Autophosphorylation Is Required for High Kinase Activity and Efficient Transformation Ability of Proteins Encoded by Host Range Alleles of *v-src*

KATHLEEN M. WOODS¹ AND MICHAEL F. VERDERAME^{1,2*}

*Cell and Molecular Biology Graduate Program¹ and Department of Microbiology and Immunology,²
College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033*

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pp60^{v-src} is a nonreceptor protein tyrosine kinase that can transform both chicken and rodent fibroblasts. The *src* homology 2 (SH2) domain of this protein serves a critical role in the regulation of protein tyrosine kinase activity. The host range proteins pp60^{v-src-L}, which contains a deletion of a highly conserved residue (Phe-172) in the SH2 domain, and pp60^{v-src-PPP}, which contains a change from a Leu to a Phe at amino acid 186 in the SH2 domain, transform chicken but not rat cells and have slightly reduced kinase activity measured in vitro. The data presented here show that these altered proteins require autophosphorylation on Tyr-416 for high kinase activity and transforming ability. In the absence of autophosphorylation, there is a further decrease of at least threefold in in vitro kinase activity relative to the phosphorylated host range parental protein, no morphological transformation, a reduction in anchorage independent growth, and no disruption of the actin cytoskeleton. In addition, these SH2 mutations abolish the ability of the SH2 domain to bind a phosphorylated peptide that corresponds to the autophosphorylation site of pp60^{v-src}. Thus, like mutant alleles of *c-src* encoding transformation competent proteins, and unlike *v-src*, transformation by pp60^{v-src-F172Δ} and pp60^{v-src-L186F} is dependent on phosphorylation of Y-416 for high kinase activity and transformation ability. The dependence of transformation on phosphotyrosine is not a reflection of an intramolecular interaction between the autophosphorylation site and the SH2 domains since purified SH2 domains are incapable of binding phosphorylated autophosphorylation site peptides in vitro.

v-src, the transforming gene of Rous sarcoma virus, encodes a protein tyrosine kinase capable of transforming both chicken and rodent fibroblasts to a neoplastic phenotype. The catalytic domain in the carboxyl-terminal half of the protein is highly conserved among all members of the protein tyrosine kinase family (14). The SH2 domain, a second region of homology in most nonreceptor protein tyrosine kinases, plays a role in the regulation of kinase activity (4, 8, 12, 17, 28, 35, 41, 53). SH2 domains also are found in signal transduction proteins such as *ras*-GTPase-activating protein (50, 55), phospholipase C- γ (48, 49), and the regulatory subunit of phosphatidylinositol-3-kinase (11, 37, 45). The SH2 domains of these proteins interact with other proteins in the signal transduction cascade by binding tyrosine-phosphorylated proteins (32-34). Changes in pp60^{v-src} SH2 domains have a variety of effects including activation of pp60^{c-src} transformation potential (17, 35), abolition of pp60^{v-src} transformation ability (10, 54), and generation of host range alleles of *v-src* (10, 16, 28, 53, 54). The particular phenotype arising from any SH2 mutation is unpredictable. For example, changing the tryptophan at position 148 to a glutamic acid (W-148-E) in a transforming variant of pp60^{c-src} has relatively little effect on kinase and transforming activities, whereas W-148-Y has decreased kinase activity and transforming ability (17).

The host-range mutant *v-src-L*, which helped to draw attention to the SH2 domain, has a deletion of phenylalanine 172 (F-172) (53); F-172 is found at this position in all but one

SH2 domain (25, 56). (This allele will now be referred to as *v-src-F172Δ*.) In another host range allele with a mutation in the SH2 domain, *v-src-PPP*, leucine is changed to phenylalanine at position 186 (54). (This allele will now be referred to as *v-src-L186F*.) By several criteria, pp60^{v-src-F172Δ} and pp60^{v-src-L186F} transform chicken embryo fibroblasts (CEFs) but not established rat fibroblasts (51, 53, 54). Studies of whole-cell tyrosine phosphorylation patterns suggest a substantial decrease in pp60^{v-src-F172Δ} kinase activity in vivo in rat cells (53), and a somewhat smaller decrease for pp60^{v-src-L186F} in rat cells (54). Consistent with these observations, pp60^{v-src-F172Δ} and pp60^{v-src-L186F} from rat cells have a specific kinase activity measured in vitro that is low compared with that of the same proteins isolated from chicken cells (36, 54). These changes in kinase activity and transformation potential correlate with the state of phosphorylation of Y-416, the major site of pp60^{v-src} autophosphorylation; Y-416 phosphorylation is low in non-transformed rat cells but is high in transformed CEFs for both of these proteins (36, 51, 53).

Given the correlation between autophosphorylation and transforming ability in the two hosts expressing *v-src-F172Δ* or *v-src-L186F*, we predicted that the transforming ability of these proteins in CEFs would be lost if residue 416 could not be phosphorylated. To test this hypothesis, Y-416 was replaced with phenylalanine, and the mutant alleles were expressed in CEFs. The new alleles encoded proteins with decreased kinase activity in vitro and in vivo and decreased ability to transform CEFs. The requirement for autophosphorylation is not due to an interaction between the SH2 domain and the autophosphorylation site because these SH2 mutations abolish the ability of the purified SH2 domains from pp60^{v-src} to bind phosphorylated peptides derived from the autophosphorylation site region in vitro.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, College of Medicine, The Pennsylvania State University, Hershey, PA 17033. Phone: (717) 531-5467. Fax: (717) 531-6522. Electronic mail address: mverdera@cor-mail.biochem.hmc.psu.edu.

MATERIALS AND METHODS

Expression vectors. The B31 clone (31) of the Bratislava 77 allele of *v-src* and the host range mutant allele, *v-src*-F172Δ (53), were subcloned as *Xho*I-*Eco*RI fragments into M13mp19. Site-directed mutagenesis was performed as described previously (26) by using reagents from Bio-Rad Laboratories (Hercules, Calif.); a phenylalanine codon was substituted for a tyrosine codon at position 416 of both wild-type pp60^{v-src} and pp60^{v-src}-F172Δ (by using the mutagenic oligonucleotide, 5'-CCGTGCTGTGAACCTCGTTGTC-3'). Successful mutagenesis was confirmed by the dideoxy sequencing method of Sanger et al. (43), with Sequenase (U.S. Biochemical; Cleveland, Ohio). The B31 allele of *v-src* (wild type), *v-src*-F172Δ, *v-src*-L186F, and two derivatives containing site-directed mutations at Y-416 (*v-src*-Y416F and *v-src*-F172Δ-Y416F) were cloned into the Cla12Nco adapter plasmid (18) as *Nco*I-*Eco*RI fragments. To create an adapter plasmid containing a *v-src* allele with both L-186-F and Y-416-F mutations, the Cla12Nco vector containing *v-src*-L186F was digested with *Alw*NI and *Eco*RI and the one containing *v-src*-F172Δ/Y416F was digested with *Alw*NI and *Nco*I, and the resulting fragments were separated on agarose gels. The fragment encoding the SH2 domain of *v-src*-L186F was ligated to the fragment encoding the kinase domain of *v-src*-F172Δ/Y416F (which contains only the Y-416-F mutation). The resulting construct contained a *v-src* allele which had both the L-186-F mutation of *v-src*-L186F and the autophosphorylation site mutation at position 416 and was called *v-src*-L186F/Y416F. The *v-src* alleles were then subcloned as *Cla*I fragments into the replication-competent Rous sarcoma virus vector RCAS (18).

Cell culture. CEFs were prepared from 11-day-old *Gallus domesticus* chicken embryos (SPAFAS, Inc.) (19). The embryos were from a C/O flock (M180G Linell) that was negative for chicken helper factor and for *gs* antigen. CEFs were grown in medium 199 supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, 1% chicken serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Transfections. CEFs to be transfected were refed with Dulbecco's modified Eagle's medium containing 10% fetal calf serum 4 h before transfection. Twenty micrograms of pRCAS DNA or pRCAS carrying a *v-src* allele was transfected into CEFs by a calcium phosphate precipitation procedure essentially as described previously although there was no dextrose in the HEPES-buffered saline (42). Virus production was assayed by reverse transcription (13) and protein blot of viral proteins in the culture medium with anti-Rous sarcoma virus antibodies (antibodies generously provided by John Wills, College of Medicine, The Pennsylvania State University, Hershey). Cells were passaged three times to ensure extensive virus spread, and virus spread was monitored by morphological transformation for cells expressing transformation-competent *v-src* alleles.

Protein blots. Whole-cell detergent lysates were prepared by lysing cells in radioimmunoprecipitation assay buffer (2) containing 300 µg of phenylmethylsulfonyl fluoride per ml, 20 µg of aprotinin per ml, 100 µM Na₃VO₄, and 10 µg of leupeptin per ml. Protein concentrations were assayed according to the method of Lowry et al. (30), and the extracts were diluted to equal protein concentrations. Equal amounts of protein from each lysate were separated on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS); proteins were subsequently transferred to nitrocellulose. Antibodies were used to detect specific proteins in the protein blots as previously described (23). pp60^{v-src} was detected with monoclonal antibody 127 (29) (antibody 127 was a gift of Joan Brugge, Ariad

Pharmaceuticals, Cambridge, Mass.), which does not detect avian pp60^{c-src}. Phosphotyrosine-containing proteins were detected by polyclonal anti-phosphotyrosine antibodies (58) (a gift from Jean Wang, University of California—San Diego, San Diego). The relative amount of pp60^{v-src} in each lysate was determined by soaking the nitrocellulose in Immersionoel (Zeiss, Oberkochen, Germany), which renders the nitrocellulose transparent, and quantitating the bands with a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.). The linear range of this method was determined by blotting known amounts of lysate to nitrocellulose and determining the relative amount of pp60^{v-src} in each lysate by protein blot. The amounts of pp60^{v-src} in all experiments were in the linear range.

Kinase assays. CEFs were lysed in kinase lysis buffer (33 mM Sørensen's phosphate buffer [pH 7.2], 200 mM KCl, 1% Nonidet P-40) containing 300 µg of phenylmethylsulfonyl fluoride per ml, 20 µg of aprotinin per ml, and 10 µg of leupeptin per ml (38). Protein concentrations were assayed according to the method of Lowry et al. (30), and the extracts were diluted to equal protein concentrations. A portion of each lysate was analyzed by protein blot to measure pp60^{v-src} levels as described above. pp60^{v-src} was immunoprecipitated from the remainder of each lysate. Monoclonal antibody 127 (29) was used for both assays; this antibody precipitates each of the mutant proteins equally well (data not shown). pp60^{v-src} immunoprecipitates used in kinase assays were washed once in kinase lysis buffer and then twice in reaction buffer {100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 6.8], 20 mM MnCl₂}. In order to compare the kinase activity of each protein, the *K_m*s for acid-denatured enolase (7) of wild-type pp60^{v-src}, pp60^{v-src}-F172Δ, and pp60^{v-src}-F172Δ/Y416F were determined to be 0.75, 0.80, and 0.59 µM, respectively (data not shown). These values were not significantly different. Kinase assays were performed by using, per reaction, 10 µM acid-denatured enolase, 20 µM ATP, and 10 µCi of [γ -³²P]ATP (3,000 Ci/mmol). Reactions were allowed to proceed for 60 s at 25°C (the rate of incorporation of ³²P into enolase is linear up to 60 s) and were stopped by the addition of concentrated Laemmli sample buffer (27). Reaction products were separated on 10% polyacrylamide gels containing SDS. The amount of radioactivity in each lane was quantitated by using a Betascope 603 blot analyzer (Betagen). The specific kinase activity was calculated by quantitating the amount of radioactivity incorporated into a substrate and normalizing this to the amount of *v-src* protein in each lysate.

Anchorage-independent growth. Infected CEFs were assayed for their ability to form colonies in 0.36% agar. Cells (10⁴ per 35-mm-diameter plate) were plated in 0.36% agar with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, 1% chicken serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Colonies greater than 0.2 mm in size were counted.

Actin filament organization. Infected CEFs were plated on coverslips at a density of 3 × 10² cells per cm² and maintained for 24 h in standard growth medium. The cells were washed three times with phosphate-buffered saline (PBS), fixed for 10 min at room temperature with 3% paraformaldehyde, and permeabilized with 0.4% Triton X-100 for 10 min at room temperature. Actin filaments were stained with rhodamine-conjugated F1-phalloidin (1 µg/ml in PBS; F1-phalloidin was the generous gift of T. Wieland, Max Planck Institute, Heidelberg, Germany) for 1 h at 37°C, mounted in Aqua-Poly/mount (Polysciences, Inc.; Warrington, Pa.), and visualized with a Nikon Labphot-2. Actin organization was scored as described

previously (52). Briefly, cells that contained many thick cables were placed into category I. Cells with few thick cables were scored as category II. Cells placed in category III contained many thin actin filaments, and cells without detectable actin filaments were scored in category IV.

Phosphotyrosine 416 binding to SH2 domains. DNA fragments encoding the SH2 domains (amino acids 145 to 251) of wild-type *v-src*, *v-src*-F172Δ, and *v-src*-L186F were isolated from PCRs with primers 5'-CCCGAATTCGCTGAAGAGTGGTACTTTGGG and 5'-GACGGGGTGCAGGTTCCGGG GTCATCATCCTTAAGCC. Each primer contains the recognition sequence for *Eco*RI, and the latter primer also contains two stop codons. PCR products were cloned into the *Eco*RI site of pGEX-1 (Pharmacia Biotech, Inc., Piscataway, N.J.), and the constructs were transformed into DH5α cells.

Saturated cultures of DH5α expressing each of the fusion proteins were diluted 1:50 and grown for 1 h at 32°C. Production of the glutathione *S*-transferase (GST) fusion proteins was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 32°C. Cells were washed once in 25 mM Tris-HCl (pH 8.0)–150 mM NaCl–1 mM EDTA and then resuspended in 25 mM Tris-HCl (pH 8.0)–150 mM NaCl–1 mM EDTA containing 100 μg of lysozyme per ml, 300 μg of phenylmethylsulfonyl fluoride per ml, 20 μg of aprotinin per ml, and 10 μg of leupeptin per ml. The mixture was incubated for 15 min on ice, and then the cells were lysed in 3.1% Triton X-100 in a cold-bath sonicator for 5 min. Protein concentrations were determined by the method of Lowry et al. (30) and equalized with lysis buffer. Since the different fusion proteins were expressed to different levels (data not shown), the lysate from DH5α cells was used to dilute some of the samples to approximately the same fusion protein concentration while the total protein concentration was kept constant.

One-third of each lysate was mixed with glutathione-Sepharose 4B beads (Pharmacia Biotech, Inc.) for 30 min at room temperature. The other two-thirds of the lysate was precleared for 30 min at 4°C with Sepharose 4B beads (Pharmacia Biotech, Inc.) coupled to the following irrelevant peptide: M-Q-G-P-S-E-S-F-V-D-F-A-N-R-L-I-K (a gift of John Wills). One-half of the precleared lysates was mixed with Sepharose 4B beads coupled to phosphorylated pp60^{v-src} autophosphorylation site peptides (R-L-I-E-D-N-E-PTYR-T-A-R-Q-G-A-K), and the other half was mixed with Sepharose 4B beads coupled to the nonphosphorylated version of the same peptide for 1 h at 4°C (both autophosphorylation site peptides were a gift from Andrew Laudano, University of New Hampshire). All beads were washed four times with cold PBS and resuspended in Laemmli sample buffer (27). Proteins eluted from the beads were separated on 15% polyacrylamide gels containing SDS, blotted to nitrocellulose, probed with anti-GST antibodies (SC-138; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) followed by secondary antibody conjugated to horseradish peroxidase, and detected by chemiluminescence. Relative amounts of SH2 fusion proteins bound to the various peptides were determined with a laser densitometer (Molecular Dynamics).

RESULTS

To test the effects of autophosphorylation on the transforming ability of proteins encoded by host range alleles of *v-src*, RCAS retroviral vectors containing double mutant alleles *v-src*-F172Δ/Y416F and *v-src*-L186F/Y416F along with various control alleles were transfected into CEFs to establish infected cultures (Fig. 1). As expected, expression of wild-type *v-src* or *v-src*-Y416F transformed CEFs to a round, refractile morphol-

ogy, and expression of *v-src*-F172Δ or *v-src*-L186F induced a fusiform morphology. The morphology of cells expressing *v-src*-F172Δ/Y416F or *v-src*-L186F/Y416F was indistinguishable from that of mock-transfected CEFs or cells transfected with the vector alone. Because the cells expressing *v-src*-F172Δ/Y416F or *v-src*-L186F/Y416F had the same morphology as mock-transfected cells, the presence of pp60^{v-src} in these cultures was confirmed by a protein blot of whole-cell lysates from each of the CEF cultures that was probed with anti-pp60^{v-src} antibodies (Fig. 2B). All *v-src* alleles were expressed to similar levels. Thus, phosphorylation of Y-416 is required for morphological transformation by pp60^{v-src}-F172Δ and pp60^{v-src}-L186F.

It was reported previously by this laboratory (53, 54) that there are fewer detectable phosphotyrosine-containing proteins in extracts from rat cells expressing *v-src*-F172Δ or *v-src*-L186F than in cells expressing wild-type *v-src* and that the intensity of many individual bands is reduced compared with that of extracts from rat cells expressing wild-type *v-src*; Y-416 of pp60^{v-src}-F172Δ or pp60^{v-src}-L186F is not phosphorylated in rat cells. In chicken cells expressing these two alleles, the abundance of phosphotyrosine-containing proteins is indistinguishable from cells expressing wild-type *v-src*; Y-416 of pp60^{v-src}-F172Δ or pp60^{v-src}-L186F is phosphorylated in these cells. These results led to the prediction that elimination of autophosphorylation would reduce the kinase activity of pp60^{v-src}-F172Δ and of pp60^{v-src}-L186F in vivo in CEFs, reminiscent of the results from rat cells. Protein blots of lysates from infected CEF cultures were probed with anti-phosphotyrosine antibodies (Fig. 2A); all cells expressing a *v-src* allele had an increase in phosphotyrosine content compared with control cultures not expressing a *v-src* allele. As predicted, lysates from cells expressing *v-src*-F172Δ/Y416F or *v-src*-L186F/Y416F had a dramatically decreased number of detectable phosphotyrosine-containing proteins compared with extracts from cells that were expressing their parental alleles. These results indicate that abolishing phosphorylation of residue 416 in pp60^{v-src}-F172Δ and pp60^{v-src}-L186F does alter the kinase activity in vivo.

Although kinase activity in vitro does not always correlate with the pattern of phosphotyrosine-containing proteins detectable in whole-cell lysates (e.g., pp60^{v-src}-F172Δ has a threefold decrease in in vitro kinase activity in the absence of a significant decrease in the pattern of proteins containing phosphotyrosine [Fig. 1 and 2]), the previous experiment nonetheless raised the possibility of a dramatic decrease in the kinase activity of the double mutants (i.e., those with both an SH2 mutation and the Y-416-F mutation); accordingly, the specific in vitro kinase activity of each of the *v-src*-encoded proteins was determined by using acid-denatured enolase as the substrate (Fig. 3). The specific kinase activity of pp60^{v-src}-F172Δ/Y416F was reduced fivefold and the specific kinase activity of pp60^{v-src}-L186F/Y416F was reduced at least threefold compared with that of parental proteins with only the SH2 mutation. The kinase activities of both pp60^{v-src}-L186F/Y416F and pp60^{v-src}-F172Δ/Y416F were reduced at least 10-fold relative to that of the wild-type pp60^{v-src}. Thus, Y-416 phosphorylation is required for high kinase activity of pp60^{v-src}-F172Δ and pp60^{v-src}-L186F.

Since kinase activity is essential for transformation by pp60^{v-src} (5, 21, 44) and the decrease in kinase activity of pp60^{v-src}-F172Δ/Y416F and pp60^{v-src}-L186F/Y416F (Fig. 2 and 3) was associated with lack of morphological transformation (Fig. 1), two other properties frequently altered upon transformation were examined. First, CEFs expressing each of the six *v-src* alleles were tested for their ability to form colonies in soft agar.

FIG. 1. Morphology of CEFs expressing *v-src* alleles. Cultures were photographed after the cells were passaged several times to ensure complete infection. RCAS, vector alone; mock, mock-transfected cells.

Cells expressing *v-src*-F172Δ/Y416F or *v-src*-L186F/Y416F produced at least 10-fold fewer colonies than cells expressing the transforming *v-src* alleles (Fig. 4). Second, the extent of actin cytoskeletal organization was determined for each infected culture (Fig. 5). As expected, CEFs expressing morphologically transforming *v-src* alleles retained few of the large actin filaments typically seen in CEFs. In contrast, cells expressing *v-src*-F172Δ/Y416F or *v-src*-L186F/Y416F were unchanged compared with cells infected with vector alone. Therefore, phosphorylation of Y-416 of pp60^{*v-src*-F172Δ} and pp60^{*v-src*-L186F} is required for efficient anchorage-independent growth and cytoskeletal disruption.

Because the above data suggest that autophosphorylation is required for high kinase activity and transformation ability of the two SH2 domain mutants and because SH2 domains bind phosphotyrosine-containing proteins, we hypothesized that the SH2 domain interacts with Tyr-416 to control these two characteristics. To test this hypothesis, GST-SH2 fusion proteins were made by using the SH2 domains from wild-type pp60^{*v-src*}, pp60^{*v-src*-F172Δ}, and pp60^{*v-src*-L186F}. Purified fusion

proteins were tested for their ability to bind phosphorylated peptides corresponding to the autophosphorylation site (Table 1 and Fig. 6). The SH2 domain from wild-type pp60^{*v-src*} could bind the Y-416 peptide only when the peptide was phosphorylated. The SH2 domains from either of the host range mutant proteins, pp60^{*v-src*-F172Δ} and pp60^{*v-src*-L186F}, could not bind either peptide regardless of its phosphorylation state. These data suggest that SH2 binding of Y-416 is abolished by mutations that result in a host range phenotype and indicate that the simple hypothesis of SH2 binding Y-416 to control pp60^{*v-src*} activity is not correct.

DISCUSSION

The phosphorylation state of residue Y-416 has been the focus of several studies regarding the regulation of the kinase activity and transforming ability of various *v-src*- and *c-src*-encoded proteins (9, 15, 20, 24, 40, 46, 47). Changing or deleting this residue in otherwise wild-type pp60^{*v-src*} slightly reduces its kinase activity and has only minor effects on

FIG. 2. Protein blots probed with anti-pp60^{v-src} monoclonal antibody 127 or anti-phosphotyrosine antibodies. Equal amounts of total protein from lysates of CEFs were separated on a 10% polyacrylamide gel containing SDS, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies (A) or monoclonal antibody 127 (specific for pp60^{v-src}) (B). RCAS, vector alone; mock, mock-transfected cells.

morphological transformation but no effect on anchorage-independent growth (9, 24, 46, 47), a result reproduced in this study. In contrast to wild-type pp60^{v-src} with regard to the kinase activity and transforming ability, pp60^{c-src-Y527F} ("activated" c-src) requires autophosphorylation on Y-416 for increased kinase activity, morphological transformation, and anchorage-independent growth (20, 40).

Previous results with the host range proteins pp60^{v-src-F172Δ} and pp60^{v-src-L186F} correlated the state of Y-416 phosphorylation with both high kinase activity and transformation; autophosphorylation is low in nontransformed rat cells and high in transformed CEFs (36, 53, 54). In the current study, mutation of residue Y-416 revealed that these two host range pp60^{v-src} proteins, which contain changes in their SH2 domains, are dependent on the phosphorylation state of Y-416 for several biochemical and biological activities. Abolishing autophosphorylation significantly decreased *in vivo* and *in vitro* kinase activities, eliminated morphological transformation, eliminated actin cytoskeletal disruption, and decreased anchorage-independent growth in normally permissive chicken cells. Thus, the results presented here for the SH2 mutants parallel the results for activated pp60^{c-src} and stand in contrast to those for wild-type pp60^{v-src}. These data strengthen the hypothesis that phosphorylation of residue Y-416 is one of the components participating in the control of the kinase activity of src proteins. Since kinase activity is required for v-src-mediated transformation, understanding the role of autophosphorylation in the regulation of kinase activity is central to understanding

FIG. 3. *In vitro* kinase assay. Whole-cell lysates from infected CEFs were made as described in Materials and Methods. (A) Proteins from a fraction of each whole-cell lysate were separated on a 10% polyacrylamide gel containing SDS, transferred to nitrocellulose, and detected with monoclonal antibody 127. (B) pp60^{v-src} was immunoprecipitated from the remaining whole-cell lysate with monoclonal antibody 127, and kinase assays were performed. Reaction products were separated on a 10% polyacrylamide gel containing SDS and detected by autoradiography. The numbers below each lane represent the relative specific kinase activities (i.e., corrected for different levels of protein expression) normalized to wild-type activity. Relative kinase activities were obtained by averaging data from four experiments. The range of specific kinase activities (relative to that of the wild type, which was set to 100%) in each of those four experiments were 14 to 66 (pp60^{v-src-Y416F}), 8 to 79 (pp60^{v-src-F172Δ}), 3 to 16 (pp60^{v-src-F172Δ/Y416F}), 7 to 41 (pp60^{v-src-L186F}), and 1 to 18 (pp60^{v-src-L186F/Y416F}). The decrease in specific kinase activity for pp60^{v-src-F172Δ} (approximately threefold) in the absence of a decrease in the pattern of phosphotyrosine containing proteins is consistent with previous reports (53). Autophosphorylation of proteins containing the Y-416-F mutation is due to the phosphorylation of other tyrosine residues in pp60^{v-src} when kinase assays are performed at high ATP concentrations (6). RCAS, vector alone; mock, mock-transfected cells.

the host range regulation of both kinase activity and transformation induced by these alleles.

Why is Y-416 phosphorylation required in some transforming src alleles (c-src-Y527F, v-src-F172Δ, and v-src-L186F) but not others (v-src)? Currently available data provide few clues to this puzzle. As a result of the loss of Y-527 combined with the mutation T-338-I, the kinase activity of pp60^{v-src} is rendered independent of Y-416 phosphorylation (5a); T-338-I is one of three independent activating mutations in pp60^{v-src} (22). Perhaps phosphorylation of Y-416 stabilizes the active conformation of pp60^{c-src-Y527F}, and the mutation at position 338 in conjunction with the loss of Y-527 in pp60^{v-src} substitutes for this stabilization in the absence of Y-416 phosphorylation. In this scenario, mutations in SH2 counteract the stabilizing effect of the mutations at 338 and 527 by decreasing the overall stability of the folded protein. As a result, a protein with F-172Δ or L-186-F in the SH2 would regain its dependence on phosphorylation of Y-416 for conformational stability.

An alternative hypothesis to explain the dependence of these SH2 mutants on phosphorylation of Y-416 would be that direct interaction of the catalytic domain of pp60^{v-src} with a cellular protein (e.g., a critical substrate) is controlled in part by the phosphorylation state of Y-416. In this model, phosphorylated Y-416 is a required component of the interaction

FIG. 4. Anchorage-independent growth. Cells (10^4) were plated in 0.36% top agar and allowed to grow for 21 days. Colonies greater than 0.2 mm in size were counted. RCAS, vector alone; mock, mock-transfected cells.

with pp60^{c-src}-Y527F. The T-338-I mutation would create a novel domain of interaction between pp60^{v-src} and cellular proteins (or constitutively expose a previously inaccessible domain), thus rendering the interaction independent of Y-416 phosphorylation. Mutations in SH2 would abolish the second interaction site and again render pp60^{v-src} dependent on Y416 phosphorylation for this event. We are currently examining the secondary structure of wild-type and mutant SH2 domains to begin addressing these questions.

A model that combines aspects of both hypotheses could also explain why SH2 mutants exhibit a host range dependence for transformation. Interaction of host range pp60^{v-src} with the relevant chicken protein(s) might stabilize an active conformation despite the presence of the mutation in SH2; this would lead to high kinase activity and transformation. In rat cells, the proper interaction is unable to occur between the host range pp60^{v-src} and the relevant rat protein (possibly because of the evolutionary distance between the rat and chicken), and the active conformation is not stabilized, consistent with the observed decrease in kinase activity and lack of transformation.

Any hypothesis explaining the dependence of high kinase activity of host range pp60^{v-src} on Y-416 autophosphorylation must also address the role of the SH2 domain, since the host range mutants map to this region. In pp60^{c-src}, the SH2 domain is believed to bind the carboxyl-terminal tail via an interaction with phosphorylated Y-527, an interaction that negatively regulates pp60^{c-src} kinase activity. The loss of Y-527 in pp60^{v-src} precludes this negative regulatory role for SH2 in the transforming protein. What then is the role of SH2 in pp60^{v-src}-mediated transformation? Whereas many experiments have been directed at understanding the SH2 domain of *src*-encoded proteins, the available data do not provide a clear explanation. Biochemical experiments in which the SH2 domain was proteolytically removed from pp60^{v-src} resulted in increased kinase activity (3), a result suggesting a negative regulatory role for SH2 in pp60^{v-src} that is distinct from its role in pp60^{c-src}. In contrast, genetic experiments suggest that SH2 has a positive regulatory role. A point mutation in this region abolishes transforming activity by dramatically decreasing the kinase activity (*v-src*-F172V [54]). Deletion of the central 54 amino acids of this 100-amino-acid domain, however, results in a protein that still transforms (albeit only at reduced temperatures) (CH119 [4]). Some host range alleles encode proteins

FIG. 5. Actin cytoskeletal organization. Actin filaments were stained with rhodamine-conjugated F1-phalloidin. One hundred cells for each cell type were scored, and cells with more organized actin cytoskeletons were assigned to lower numbered categories (classes) (see Materials and Methods for details). RCAS, vector alone; mock, mock-transfected cells.

that cannot bind phosphorylated Y-416 (i.e., pp60^{v-src}-F172Δ and pp60^{v-src}-L186F) but are nonetheless transforming provided that phosphorylated Y-416 is present (this report). Although interactions between only SH2 and low-affinity peptides were tested, mutations predicted to abolish even high-affinity SH2-peptide interactions (R-175-A and R-175-K) (56, 57) do not affect the transforming ability of wild-type *v-src*, *v-src*-F172Δ,

TABLE 1. Binding of GST-SH2 fusion proteins to Y-416-containing peptides

SH2 domain ^a	Binding of Sepharose 4B beads coupled to ^b :		
	Irrelevant peptide	P-Y-416	NP-Y-416
Wild type	1.3 (0.3–3.4)	56 (30–75)	4.4 (0.6–16)
F-172Δ	1.0 (0.7–1.2)	1.3 (0.8–1.7)	0.6 (0.6–0.6)
L-186F	1.2 (0.4–1.2)	0.6 (0.4–0.9)	0.9 (0.2–2.2)

^a Recombinant GST-SH2 fusion protein synthesized in bacteria.

^b Values are the ratios of the amount of SH2 fusion protein bound to test peptide versus the amount of fusion protein bound to glutathione-Sepharose 4B beads. Numbers in parentheses are the range of values obtained in four (wild type), two (F-172Δ), or three (L186F) experiments. P-Y-416, phosphorylated autophosphorylation site peptide; NP-Y-416, nonphosphorylated autophosphorylation site peptide.

FIG. 6. GST-SH2 fusion protein binding to autophosphorylation site peptides. Bacterial cell lysates from cells expressing GST-SH2 fusion proteins were incubated with Sepharose 4B beads coupled to various peptides (see Materials and Methods). Bound proteins were eluted in Laemmli sample buffer, separated on a 15% polyacrylamide gel containing SDS, transferred to nitrocellulose, and probed with anti-GST antibodies. The amount of GST-SH2 fusion protein in each reaction did not vary more than threefold compared with each other in any given experiment. GLUT, Glutathione Sepharose 4B; control, proteins eluted from beads coupled to irrelevant peptide; P-Y-416, beads coupled to phosphorylated autophosphorylation site peptide; NP-Y-416, beads coupled to nonphosphorylated autophosphorylation site peptide; GST, vector only.

or v-src-L186F (52a). Thus, at least for pp60^{v-src}, binding to phosphotyrosine via the SH2 domain is not essential for transformation. Clearly, given that some SH2 mutations abolish transformation by pp60^{v-src} (9, 54), this domain must play some other critical role in the regulation of transformation by pp60^{v-src}. That role is still speculative, although there is some evidence for SH2 interactions that are phosphotyrosine independent (1, 39).

In summary, the data presented here indicate that Y-416 phosphorylation is required for high levels of kinase activity and efficient transforming ability of both pp60^{v-src-F172Δ} and pp60^{v-src-L186F}, proteins with changes in the SH2 domain. Continued biological and biochemical analyses of mutant SH2 domains will be required to completely understand the role of SH2 in v-src-mediated transformation.

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