Host Range Mutants of *v-src*: Alterations in Kinase Activity and Substrate Interactions

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Host range mutants of Schmidt-Ruppin *v-src* that transform chicken embryo fibroblasts (CEF) but not Rat-2 cells were generated previously by linker insertion-deletion mutagenesis (J. E. DeClue and G. S. Martin, J. Virol. 63:542–554, 1989). One of these mutants, SRX5, in which Tyr-416 is substituted by the sequence Ser Arg Asp, retained high levels of kinase activity in vitro and in vivo, both in CEF and in Rat-2 cells. Phosphorylation of p36 (the calpactin I heavy chain) was drastically reduced in cells expressing SRX5 *src*, suggesting that the phenotype of SRX5 results from an alteration in substrate recognition by the *src* kinase. Three mutants, SPX1, SHX13, and XD6, containing linker insertions or small deletions within the *src* homology 2 (SH2) region, induced reduced levels of kinase activity in both CEF and Rat-2 cells. However, the residual levels of kinase activity in Rat-2 cells were above the threshold at which wild-type pp60^{v-src} transforms Rat-2 cells, indicating that the reduction in kinase activity was not sufficient to account for the failure to transform. Cells infected by these mutants exhibited reduced levels of phosphorylation of 120- and 62-kDa proteins. We have reported elsewhere (M. F. Moran, C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson, Proc. Natl. Acad. Sci. USA 87:8622–8626, 1990) that *ras* GTPase-activating protein GAP and associated protein p62 are not tyrosine phosphorylated in Rat-2 cells expressing SHX13 or XD6. The transformation defect in Rat-2 cells may result from the failure to phosphorylate those proteins. The fifth mutant, XD4, contains a deletion which removes all of the *src* homology 3 (SH3) and most of the SH2 sequences of *src*. The protein encoded by XD4 is active as a kinase when expressed in CEF, indicating that in CEF the SH2 and SH3 regions of *v-src* are not necessary for kinase activity and transformation. The XD4 *src* product is not tyrosine phosphorylated and is inactive as a kinase when expressed in Rat-2 cells. Thus, host cell factors can affect the tyrosine phosphorylation and activity of the *v-src* kinase in the absence of the SH2 and SH3 regions. These results indicate that the host-dependent transformation phenotype results from alterations in *src* kinase activity and substrate specificity.

The signalling pathways regulated by viral and cellular tyrosine kinases are poorly understood. A variety of proteins which contain phosphotyrosine have been identified in transformed or growth factor-stimulated cells (reviewed by Cooper and Hunter [5]). Several proteins, some of which are known to be involved in intracellular signalling, serve as substrates for or are specifically associated with tyrosine kinases. These include *ras* GTPase-activating protein GAP, an 85-kDa subunit of phosphatidylinositol 3' kinase, p85, and phospholipase C- γ 1 (reviewed by Koch et al. [30]). In addition, it has been reported that the activated platelet-derived growth factor receptor is associated with the product of the *c-src* proto-oncogene, as well as with other members of the *src* family of tyrosine kinases (31). It has been suggested that these multisubunit complexes are signalling complexes in which signalling molecules are targeted to the plasma membrane (4, 30, 34).

It is still unclear, however, whether phosphorylation of these proteins is necessary for transformation or mitogenesis. An alternative approach to examining the interaction between transforming tyrosine kinases and the host cell involves isolation of host range mutants of viral transforming genes such as *v-fps* (9) or *v-src* (8, 17, 45, 47). By comparing

mutants of pp60^{v-src} expressed in both permissive and non-permissive cell types, it may be possible to determine which of the many changes induced by *v-src* expression are necessary for transformation. In addition, characterization of these mutants can provide information about the regions within *src* that are critical for the interaction of pp60^{v-src} with cellular components. The hypothesis underlying this approach is that mutations which result in a host-dependent phenotype may affect the interaction between the transforming protein and cellular components involved in transformation. We have previously reported the use of linker insertion and deletion mutagenesis to generate host range mutants of Schmidt-Ruppin *v-src* which transform chicken embryo fibroblasts (CEF) but not Rat-2 cells. Five host range mutants were identified (8). Consistent with the working hypothesis outlined above, four of the five host range mutants identified contained insertions or deletions in the SH2-SH3 region of *v-src*. These regions, which are conserved in nontransmembrane tyrosine kinases and a variety of signalling and cytoskeletal proteins (7, 10, 36), have been shown to mediate a variety of protein-protein interactions (4, 21, 30, 34). The fifth host range mutant isolated contains a substitution of Tyr-416, the major site of autophosphorylation in pp60^{v-src}, by the sequence Ser Arg Asp.

To understand the defects responsible for the host range phenotypes, we characterized the properties of CEF and Rat-2 cells expressing the mutant *src* genes. Our results suggest that four of the five mutants display host-dependent transformation because of alterations in the interaction of

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pp60^{v-src} with critical tyrosine phosphorylation substrates. One of the five mutants displays host-dependent kinase activity in vivo and in vitro, suggesting that the phenotype of this mutant results from an alteration in the interaction of pp60^{v-src} with cellular regulatory factors. The implications of these findings for our understanding of the interactions of pp60^{v-src} with regulatory factors and host cell targets are discussed.

MATERIALS AND METHODS

Cells, viruses, and expression vectors. CEF and Rat-2 cells (44) were cultured as previously described (8). Linker insertion and deletion mutagenesis of Schmidt-Ruppin *v-src* has been described in detail elsewhere (8). Cell lines MS1 and MS7 (22) were a gift from Harold Varmus (University of California, San Francisco).

To express a mutant pp60^{v-src} protein in CEF, a *Cla*I restriction fragment containing the mutated *v-src* gene, including the splice acceptor 5' to the *src* open reading frame, was cloned into expression vector RCAN(BH). This is a replication-competent, helper-independent retroviral vector (19) modified (by J. Brugge) by substitution of the Bryan high-titer virus (BH) *pol* gene (2, 43). CEF were infected with RCAN(BH)-based viral stocks. To collect virus stocks, CEF were transfected with RCAN(BH)-based plasmids by using dimethyl sulfoxide shock (26). Cells were passed every 3 days until the cultures became transformed. Virus-containing supernatants were then harvested every 2.5 to 3 h.

To express a mutant pp60^{v-src} protein in Rat-2 cells, the *Cla*I restriction fragment containing the mutated *v-src* gene was cloned into the Moloney murine leukemia virus-based mammalian expression vector fpGV-1 (23, 40). The vector contains a segment of bacterial transposon Tn5, driven from the simian virus 40 early-region promoter, which confers resistance to the drug G418 in mammalian cells. Virus stocks were generated by transfecting fpGV-1 based plasmids into Ψ -2 cells (33). Virus-containing supernatants were harvested from G418-resistant (G418^r) Ψ -2 cell pools.

Rat-2 cell lines were generated by transfection of fpGV-1-based plasmids, using dimethyl sulfoxide shock (26), or by infection with virus stocks generated from Ψ -2 cells. Cultures were grown in medium containing G418 (Geneticin; GIBCO; effective concentration, 0.25 mg/ml). G418^r colonies were picked with sterile cloning cylinders and expanded into cell lines. Lines were screened for pp60^{v-src} expression levels by immunoblotting (see below), and those that expressed levels comparable to those observed in lines expressing wild-type pp60^{v-src} were expanded for further study. The cell lines used in these studies were fpGV1-3, SRX5-c-10, SPX1-43, SHX13-222, XD4-224, XD6-64, and *v-src*-A-4.

Focus assays. Focus assays on CEF were carried out as previously described (20). Focus assays on Rat-2 cells were carried out by transfection with fpGV-1-based plasmids. For each transfection, one set of plates was incubated in medium containing G418 to determine transfection efficiency. A duplicate set was overlaid with medium containing 0.72% agar, 1% dimethyl sulfoxide, 5% fetal bovine serum, and 5% calf serum and incubated at 37°C. After 4 days, these plates were overlaid with identical agar mixtures. Foci and G418^r colonies were counted after 14 to 21 days of incubation.

Agar colony formation. A 0.5-ml volume of growth medium containing various dilutions (7.5×10^6 to 7.5×10^2) of infected CEF or of Rat-2 cells expressing different *v-src*

mutants was added to 1 ml of agar medium equilibrated at 44°C. The agar medium contained, for CEF, 0.66% agar, 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum or, for Rat-2 cells, 0.66% agar, 5% fetal bovine serum, and 5% calf serum. The cell suspension was immediately poured into a 60-mm-diameter dish containing a 5-ml basal layer of 0.66% agar containing the identical nutrients, and the top layer was allowed to set. Colonies were counted and photographed after 14 days of incubation at 37°C.

Antibodies. Antiphosphotyrosine antibody was generated as previously described (24) and was a gift from Gary Schieven (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Wash.). Anti-*src* monoclonal antibody (Mab) 2-17 was harvested as ascites fluid from the hybridoma supplied by Microbiological Associates, Bethesda, Md.

Immunoblotting. Infected CEF or Rat-2 cells that had grown to confluence in 60-mm plates were lysed by being scraped into 0.5 ml of boiling sodium dodecyl sulfate (SDS) sample buffer without 2-mercaptoethanol (10% glycerol, 2.3% SDS, 62.5 mM Tris, pH 6.8). The lysates were boiled for 5 min. An aliquot was removed and used for protein determination, using the bicinchoninic acid method (BCA reagent; Pierce). 2-Mercaptoethanol was added to 5%, and the sample was reboiled and then quick-frozen in a dry ice bath.

Immunoblotting with antiphosphotyrosine antibody (2 μ g/ml) or Mab 2-17 (1:2,000 dilution) was carried out as previously described (13), except that transfer was carried out with transfer buffer containing no methanol or SDS (14), and membrane blocking was carried out with 5% bovine serum albumin (ICN). A 125- μ g portion of sample protein was loaded in each gel lane for immunoblotting with antiphosphotyrosine antibody, and a 50- μ g portion was used for immunoblotting with Mab 2-17.

Relative levels of pp60^{v-src} proteins in cell lysates or in immunoprecipitates used for in vitro kinase assays were determined by immunoblotting and autoradiography using preflashed film (X-Omat). The radiolabel in the pp60^{v-src} band was quantified by scanning densitometry (Hoefer Scientific). The exposure of the autoradiogram was adjusted so that the relationship between the sample loading and pp60^{v-src} band area was linear.

Metabolic labeling and immunoprecipitation. CEF were labeled with ³²P_i as previously described (9). p36 was immunoprecipitated as previously described (47).

Kinase assays. Infected CEF or Rat-2 cells were lysed in 0.5 ml of RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.05% SDS, 1 mM sodium orthovanadate, 100 μ g of leupeptin per ml, 20 μ g of aprotinin per ml [pH 7.4]). Lysates were clarified at 14,000 $\times$ g. An aliquot was removed for determination of protein content, and 0.66 μ l of ascites fluid containing Mab 2-17 was added to the remainder. After 2 h at 4°C, 20 μ l of a 10% suspension of fixed *Staphylococcus aureus* (IgG-sorb; The Enzyme Center) was added. After 45 min at 4°C, the adsorbed immune complexes were washed in detergent wash buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.5% Nonidet P-40, 1% sodium deoxycholate, 10 μ g of leupeptin per ml [pH 7.4]) and then suspended in kinase wash buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 5 mM MgCl₂, 5 mM MnCl₂ [pH 7.4]). The suspension was then divided into two portions. One portion was pelleted and then boiled in 60 μ l of SDS sample buffer; the immunoprecipitated proteins were then resolved by SDS-polyacrylamide gel electrophoresis

FIG. 1. Positions of linker insertion-deletion mutations in the *v-src* open reading frame. For linker insertions (SPX1, SHX13, and SRX5), numbers refer to the pp60^{v-src} residue preceding the insertion (with M as residue 1). Linker sequences resulted in insertion of residues LE (SPX1), RA (SHX13), or SRD (SRX5). The mutation in SRX5 removes the codon for Tyr-416. For deletions (XD4 and XD6), the numbers indicate the first and last pp60^{v-src} residues deleted. Linker sequences resulted in insertion of residues DLE (XD4) or SRG (XD6).

and subjected to immunoblotting with MAb 2-17 to quantify the amount of pp60^{v-src} in the immunoprecipitate. The second portion was pelleted and suspended in 30 μ l of kinase assay buffer (20 mM HEPES, 5 mM MgCl₂, 5 mM MnCl₂, 0.33 mg of acid-denatured enolase per ml [pH 7.4]) containing 1 μ M [γ -³²P]ATP (specific activity, 1,000 Ci/mmol). After 5 min at 30°C, assays were terminated by addition of 6 μ l of 6 $\times$ -concentrated sample buffer and EDTA (to prevent non-enzymatic tyrosine phosphorylation [41]) to 5 mM; the samples were then boiled. Radiolabeled proteins were detected by SDS-polyacrylamide gel electrophoresis and autoradiography. The bands corresponding to labeled enolase were excised, digested overnight at 70°C in 0.75 ml of 30% H₂O₂, and the radioactivity incorporated was determined by liquid scintillation counting. Incorporation of radiolabel into enolase was linear for 5 min or more. Total kinase activity was calculated as radiolabel incorporated into enolase divided by the total micrograms of protein in the lysate from which *src* was immunoprecipitated. To calculate the specific activity of pp60^{v-src} kinase, radiolabel incorporated into enolase was divided by the amount of pp60^{v-src} immunoprecipitated (determined by scanning densitometry as described above). The values obtained for both total and specific kinase activities were normalized to the values obtained for wild-type pp60^{v-src} from the same cell type.

RESULTS

Phenotype of CEF and Rat-2 cells expressing mutant *v-src* genes. Five linker insertion or deletion mutants (Fig. 1) were previously identified as host range mutants because CEF expressing the mutant pp60^{v-src} genes were morphologically transformed whereas Rat-2 cells expressing these mutant genes were morphologically normal or nearly normal. To confirm and quantify this phenotype, CEF or Rat-2 cells expressing these mutant pp60^{v-src} genes were tested for the ability to form foci under agar. Focus assays were performed on supernatants harvested from CEF after transfection with RCAN(BH)-based virus vectors carrying the mutant pp60^{v-src} genes. As shown in Table 1, these virus stocks had focus-forming titers equal to those of wild-type pp60^{v-src} stocks. Thus, all of the mutants were fully transforming in CEF. However, it should be noted that the transformed cells

TABLE 1. Quantitation of the host range phenotype

Mutant	Virus titer on CEF (FFU ^a /ml)	Rat-2-cells		
		G418 ^r colonies ^b	Foci ^c	Foci/G418 ^r colony
Vector	<1.0 $\times 10^1$	824	0	0.00
SPX1	1.5 $\times 10^7$	471	0	0.00
SHX13	1.0 $\times 10^7$	582	48	0.08
SRX5	1.9 $\times 10^7$	274	5	0.02
XD4	2.3 $\times 10^7$	313	0	0.00
XD6	6.6 $\times 10^7$	543	0	0.00
<i>v-src</i>	1.4 $\times 10^7$	616	466	0.76

^a FFU, focus-forming units.

^b Total number of G418^r colonies on nine 100-mm dishes.

^c Total number of foci on nine 100-mm dishes.

resulting from infection by mutants with lesions in SH2 sequences were fusiform (8).

To quantify the transformation of Rat-2 cells, the cells were transfected with the fpGV-1 expression vector carrying the mutant pp60^{v-src} genes. Pools of transfected cells were split into two; one half was overlaid with agar to quantify focus formation, while the other was grown in liquid medium supplemented with G418 to determine transfection efficiency. The ratio of foci to G418-resistant colonies was taken as a measure of transformation efficiency. The five mutants were partially (SHX13 and SRX5) or wholly (SPX1, XD4, and XD6) transformation defective in Rat-2 cells (Table 1). These observations confirm that these mutants were able to transform CEF but not Rat-2 cells.

Isolation of Rat-2 clones expressing mutant *v-src* genes. Not all G418^r colonies derived after transfection and selection expressed pp60^{v-src} protein. It was therefore necessary to derive clonal cell lines of Rat-2 cells expressing the host range mutants for further study. Rat-2 cells were transfected with fpGV-1-based plasmids or infected with virus stocks produced by transfection of Ψ -2 cells and grown in the presence of G418. G418^r colonies were picked and expanded into cell lines. The level of mutant pp60^{v-src} expression was examined by immunoblotting with anti-pp60^{v-src} antibody (Fig. 2). Cell lines expressing levels of pp60^{v-src} protein comparable to that seen in mass-infected CEF were chosen for further study. The morphologies of these cell lines were flat or slightly refractile. In all cases, these cells were

FIG. 2. Level of pp60^{v-src} expression. Whole-cell lysates from infected CEF or Rat-2 cell lines were subjected to anti-pp60 immunoblotting. In all cases, the level of mutant pp60 expression is comparable to that in cells expressing wild-type *v-src*.

TABLE 2. Soft agar colony formation by cells expressing host range mutant *v-src* proteins

Mutant	No. of soft agar colonies of:	
	CEF ^a	Rat-2 cells ^b
Vector	—	<1.3 × 10 ⁻⁶
SPX1	1.0 × 10 ⁻²	1.4 × 10 ⁻¹
SHX13	6.9 × 10 ⁻³	1.2 × 10 ⁻¹
SRX5	3.6 × 10 ⁻³	9.5 × 10 ⁻²
XD4	2.4 × 10 ⁻³	1.3 × 10 ⁻⁵
XD6	4.4 × 10 ⁻³	1.4 × 10 ⁻¹
<i>v-src</i>	3.7 × 10 ⁻³	1.2 × 10 ⁻¹

^a Number of colonies in soft agar per focus-forming unit. —, not applicable.^b Number of colonies in soft agar per cell plated.

phenotypically distinct from *v-src*-transformed Rat-2 cells (Fig. 3).

Agar colony formation. The ability of CEF and Rat-2 cells expressing the mutant *src* proteins to form colonies in soft agar was examined. CEF infected with the host range mutants were as efficient in forming soft agar colonies as CEF infected with wild-type pp60^{v-src} (Table 2). In addition, the morphologies of the colonies induced by the host range mutants were indistinguishable from those resulting from wild-type pp60^{v-src} expression (data not shown).

Although Rat-2 cell lines expressing mutant pp60^{v-src} proteins did not form foci under agar (see above and Table 1), four of the five cell lines expressing the mutant pp60^{v-src} proteins (SHX13-222, SPX1-43, SRX5-c-10, and XD6-64) formed an appreciable number of soft agar colonies (Table 2). Rat-2 cells expressing XD4 (XD4-224) formed only few colonies in soft agar (Table 2). However, the soft agar colonies formed by the Rat-2 cell lines expressing mutant pp60^{v-src} proteins were considerably smaller than those formed by Rat-2 cells transformed by wild-type pp60^{v-src} (Fig. 4) although cells expressing SHX13 yielded both small and large colonies. It is likely that the small colonies formed by these Rat-2 cell lines resulted from residual function of the mutant pp60^{v-src} proteins, i.e., to "leakiness" of the defect in Rat-2 cells.

Protein tyrosine phosphorylation in vivo. The pattern of protein tyrosine phosphorylation was examined in cells expressing the mutant pp60^{v-src} kinases. Lysates of infected CEF or Rat-2 cell lines were analyzed by immunoblotting with antiphosphotyrosine antibody (Fig. 5). The overall levels of phosphotyrosine detected by this analysis were lower in Rat-2 cells than in CEF. The mutants were classified into three groups based on comparison of the patterns observed in cells expressing the mutant pp60^{v-src} kinases with those observed in the corresponding host cells expressing wild-type pp60^{v-src}.

The first group consisted of SPX1, SHX13, and XD6. Lysates from cells expressing these mutants showed reduced levels of tyrosine phosphorylation in two molecular mass regions, one centered around 120 kDa and another at 62 to 64 kDa (Fig. 5). These alterations were evident in both CEF and Rat-2 cells expressing the mutant *v-src* proteins. In addition, in XD6-expressing CEF and Rat-2 cells, tyrosine phosphorylation of proteins in most other regions of the gel was also considerably reduced. All of the mutants in this group are linker insertions or deletions in the SH2 region (Fig. 1).

The second type consisted of the SRX5 mutant. The linker insertion in SRX5 removes the codon for Tyr-416 (Fig. 1). In

cells expressing SRX5 *src*, the tyrosine phosphorylation of the band at 60 kDa, which corresponds to pp60^{v-src}, is accordingly reduced. The pattern of protein tyrosine phosphorylation resulting from SRX5 expression was, in general, similar to that induced by wild-type pp60^{v-src}. However, the tyrosine phosphorylation of a 36-kDa protein was reduced in both CEF and Rat-2 cells expressing SRX5; the difference between SRX5 and wild-type pp60^{v-src} in Rat-2 cells was more dramatic than the difference observed in CEF. The results of partial proteolysis by *S. aureus* protease V-8 indicated that the 36-kDa tyrosine-phosphorylated protein was related or identical to p36 (the calpactin I heavy chain), a prominent substrate for phosphorylation by pp60^{v-src} (38) (data not shown). Phosphorylation of p36 was therefore examined by immunoprecipitation of p36 from cells metabolically labeled with ³²P_i. The results (Fig. 6) confirmed that p36 phosphorylation was drastically reduced in cells expressing SRX5.

The third type consisted of XD4. When expressed in CEF, this mutant pp60^{v-src} had protein tyrosine kinase activity. The pattern of protein tyrosine phosphorylation in CEF expressing XD4 was similar to that observed in CEF infected by mutants of group 1 (SH2 mutants), with reduced levels of tyrosine phosphorylation of proteins in two regions of the gel, centered at 120 and 62 to 64 kDa. However, when expressed in Rat-2 cells the XD4 mutant pp60^{v-src} did not induce detectable levels of protein tyrosine phosphorylation, even when expressed at levels comparable to or higher than the levels of pp60^{v-src} observed in wild-type *src*-transformed Rat-2 cells. Thus, not only did the XD4 mutation result in host-dependent transformation, the XD4 protein itself displayed host-dependent kinase activity as assayed by protein tyrosine phosphorylation in vivo.

Immune complex kinase activities of mutant *src*-encoded proteins. Immune complex kinase assays were performed to quantify the kinase activities of the pp60^{v-src} mutants expressed in either CEF or Rat-2 cells. Mutant pp60^{v-src} proteins were immunoprecipitated from infected CEF and Rat-2 cell lines by using MA b 2-17, and their kinase activity was measured by using acid-denatured enolase as the substrate. As shown in Fig. 7, SRX5, SPX1, SHX13, and XD6 yielded comparable total kinase activities, whether isolated from CEF or Rat-2 cells. However, all displayed reduced total kinase activities compared with that of wild-type pp60^{v-src} expressed in the same cell type. For both CEF and Rat-2 cells, thresholds of wild-type Schmidt-Ruppin pp60^{v-src} activity required for transformation have been estimated. Jakobovits et al. (22), using the mouse mammary tumor virus long terminal repeat to drive *v-src* expression in Rat-2 cells, estimated that a level of 5 to 9% of the level at which wild-type Schmidt-Ruppin pp60^{v-src} kinase activity was expressed was sufficient to transform Rat-2 cells. DeClue and Martin (8), using the RCAN vector to express a variety of different *v-src* mutants in CEF, estimated that a level of 3 to 4% of the level at which wild-type Schmidt-Ruppin pp60^{v-src} kinase activity was expressed was sufficient to transform CEF. To compare the level of pp60^{v-src} activity in Rat-2 lines expressing SRX5, SPX1, SHX13, and XD6 with the threshold level for transformation, we examined pp60^{v-src} expression and kinase activity in two cell lines, MS1 and MS7, which express low levels of Schmidt-Ruppin *v-src* (22). Under the conditions of the assay, MS1, which is morphologically transformed, expressed 14.4% of the *src* kinase activity found in the cell line *v-src*-A-4, which is transformed by wild-type *v-src*. MS7, which is morphologically normal, expressed 4.0% of the *src* kinase activity found

FIG. 3. Morphology of Rat-2 cell lines expressing mutant *src* proteins. Cells (7.5×10^5) were plated into 60-mm dishes and photographed 18 h later. Rat-2 cell lines expressing mutant pp60^{v-src} proteins were flat or slightly refractile, in contrast to the morphology of a Rat-2 cell line expressing wild-type pp60^{v-src}.

FIG. 4. Morphology of colonies formed in soft agar by Rat-2 lines expressing mutant *src* proteins. Cells expressing the vector alone (fpGV1-3) and the XD4 cell line XD4-224 formed pinpoint, microscopic colonies. Rat-2 cell lines SRX5-c-10, SPX1-43, SHX13-222, XD6-64, and *v-src*-A-4 were able to form colonies in soft agar. Cells expressing SRX5, SPX1, and XD6 formed small but macroscopically detectable colonies. Cells expressing SHX13 formed both compact and large colonies. The colonies induced by the mutant *v-src* proteins were all clearly distinguishable from the colonies induced by wild-type *pp60^{v-src}*.

FIG. 5. Protein tyrosine phosphorylation in CEF and Rat-2 cells. Lysates of either infected CEF or Rat-2 lines were subjected to antiphosphotyrosine immunoblotting. Arrowheads indicate regions deficient in tyrosine phosphorylation as described in the text. Molecular sizes are given on the left in kilodaltons.

in the *v-src*-A-4 line. The Rat-2 cell lines expressing SRX5, SPX1, SHX13, and XD6 *src* proteins all yielded kinase activities equal to or greater than the level expressed in transformed line MS1. These observations suggest that the reduction in kinase activities alone cannot explain the failure to transform Rat-2 cells.

XD4 displayed host-dependent kinase activity in vitro: XD4 pp60^{v-src} isolated from Rat-2 cells had little detectable kinase activity, while XD4 pp60^{v-src} isolated from CEF had kinase activity equivalent to that of wild-type pp60^{v-src}. These findings are in agreement with the observations on protein tyrosine phosphorylation in cells expressing XD4. XD4 mutant pp60^{v-src} displayed host-dependent protein tyrosine phosphorylation in vivo, host-dependent kinase activity in vitro, and host-dependent transformation.

FIG. 6. Calpactin 1 (p36) phosphorylation in cells expressing wild-type (wt) or SRX5 *src*. Infected CEF were labeled with ³²P_i, and p36 was immunoprecipitated as described in Materials and Methods.

FIG. 7. Total (a) and specific (b) kinase activities of *src* proteins determined as described in Materials and Methods in immune complex kinase assays. Activities are expressed relative to that of wild-type pp60^{v-src} isolated from the same cell type. Values are averages of four determinations.

DISCUSSION

Mutations in *v-src* that engender the host range phenotype can occur at a number of sites. Young et al. (47) have characterized *tsLA33-1*, a host-dependent, temperature-sensitive mutant. The defect responsible for this phenotype has been mapped to two amino acid changes in the kinase region (15). A single amino acid deletion in the conserved FLVR SH2 sequence is responsible for the host range phenotype of the *v-src*-L mutant (45). Hirai and Varmus (17) have used oligonucleotide-directed mutagenesis, as well as deletion mutagenesis of SH2 and SH3 sequences, to generate host range mutants of Y527F-activated pp60^{c-src}. Likewise, a host range mutant of *v-fps*, AX9m, was generated by linker insertion into SH2 (9). Of the five host range mutants described here, four (SPX1, SHX13, XD4, and XD6) resulted from alterations in SH2 sequences (and also, in the case of XD4, SH3 sequences). This is consistent with the notion that this region has a regulatory or modulatory role (see below). The fifth mutant (SRX5) resulted from a linker insertion in the kinase domain. Thus, the results described here, in conjunction with the reports cited above, indicate

that the host range phenotype can result both from mutations in the SH2 and SH3 regions and from mutations in the kinase domain.

The mutants described here were fully transforming in CEF, except that cells expressing the SH2 mutants had a fusiform rather than a round morphology, but all were partially or completely transformation defective in Rat-2 cells. The phenomenon of host-dependent transformation might be explained by hypothesizing that different levels of pp60^{v-src} activity are required for transformation of different cell types. In the case of mutant XD4, the biochemical properties of the mutant protein are clearly different in CEF and Rat-2 cells: the kinase activity of XD4 *src* is well above the transformation threshold in CEF and well below the transformation threshold in Rat-2 cells. In the case of mutant SRX5, the kinase activity of the mutant protein, measured on enolase as the substrate, is only slightly reduced relative to the level exhibited by the wild-type protein and is well in excess of the transformation threshold both in CEF and in Rat-2 cells. Thus, a difference in transformation threshold cannot explain the phenotype of this mutant. In the case of mutants SPX1, SHX13, and XD6, which contain linker insertions or small deletions within the SH2 domain, the kinase activities of the mutant proteins, with enolase as the substrate, were equal to that of a clone (MS1) transformed by low levels of wild-type Schmidt-Ruppin *v-src*. Although there may be some clonal variation in the transformation threshold, this observation suggests that a difference in transformation threshold by itself cannot explain the host-dependent phenotype of these mutants. However, it is possible that if the assays had been carried out on a physiologically relevant substrate the level of kinase activity exhibited by the SPX1, SHX13, and XD6 proteins might have been higher than the transformation threshold in CEF but lower than the transformation threshold in Rat-2 cells. Thus, differences in the transformation threshold may contribute to the host-dependent phenotype, but other factors, such as alterations in substrate specificity, must also be involved.

The phenotype of the XD4 mutant raises two questions about the role of the SH2 and SH3 regions in kinase function and transformation. The mutation in XD4 deletes all of SH3 and most of SH2, yet XD4 is fully transforming in CEF. Mutants of pp60^{v-src} with large deletions in SH2 that retain transforming ability in CEF have been described by others, e.g., deletion of residues 173 to 227 (*tsCH119* [3]) and deletion of 168 to 226 (*tsCH120* [39]). Hirai and Varmus (18) have similarly described a number of such mutations in Y527F-activated *c-src*: deletion of residues 187 to 197 (D8), deletion of 198 to 205 (D9), deletion of 206 to 226 (D10), and deletion of 176 to 226 (D13). It seems likely that SH2 sequences play an essential role in the function of pp60^{c-src} (4, 30). In the case of pp60^{v-src}, it appears that the requirement for SH2 and/or SH3 sequences is abrogated as a result of the high level of *src* kinase activity: it is possible that other proteins containing SH2 or SH3 sequences can complex with proteins phosphorylated by pp60^{v-src} and thus complement the *src* mutations in *trans*.

The pp60^{v-src} encoded by XD4 displayed host-dependent kinase activity. Hirai and Varmus (17) have described host range mutants of Y527F-activated pp60^{v-src} which have reduced kinase activities when isolated from NIH 3T3 cells compared with those isolated from CEF. A host range mutant of Fujinami sarcoma virus, AX9m, encodes a tyrosine kinase that displays reduced activity when isolated from Rat-2 cells (9). The XD4 mutant, however, is almost

totally inactive when isolated from Rat-2 cells yet is fully active when isolated from CEF. The XD4 *src* protein is tyrosine phosphorylated in CEF but not in Rat-2 cells (Fig. 5 and reference 32). The activity of pp60^{v-src} is not dependent on Tyr-416 phosphorylation (42), whereas that of Y527F-activated pp60^{c-src} is dependent on Tyr-416 phosphorylation and the Y416F mutation decreases the transforming and kinase activities of Y527F-activated pp60^{c-src} (27, 37). It is possible that the XD4 deletion, which removes activating mutations in pp60^{v-src}, renders the activity of the XD4 kinase dependent on Tyr-416 phosphorylation. Thus, if host factors regulate the phosphorylation of Tyr-416 in XD4 *src*, the kinase activity of the XD4 protein would be host cell dependent. Evidence in favor of this model will be presented elsewhere (32).

The linker insertion in SRX5 removes the major autophosphorylation site, Tyr-416, and inserts three amino acids (SRD). Removal of Tyr-416 by itself is unlikely to be the basis of the host range phenotype, since the Y416F mutation does not affect transformation of mammalian fibroblasts by pp60^{v-src} (42). Although SRX5 pp60^{v-src} retains kinase activity in vitro when assayed on enolase as the substrate, the level of p36 (the calpactin I heavy chain) phosphorylation in vivo was drastically reduced. This suggests that the phenotype of SRX5 results from an alteration in substrate recognition. Consistent with this hypothesis is the fact that a reduction in p36 phosphorylation was also observed in cells infected by the partially transforming mutant CU2, which contains mutations (A403T, D413G, and V461M) in this region of the *src* molecule (6, 35). The crystal structure of a complex of the catalytic subunit of the cyclic AMP-dependent protein and a peptide inhibitor (28, 29) indicates that a hydrophobic pocket involved in peptide binding immediately flanks the autophosphorylation site, Thr-197, which occupies a site corresponding to that of Tyr-416 in pp60^{v-src}. This phosphothreonine interacts with a variety of residues in the catalytic center of the protein (28, 29). Thus, an alteration in this region could have direct or indirect effects on substrate recognition.

Mutations SPX1, SHX13, and XD6, which are located within SH2, resulted in reduced tyrosine phosphorylation of 120- and 62- to 64-kDa bands in both CEF and Rat-2 cells; the same alterations were seen in CEF expressing XD4. Other deletion mutations in pp60^{v-src} SH2 have been reported to reduce the phosphorylation of proteins ranging in size from 110 to 150 kDa (25, 46). Mutations within the SH2 and SH3 regions of activated *c-src* have been reported to affect tyrosine phosphorylation and pp60^{v-src} association of two proteins, p110 and p130 (25). One of the 120-kDa proteins detected by antiphosphotyrosine antibody in lysates of *src*-transformed cells is *ras*-GAP, which is tyrosine phosphorylated in cells transformed by pp60^{v-src} (11). In addition, *ras*-GAP complexes with a highly tyrosine-phosphorylated 62-kDa protein in these cells (11). The band at 62 to 65 kDa that is detected by antiphosphotyrosine antibody in lysates of *src*-transformed cells appears to represent this protein (1, 12, 34).

Both p120 *ras*-GAP and p62 exhibit reduced levels of tyrosine phosphorylation in Rat-2 cell lines expressing mutants SPX1, XD6, and SHX13 (12, 34). The results of immunoblotting of cell lysates with antiphosphotyrosine antibody indicate that the tyrosine phosphorylation of 120- and 62- to 64-kDa proteins is also reduced in CEF. If these proteins are related to *ras*-GAP and p62, these findings suggest that *ras*-GAP and p62 tyrosine phosphorylation is not necessary for transformation in CEF or that low levels of GAP and p62 tyrosine phosphorylation are sufficient for

transformation. In XD6-expressing CEF, tyrosine phosphorylation of several other species was also considerably reduced. Again, either tyrosine phosphorylation of these proteins is unnecessary for transformation or low levels of phosphorylation are sufficient. Further characterization of p120 and p62 phosphorylation in CEF will be necessary to resolve these issues.

In conclusion, a variety of factors contribute to the host-dependent phenotype, including differences in transformation thresholds, alterations in the regulation of *src* kinase activity, and alterations in the substrate specificity of pp60^{src}. The properties of the SH2 mutants described here suggest that SH2 sequences may be important both in intramolecular interactions which regulate kinase activity and in intermolecular interactions with substrates. The failure of the SH2 mutants to phosphorylate *ras*-GAP and p62 (34; this report) suggests that the SH2 region is involved in recognition of these substrates. Similarly, the reduced level of phosphorylation of p36 (the calpactin I heavy chain) in SRX5-expressing cells suggests that the region of pp60^{src} adjacent to Tyr-416 is also involved in substrate recognition. Alterations in the pattern of tyrosine phosphorylation might also result from changes in the intracellular location of pp60^{src}, as mutations in SH2 appear to affect the interaction of pp60^{src} with the detergent-insoluble cytoskeleton (16). Experiments to distinguish between these models are in progress.

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