4- to 6-fold (29). For the insulin receptor, "autophosphorylation" at several sites, including either of two adjacent tyrosines at the position of Tyr-416 in $p60^{c\text{-}src}$, renders the receptor independent of insulin for activity (30–32). Recent evidence suggests that insulin receptor autophosphorylation occurs in an intersubunit reaction within a single heterodimer of the receptor—i.e., one catalytic site phosphorylates its partner (33). The calcium and calmodulin-regulated multifunctional protein-serine kinase is an oligomer of 10–12 catalytic subunits that appears to be regulated by intersubunit autophosphorylation. Phosphorylation of a few subunits is sufficient to allow calcium-independent phosphorylation of the remaining subunits and of exogenous substrates (34). Different sites are autophosphorylated if calcium is withdrawn and these sites of phosphorylation appear to inhibit activity (35). In each of these samples, a parallel may be drawn with phosphorylation in $p60^{c\text{-}src}$, in that phosphorylation of one catalytic domain by another could be important for regulation.

Reversibility is a necessary part of any signal–response system, allowing for response to a second stimulus. Intermolecular phosphorylation may serve to reverse activation of $p60^{c\text{-}src}$ if it is activated in the cell by dephosphorylation of Tyr-527. If active, dephosphorylated $p60^{c\text{-}src}$ is the only kinase able to phosphorylate Tyr-527, then the rate of down-regulation would follow first- or second-order kinetics. Intramolecular autoinhibition would be a zero-order reaction. Similarly, if phosphorylation of Tyr-416 stimulates $p60^{c\text{-}src}$, then intermolecular phosphorylation of this residue may serve as a positive feedback system.

While our experiments were in progress, Jove *et al.* (36) reported that a kinase-negative mutant of $p60^{c\text{-}src}$ ($p60^{M295}$) was not phosphorylated at tyrosine when expressed in yeast but was extensively phosphorylated at Tyr-527 when expressed in fibroblasts. Similarly, we have found that Tyr-527 is phosphorylated in $p60^{R295}$ from fibroblasts (unpublished results). Our present observation that one $p60^{c\text{-}src}$ can phosphorylate another at Tyr-527 in yeast cells raises the possibility that $p60^{M295}$ and $p60^{R295}$ are phosphorylated at Tyr-527 in fibroblasts by the resident $p60^{c\text{-}src}$, and could help explain the ability of certain mutations in the kinase domain to reduce phosphorylation of Tyr-527 in fibroblasts (11, 12).

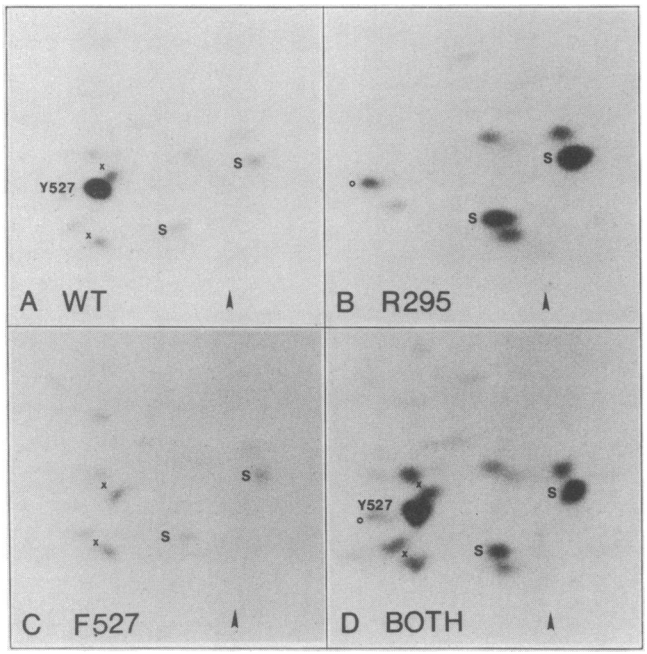

FIG. 3. Sites of *in vivo* phosphorylation of $p60^{c\text{-}src}$ in yeast cells expressing $p60^{R295}$ and $p60^{F527}$ separately or together. $p60^{c\text{-}src}$ was immunoprecipitated from $^{32}P_i$-labeled yeast cells expressing plasmids pUWT (*A*), pLR295:pU (*B*), pL:pUF527 (*C*), and pLR295:pUF527 (*D*) and digested with chymotrypsin, and phosphopeptides were analyzed. A minor phosphoserine-containing peptide that runs to the left of the Tyr-527 phosphopeptide is indicated with a small circle in *B* and *D*. Notice that the map of the mixture of $p60^{R295}$ and $p60^{F527}$ (*D*) contains all peptides phosphorylated when each protein is expressed separately (*B* and *C*), with the addition of the Tyr-527 phosphopeptide.

Why is Tyr-527 phosphorylated more extensively in fibroblasts than in yeast, even though the concentrations are similar? Phosphorylation could be faster, or dephosphorylation could be slower, in fibroblasts. Fibroblasts might provide any of the following components that may be missing from yeast: protein-tyrosine kinases (besides $p60^{c\text{-}src}$) that are able to phosphorylate Tyr-527; a factor that promotes intermolecular phosphorylation between $p60^{c\text{-}src}$ molecules; or a protein that inhibits dephosphorylation of Tyr-527. Candidate kinases for Tyr-527 include other members of the src family of protein-tyrosine kinases, which are also regulated by C-terminal phosphorylation (37, 38). Polyoma virus middle-sized tumor antigen is an example of a factor that is not itself a kinase but modulates the balance of phosphorylation between Tyr-416 and Tyr-527.

Since both Tyr-416 and Tyr-527 can be phosphorylated in trans, they must be accessible at the surface of $p60^{c\text{-}src}$ and not be buried. Sequence homologies with the catalytic subunit of the cAMP-dependent protein kinase imply that Tyr-416 may be involved in binding substrate (23). Therefore, phosphorylation at Tyr-416 may involve reciprocal binding between two Tyr-416 regions. Phosphorylation of Tyr-527 requires the C terminus of one $p60^{c\text{-}src}$ to be near the active site of another, perhaps in a symmetrical configuration. After phosphorylation, the C terminus of one molecule may act to inhibit the kinase domain of the other molecule (4). Thus, we can postulate two different arrangements of pairs of $p60^{c\text{-}src}$ molecules; one with Tyr-416 regions apposed, and one with the Tyr-527 of one molecule close to the active site of the other. Further characterization of interactions between $p60^{c\text{-}src}$ molecules is needed.

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