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pp60^{v-src} Transformation of Rat Cells but Not Chicken Cells Strongly Correlates with Low-Affinity Phosphopeptide Binding by the SH2 Domain

Michael F. Verderame

Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, Department of Microbiology and Immunology, Program in Cell and Molecular Biology, and Program in Genetics, College of Medicine, Pennsylvania State University, Hershey, Pennsylvania 17033

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Substrates critical for transformation by pp60^{v-src} remain unknown, as does the precise role of the *src* homology 2 (SH2) domain in this process. To continue exploring the role of the SH2 domain in pp60^{v-src}-mediated transformation, site-directed mutagenesis was used to create mutant v-*src* alleles predicted to encode proteins with overall structural integrity intact but with reduced ability to bind phosphotyrosine-containing peptides. Arginine-175, which makes critical contacts in the phosphotyrosine-binding pocket, was mutated to lysine or alanine. Unexpectedly, both mutations created v-*src* alleles that transform chicken cells with wild-type (wt) efficiency and are reduced for transformation of rat cells; these alleles are host dependent for transformation. Additionally, these alleles resulted in a round morphological transformation of chicken cells, unlike 12 of the 13 known host-dependent *src* SH2 mutations that result in a fusiform morphology. Analysis of phosphopeptide binding by the mutant SH2 domains reveal that the in vitro ability to bind phosphopeptides known to have a high affinity for wt *src* SH2 correlates with wt (round) morphological transformation in chicken cells and the in vitro ability to bind phosphopeptides known to have a low affinity for wt *src* SH2 correlates with rat cell transformation. These results suggest that the search for critical substrates in rat cells should be among proteins that interact with pp60^{v-src} with low affinity.

INTRODUCTION

The cellular protein tyrosine kinase pp60^{c-src} has been strongly implicated in human colon carcinogenesis (Bolen *et al.*, 1987; Cartwright *et al.*, 1990, 1991; Garcia *et al.*, 1991; Novotnysmith and Gallick, 1992). The retroviral oncoprotein pp60^{v-src}, a mutated counterpart of chicken pp60^{c-src}, has long been used as a model system for understanding neoplastic transformation and is capable of transforming avian or rodent fibroblasts to a neoplastic phenotype (Wyke and Stoker, 1987; Parsons and Weber, 1989). Studies on a large collection of mutant alleles clearly reveal that tyrosine kinase activity is necessary but not sufficient for transformation (Parsons and Weber, 1989). Despite intensive efforts, a complete understanding of the biochemical mechanism of transformation by pp60^{v-src} is still lacking. Nonetheless, it is clear that in addition to the catalytic domain pp60^{v-src} contains several other well-defined domains that make critical contributions to transformation.

The extreme N terminus of pp60^{v-src} contains the myristylation domain (also known as *src* homology domain 4; Resh, 1993) that is responsible for the post-translational addition of the 14-carbon fatty acid myristate to the glycine at position 2 (Kamps *et al.*, 1985). This fatty acid plus nearby acidic amino acids (Silverman and Resh, 1992) form a membrane-binding domain that is essential for transformation by pp60^{v-src} (Buss *et al.*, 1986; Kamps *et al.*, 1986). pp60^{v-src} also contains a *src* homology domain 3 (amino acids 86–139) that binds the regulatory subunit of phosphatidylinositol-3′-kinase (Liu *et al.*, 1993a), an enzyme whose role in transformation remains to be clearly defined.

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Amino acids 148–244 constitute the *src* homology 2 domain (SH2)[1] that binds proteins containing phosphotyrosine (P-Tyr; Koch *et al.*, 1992) with high affinity (Songyang *et al.*, 1993) and high specificity (Songyang *et al.*, 1993; reviewed in Marengere and Pawson, 1994; Schaffhausen, 1995). Affinity and specificity are determined in part by the amino acids surrounding the P-Tyr, particularly the three amino acids to the carboxyl terminal of the tyrosine (Songyang *et al.*, 1993). The SH2 domain, which has been intensely studied over the last several years in a wide variety of metazoan signal transduction systems, clearly acts as a protein–protein interaction domain that assembles critical signaling complexes (Marengere and Pawson, 1994; Schaffhausen, 1995). Additionally, this domain can serve a direct negative regulatory function. For example, in pp60$^{c\text{-}src}$, which does not have transforming activity or high kinase activity (Parker *et al.*, 1984), the SH2 domain negatively regulates kinase activity and transformation (Nemeth *et al.*, 1989; Hirai and Varmus, 1990a; O'Brien *et al.*, 1990; Seidel-Dugan *et al.*, 1992) by binding to the P-Tyr at position 527 and stabilizing an inactive conformation (Liu *et al.*, 1993b; reviewed in Cooper and Howell, 1993). The loss of Tyr-527 in pp60$^{v\text{-}src}$ is partly responsible for its transforming ability (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; reviewed in Sefton and Hunter, 1986).

In pp60$^{v\text{-}src}$, however, the SH2 domain also serves a positive role, demonstrated by a substantial collection of transformation-defective v-*src* alleles with mutations in SH2 (Parsons and Weber, 1989). Thirteen of 17 mutant alleles of pp60$^{v\text{-}src}$ that are host dependent for transformation contain lesions in SH2 (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990b; Foster and Martin, 1992; Verderame and Varmus, 1994). In 11 of the 13 host-dependent alleles resulting from SH2 mutations, host dependence is accompanied by fusiform morphological transformation in chicken cells rather than wild-type (wt) round morphological transformation (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990b; Verderame and Varmus, 1994). Understanding how SH2 regulates both host dependence and morphological transformation should provide insight on how pp60$^{v\text{-}src}$ transforms fibroblasts and what role SH2 plays in that process. For example, using host-dependent alleles encoding pp60$^{v\text{-}src}$ that are deficient for P-Tyr binding due to mutations in SH2, this laboratory recently provided genetic evidence that SHC may be an important downstream target (Verderame *et al.*, 1995). Along with pursuing the biochemical pathways implicated by those results, additional mutations in SH2 were generated to further explore the role SH2 serves in transformation; such mutations are the subject of this report.

Of the 13 previously reported host-dependent alleles with SH2 domain mutations, only 3 are single amino acid substitutions (the rest are insertions or deletions) and none of these three substitutions are biochemically conservative: L186F (Verderame and Varmus, 1994); F172P (Hirai and Varmus, 1990b); R175L (Hirai and Varmus, 1990b). Thus, the consequences of subtle changes in P-Tyr binding by pp60$^{v\text{-}src}$ have not yet been extensively explored as they have in, e.g., ABL (Mayer *et al.*, 1992).

Recently, the three-dimensional structure of the c-*src* SH2 domain bound to both a low-affinity (Waksman *et al.*, 1992) and a high-affinity (Waksman *et al.*, 1993) phosphopeptide was determined. Using this information, pp60$^{v\text{-}src}$ SH2 domains predicted to have minimal overall structural alterations but reduced (or absent) P-Tyr binding were created by mutating the highly conserved arginine-175 (R175; known as βB5 in the generic structure of SH2 domains; Eck *et al.*, 1993) to lysine or to alanine. Lysine is structurally conservative, retaining a charged side chain, whereas alanine will eliminate the side chain charge but is predicted not to disrupt the overall folded structure. This report shows that these mutations in pp60$^{v\text{-}src}$ created novel host-dependent alleles with unusual properties that have provided insight into its mechanism of transformation. The results demonstrate that the ability to interact with so-called "high-affinity" peptides correlates with traditional wt (round) morphological transformation in chicken cells and the ability to interact with so-called "low-affinity" P-Tyr peptides correlates with the ability to transform rodent cells.

[1]Abbreviations: GST, glutathione *S*-transferase; P-Tyr, phosphotyrosine; SH2, *src* homology 2 domain.

MATERIALS AND METHODS

Mutagenesis

Site-directed mutagenesis was performed according to the method of Kunkel (1985) using reagents from Bio-Rad (Richmond, CA). To mutate R175, the following oligonucleotides were used: for R175K, 5'-GTCTCGCTCTTCTTGACCAAGAAGG-3'; for R175A, 5'-GTCTCGCTCTTCGCGACCAAGAAGG-3' (mutations are underlined). Clones were identified by screening for loss of a natural *Bsp*EI site (R175K) or gain of a novel *Nru*I site (R175A) and confirmed by sequencing.

Recombinant DNA

All cloning was accomplished with standard procedures (Sambrook *et al.*, 1989) using the following previously described eukaryotic expression vectors: the RCAS avian retroviral vector for expression in chicken cells (Hughes *et al.*, 1987) and MX2122 expression vector for rat cells (Brown and Scott, 1987). To ensure that results were not due to cloning artifacts, two independent molecular clones of the mammalian expression vector were used for both v-*src*-R175A and v-*src*-R175K.

Cell Culture

General cell culture and transfection procedures have been described previously (Verderame *et al.*, 1989; Verderame and Varmus, 1994; Woods and Verderame, 1994).

Transformation in rodent cells was assessed by using the RAT-2 cell line. To isolate individual rat cell lines expressing mutant v-*src* alleles, 4-mm (inside diameter) cloning cylinders were placed over colonies of uniform morphology (either all flat or all transformed by microscopic examination) that were well separated (>1 cm) from all adjacent colonies. Cells were trypsinized, replated, and expanded. All clones were maintained in the presence of G418. In the first round of transfections, most (11/12) clones expressing v-*src*-R175K had a transformed morphology when first picked (see RESULTS), whereas most clones (9/12) of v-*src*-R175A did not. All clones were subcloned to ensure pure populations.

Biochemical Analysis

Protein analysis procedures and kinase assays have been described previously (Verderame *et al.*, 1989; Verderame and Varmus, 1994; Woods and Verderame, 1994).

SH2–Phosphopeptide Interactions

All SH2 domains were cloned into pGEX1 as described (Woods and Verderame, 1994); identity was verified in all cases by restriction analysis. Fusion proteins were assayed from whole bacterial lysates as described (Woods and Verderame, 1994) or purified and assayed as described (Bibbins *et al.*, 1993). Glutathione *S*-transferase- (GST) SH2 fusion proteins were detected by protein blotting with polyclonal anti-GST antiserum (generous gift from Cliff Lowell, University of California, San Francisco, CA) and enhanced chemiluminescence detection.

A peptide (P-416) corresponding to the pp60$^{v\text{-}src}$ autophosphorylation site coupled to Sepharose beads (Woods and Verderame, 1994) was generously provided by Andy Laudano (University of New Hampshire, Durham, NH). A peptide (P-EEI) with high affinity for the *src* SH2 domain (Songyang *et al.*, 1993) was synthesized both with and without a phosphate group on the unique tyrosine by the Pennsylvania State University College of Medicine Macromolecular Core Facility. The sequence of this peptide is: EPQY*EEIPI. The asterisk indicates the phosphorylated tyrosine.

RESULTS

Rationale for Targeting R175 (βB5)

Previous results have shown that the pp60$^{v\text{-}src}$ SH2 domain is important for transformation of rodent cells (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990b; Verderame and Varmus, 1994). Because previous experiments from this laboratory had been conducted with SH2-F172Δ and SH2-L186F, which are both totally defective for P-Tyr binding [Woods and Verderame, 1994; most likely due to significant unfolding of the domain given the important packing role of phenylalanine-172 (F172) in surrounding the hydrophobic core that contains leucine-186 (L186; Waksman *et al.*, 1992, 1993], additional mutant alleles were constructed that are predicted to preserve the tertiary structure of the SH2 domain but specifically interfering with P-Tyr binding. Based on the structural information available for the *src* SH2 domain, R175 of pp60$^{v\text{-}src}$ (generically described as position βB5 in folded SH2 domains; Eck *et al.*, 1993) was selected. The guanidino group of this arginine (which is invariant in SH2 domains; Marengere and Pawson, 1992) makes important bidentate contacts with the phosphate group of the P-Tyr (Waksman *et al.*, 1992, 1993). Furthermore, mutation of the equivalent arginine to lysine in the protein encoded by v-*abl* abolishes P-Tyr binding completely (Mayer *et al.*, 1992).

Site-directed mutagenesis was used to change R175 to lysine or to alanine. Lysine's amino group cannot make bidentate contacts to the phosphate as can the guanidino group of arginine, and the lysine side chain is one atom shorter; this mutation is predicted to decrease the ability of the *src* SH2 domain to interact with P-Tyr-containing proteins. Alanine lacks a charged group entirely and is predicted to decrease P-Tyr interactions even further.

Expression of v-src-R175K and v-src-R175A in Chicken Cells

Both R175K and R175A mutations were built into wt v-*src* to determine their effect on transformation and into the host-dependent alleles v-*src*-F172Δ (Verderame *et al.*, 1989) and v-*src*-L186F (Verderame and Varmus, 1994) to determine whether the double mutants would be more seriously effected than either single mutant. All mutant alleles were confirmed by sequencing, cloned into the avian retroviral vector RCAS (Hughes *et al.*, 1987), and transfected into chicken embryo fibroblasts; virus was allowed to spread throughout the culture.

Both v-*src*-R175K and v-*src*-R175A alleles were clearly able to induce the typical round morphological transformation associated with wt pp60$^{v\text{-}src}$ (Figure 1). This result was unexpected because many other mutations in SH2 that are known to disrupt P-Tyr binding (e.g., L186F; Woods and Verderame, 1994) result in fusiform morphology. Additionally, each of the double mutants (v-*src*-F172Δ/R175K, v-*src*-F172Δ/R175A, v-*src*-L186F/R175K, and v-*src*-L186F/R175A) was indistinguishable from its parental host-dependent allele, implying the double mutants are no more defective than the host-dependent alleles. This result is consistent with previous results demonstrating no P-Tyr binding by the original host-dependent mutant domains (Woods and Verderame, 1994). All of the alleles with mutations at R175 were able to induce anchorage-independent growth equal to wt pp60$^{v\text{-}src}$ (Figure 2).

Mutations in the SH2 domain can have effects on the kinase activity of pp60$^{v\text{-}src}$ (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990b; Verderame and Varmus, 1994). To examine this possibility with R175K and R175A, the kinase activity and steady-state expression level of the various pp60$^{v\text{-}src}$ proteins was examined (Figure 3). For both R175K

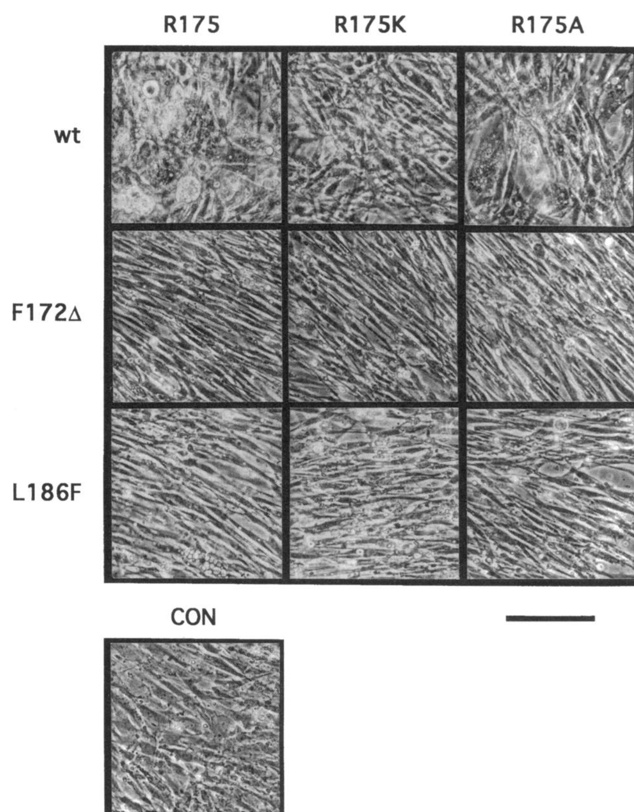

Figure 1. Morphology of chicken cells expressing v-*src*-R175K or v-*src*-R175A. Chicken cells transfected with RCAS retroviral vectors carrying indicated v-*src* alleles were passaged three times to ensure viral spread and photographed. Bar, 500 μm. CON, chicken embryo fibroblasts transfected with RCAS plasmid without any v-*src* allele.

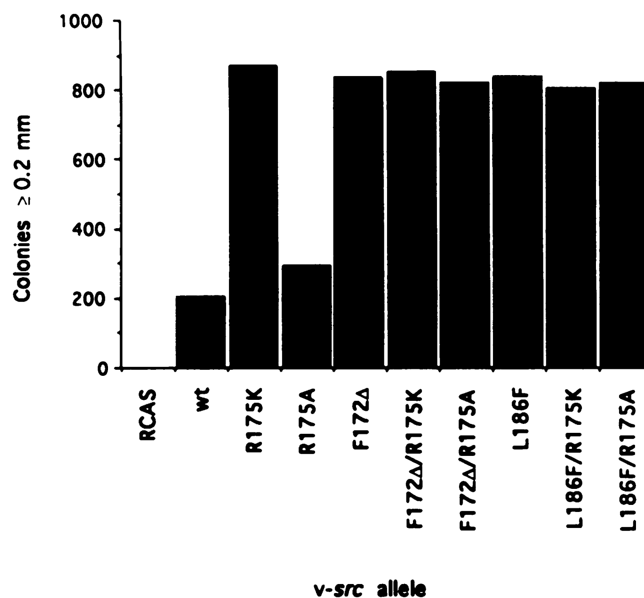

Figure 2. Anchorage-independent growth of chicken cells expressing v-*src*-R175K or v-*src*-R175A. Approximately 5×10^5 chicken cells fully infected with RCAS viruses expressing the various v-*src* alleles were plated in 0.35% agar in 60-mm dishes. Cells were stained 15 d later and colonies ≥0.2 mm were counted. The slight reduction seen with wt and R175A is due to cell crowding at this density; microscopic examination revealed that all cultures expressing a v-*src* gene exhibited significant growth compared with the control cells not expressing v-*src* with little difference between any of the cells expressing v-*src*. Similar results were seen when 5×10^4 cells were used (my unpublished results). RCAS, chicken cells transfected with RCAS plasmid without any v-*src* allele.

and R175A single mutations, the level of kinase activity was identical to wt $pp60^{v\text{-}src}$. Similarly, the double-mutant alleles encoded proteins that had kinase activities similar to $pp60^{v\text{-}src\text{-}F172\Delta}$ and $pp60^{v\text{-}src\text{-}L186F}$. [The reduction in kinase activity of $pp60^{v\text{-}src\text{-}F172\Delta}$ and $pp60^{v\text{-}src\text{-}L186F}$ has been reported previously (Woods and Verderame, 1994) and is consistent with decreased P-Tyr detected in vivo (Verderame *et al.*, 1989; Verderame and Varmus, 1994).] Because the steady-state level of the various proteins also was indistinguishable from the appropriate control in all cases (Figure 3B), neither R175K nor R175A effects the specific kinase activity of $pp60^{v\text{-}src}$. Protein blots of whole-cell lysates probed with anti-P-Tyr antibody PY20 revealed no differences in the overall pattern of P-Tyr-containing proteins (my unpublished results). Thus, by all in vitro measures, R175K and R175A have no detectable impact on $pp60^{v\text{-}src}$-mediated transformation in chicken cells.

Expression of v-src-R175K and v-src-R175A in Rat Cells

To examine the ability of v-*src*-R175K and v-*src*-R175A to transform rat cells, these alleles were cloned into the mammalian expression vector MX2122, which allows for selection of transfected cells in G418 (Brown and Scott, 1987). RAT-2 cells were transfected with these vectors (or with control vectors carrying no *src* gene or wt v-*src*) to perform a focus assay. Neither v-*src*-R175K nor v-*src*-R175A gave rise to many transformed foci in rat cells (Figure 4) apparently defining two new host-dependent alleles of v-*src*. To analyze properties of cells expressing these mutant alleles in detail, RAT-2 cells were transfected with the various expression plasmids and selected with G418 to isolate clonal cell lines. Two independent plasmids of each mutant allele were transfected: R175K-1, R175K-5, R175A-10, and R175A-11. Six colonies (labeled A through F) were picked from each transfection.

In contrast to the focus assay, the isolated rat cell colonies expressing v-*src*-R175K in general were moderately transformed (my unpublished results) and were obviously transformed upon subcloning and expansion (Figure 5). Of the 11 rat cell clones isolated

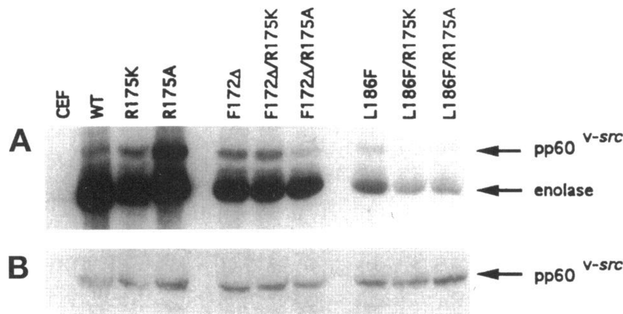

Figure 3. Kinase activity of pp 60$^{v\text{-}src}$ from chicken cells expressing v-*src*-R175K or v-*src*-R175A. (A) Cells expressing the various v-*src* alleles were lysed; pp60$^{v\text{-}src}$ was immunoprecipitated with monoclonal antibody 127 and subjected to a kinase assay with acid-denatured enolase as a substrate. Reaction products were separated on a 10% polyacrylamide gel. (B) Whole-cell lysate (120 μg) from the same extracts used in the kinase assay was separated by SDS-PAGE on a 10% gel and blotted to nitrocellulose, and the blot was probed with monoclonal antibody 127 to reveal the steady-state level of pp60$^{v\text{-}src}$.

after transfection with v-*src*-R175A (R175A-11-F was lost during expansion), 8 were morphologically flat (my unpublished results), more in line with the results of the focus assay. However, only four of these clones (including the three transformed clones) expressed detectable pp60$^{v\text{-}src}$ (cell clones R175A-10-B, -10-C, -11-C, and -11-E). To increase the number of clones expressing pp60$^{v\text{-}src\text{-}R175A}$ available for study, four additional cell clones were isolated (R175A-11-G, -I, -J, and -K). One such clone that originally appeared morphologically flat (R175A-11-I) gave rise to both flat and transformed colonies upon subcloning; one clone of each type was expanded for further analysis. These clones have subsequently been stable for the duration of the experiments.

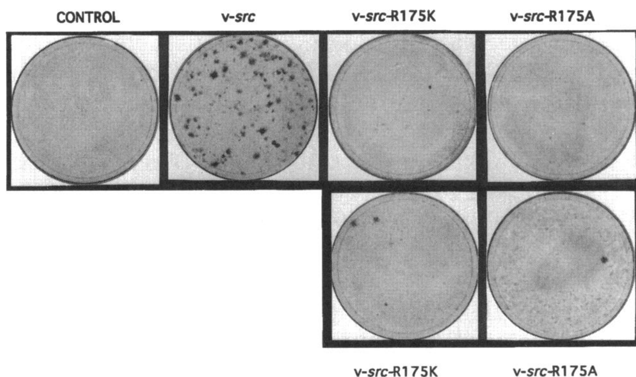

Figure 4. Focus assay on Rat-2 cells transfected with v-*src*-R175K or v-*src*-R175A. Mammalian expression vectors carrying wt v-*src*, v-*src*-R175K, or v-*src*-R175A were transfected into Rat-2 cells; cells were fed twice weekly and stained 18 d later. Duplicate dishes of v-*src*-R175A and v-*src*-R175K are shown.

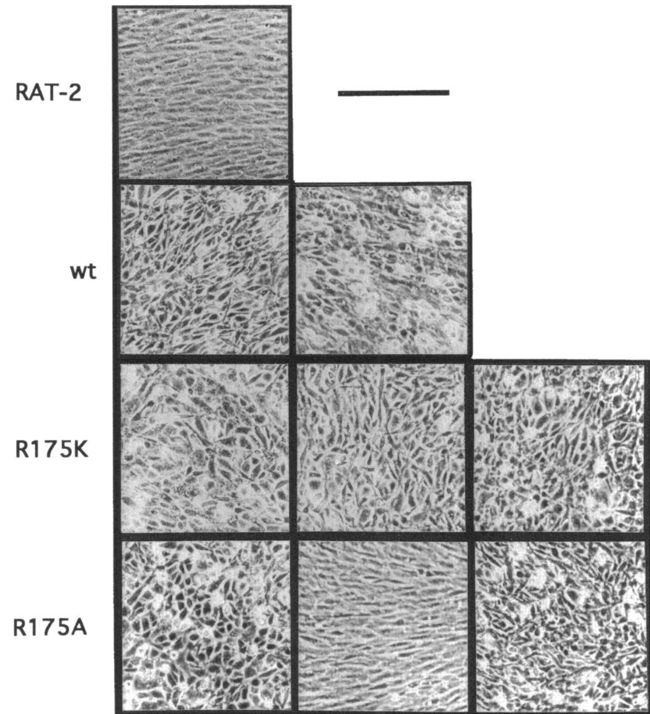

Figure 5. Morphology of clonal Rat-2 cell lines expressing v-*src*-R175K or v-*src*-R175A. Clonal cell lines were plated on 60-mm culture dishes, allowed to reach confluence, and photographed under phase microscopy. The row labeled wt shows clonal cell lines A and D (left to right) derived from cells transfected with wt v-*src*. The row labeled R175K shows clonal cell lines 1-B, 1-C, and 5-G derived from cells transfected with v-*src*-R175K. The row labeled R175A shows clonal cell lines 10-B, 11-I (flat), and 11-I (tx) derived from cells transfected with v-*src*-R175A. Rat-2 is the parental cell line. Bar, 1 mm.

All of the R175K and a selection of the R175A clonal cell lines were examined for anchorage-independent growth (Figure 6) and for steady-state expression of pp60$^{v\text{-}src}$ (Figure 7). All of the rat cell lines that express v-*src*-R175K are capable of growing without anchorage, although the steady-state level of pp60$^{v\text{-}src\text{-}R175K}$ was routinely higher than the steady-state level in cells transformed by wt pp60$^{v\text{-}src}$. Similarly, the few v-*src*-R175A clones that are anchorage independent express higher levels of pp60$^{v\text{-}src}$ than clones expressing the wt protein that are anchorage independent. Particularly revealing in this regard are the two subclones derived from a single original population: R175A-11-IF and R175A-11-IT. The subclone that grows well in agarose expresses pp60$^{v\text{-}src\text{-}R175A}$ to a very high level (indeed significantly higher than wt or R175K), whereas the morphologically flat subclone does not grow in agarose and has a low steady-state level of pp60$^{v\text{-}src}$. Importantly, the level of expression of R175A in the anchorage-dependent subclone R175A-11-IF is roughly equal to the level of expression of wt v-*src* in the anchorage-independent subclone

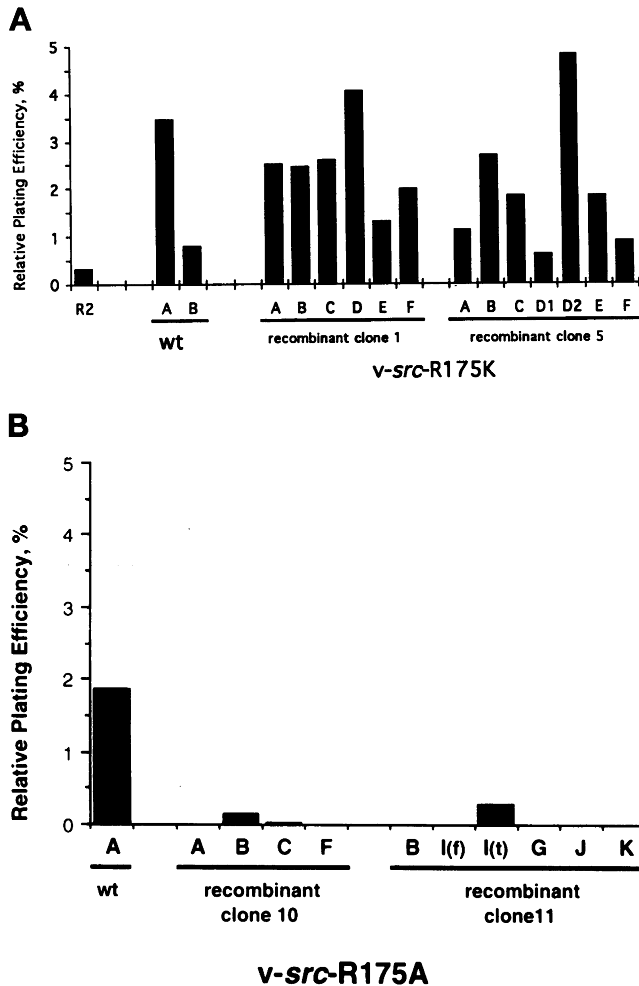

Figure 6. Anchorage-independent growth of Rat-2 cells expressing v-*src*-R175K or v-*src*-R175A. (A) Approximately 5×10^3 cells from independent clonal cell lines expressing v-*src*-R175K [and control cell lines expressing no v-*src* (RAT-2) or wt v-*src*] were plated in 0.35% agarose in 60-mm dishes. Cells were stained 19 d later and colonies ≥0.3 mm were counted. Similar results were seen when 2.5×10^4 cells were used (my unpublished results). In cell clone R175K-5-D, two distinct morphologies were observed. Subclones representing both morphologies were expanded and analyzed. Clone D2, which had a stronger morphological change, grew slightly better in agarose; however, no differences in $pp60^{v\text{-}src}$ expression were noted (see below). (B) Approximately 2×10^4 cells from independent clonal cell lines expressing v-*src*-R175A (and appropriate control cell lines) were plated in 0.35% agarose in 60-mm dishes. Cells were stained 14 d later and colonies ≥0.2 mm were counted. Similar results were seen when 5×10^3 cells were used (my unpublished results). f, flat subclone; t, transformed subclone.

R2-wt-B (Figure 6A). Thus, the threshold for rodent cell transformation is higher for R175A than wt v-*src*. Protein blots of whole-cell lysates probed with anti-P-Tyr antibody PY20 revealed no consistent differences in the overall pattern of P-Tyr-containing proteins among these cell lines (my unpublished results).

P-Tyr Binding of Mutant SH2 Domains

Given these surprising results, it was important to directly test the P-Tyr binding ability of the SH2 domains. Recombinant GST fusion proteins expressed in bacteria as described previously (Woods and Verderame, 1994) were incubated with Sepharose beads covalently coupled to either a high-affinity phosphopeptide (P-EEI, revised estimated K_d 600 nM; Ladbury *et al.*, 1995) or low-affinity phosphopeptide (P-416, corresponding to the autophosphorylation site of $pp60^{v\text{-}src}$, the estimated K_d is ~10^4-fold higher; Bibbins *et al.*, 1993). After removing unbound proteins, proteins bound to the P-Tyr beads were analyzed by protein blot with antibodies to GST (Figure 8). The SH2 domain with R175A (SH2-R175A) was unable to bind the low-affinity phosphopeptide but unexpectedly retained its ability to bind the high-affinity phosphopeptide. The SH2 domain with the R175K mutation was indistinguishable from wt in its binding to both low- and high-affinity phosphopeptides. This latter result is somewhat surprising given results with the analogous mutation in *abl* (Mayer *et al.*, 1992) and the clear biological effect of the mutation (this report). As a negative control, the ability of SH2 domains containing F172Δ or L186F to bind high-affinity peptides was also tested; neither of these proteins bound high-affinity P-Tyr-containing peptides (Figure 8B).

The crude lysate assay may mask subtle differences in binding affinity, particularly if the fusion protein concentration is high. Accordingly, recombinant GST-SH2 proteins were purified on a glutathione-agarose column and tested for their ability to bind P-416 and P-EEI at various concentrations of recombinant protein (Figure 9A). Although this kind of experiment cannot be used to accurately determine the K_d of the interaction, the results are unambiguous: SH2-R175K and SH2-R175A bind low-affinity P-Tyr peptides less well than wt. In contrast, in repeated experiments testing the ability to bind high-affinity phosphopeptides, no decrease in binding could be detected with either SH2-R175K or SH2-R175A (Figure 9B). These results are consistent with those previously reported by other laboratories. Using a *src*-transformed cell lysate assay, Okamura and Resh (1994) reported that R175K (in c-*src* SH2) has reduced but detectable P-Tyr binding. Bibbins *et al.* (1993) have shown that R175L (in c-*src* SH2) binds high- but not low- affinity P-Tyr peptides.

These results, and previously published results from this laboratory (Verderame *et al.*, 1989; Verderame and Varmus, 1994; Woods and Verderame, 1994) and the Varmus laboratory (Hirai and Varmus, 1990b; Bibbins *et al.*, 1993), revealed that *src* alleles with mutations affecting the ability of the SH2 domain to bind low-affinity phosphopeptides exhibited host dependence for transformation; i.e., they were diminished in their

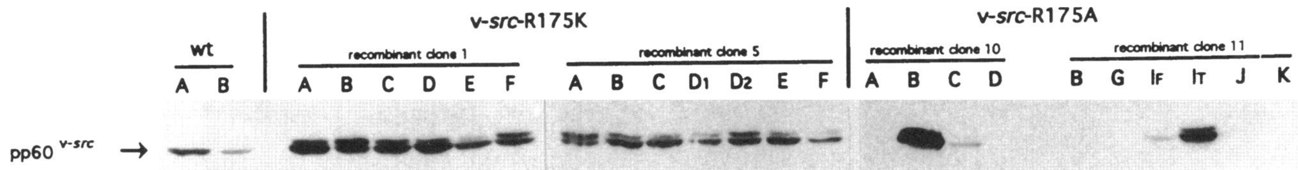

Figure 7. Steady-state pp60$^{v\text{-}src}$ levels from rat cells expressing v-*src*-R175K or v-*src*-R175A. Protein (40 μg) from whole-cell lysates produced from the clonal cell lines expressing wt pp60$^{v\text{-}src}$, pp60$^{v\text{-}src\text{-}R175K}$, or pp60$^{v\text{-}src\text{-}R\text{-}175A}$ were separated by SDS-PAGE on 10% gels. After blotting the proteins to nitrocellulose, pp60$^{v\text{-}src}$ was visualized by probing the blot with monoclonal antibody 127 followed by horseradish peroxidase-conjugated secondary antibody; detection was accomplished by chemiluminescence.

ability to transform rat cells but were still able to transform chicken cells. To further extend this correlation, several other mutant *src* SH2 domains with well-described biological properties were expressed as recombinant proteins and tested for their ability to bind low- (Figure 9A) or high- (Figure 9B) affinity phosphopeptides. (The results of all of these studies are summarized in Table 1.) All seven SH2 domains from *src* alleles that have reduced ability to transform rat cells [F172Δ (Verderame *et al.*, 1989), L186F (Verderame and Varmus, 1994), R175K (this report), R175A (this report), F172P (Hirai and Varmus, 1990b), R205L/K206E (Hirai and Varmus, 1990b), and R175L (Hirai and Varmus, 1990b)] have reduced ability to bind low-affinity P-Tyr peptides (Figure 9A, except for R175L; Bibbins *et al.*, 1993). Transformation of rat cells appears to require an SH2 domain capable of low-affinity P-tyr-dependent interactions.

DISCUSSION

Understanding the biochemical pathways altered by pp60$^{v\text{-}src}$ that are crucial to neoplastic transformation has been compromised by the large number of proteins phosphorylated by pp60$^{v\text{-}src}$ (Parsons and Weber, 1989). Host-range dependence for neoplastic transformation provides a unique opportunity to search for relevant biochemical pathways: the same enzyme activates the necessary pathways in one cell but not another. Because virtually all host-dependent alleles of v-*src* are the result of SH2 domain mutations, study of these mutants should also provide important clues into the role of the SH2 domain in transformation. This report describes two novel alleles of pp60$^{v\text{-}src}$ that are host dependent for focus formation, transforming chicken but not rat cells, as a result of single amino acid changes in the P-Tyr-binding pocket of the *src* SH2 domain: R175 was changed to the structurally similar lysine or the structurally neutral alanine.

It is important to recognize that the block to rat cell transformation is not absolute, however. Both v-*src*-R175K and v-*src*-R175A can give rise to transformed rat cells; indeed, v-*src*-R175K does so with a reasonable frequency when tested in a colony assay. Such transformation is correlated with a steady-state level of pp60$^{v\text{-}src}$ above that typically seen, and significantly above the threshold for transformation by wt pp60$^{v\text{-}src}$ (see Figure 7): the block to transformation of rat cells by host-dependent alleles can be overcome by sufficient expression of the mutant allele. The mutant alleles were inducing the full transformed phenotype: morphologically transformed cells expressing R175A or R175K are also capable of anchorage-independent

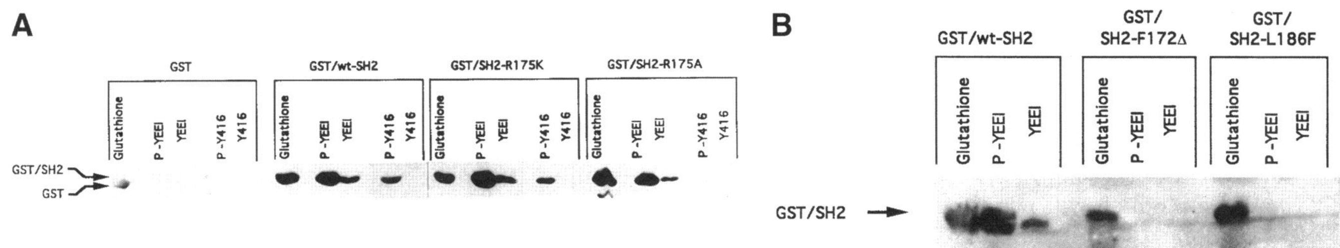

Figure 8. SH2–phosphopeptide interactions in whole bacterial lysates. Sepharose beads covalently coupled to high- (P-EEI) or low- (P-416) affinity P-Tyr-containing peptides (or control peptides) were incubated with whole bacterial lysate expressing the indicated recombinant SH2-GST fusion proteins. Unbound proteins were washed away and bound proteins were eluted in SDS-PAGE sample buffer. Eluted proteins were separated on 10% polyacrylamide SDS-PAGE gels, blotted to nitrocellulose, and detected by anti-GST antibodies using an enhanced chemiluminescence protocol. (A) Analysis of SH2 domains carrying either the R175K or R175A mutations. (B) Analysis of SH2 domains carrying either the F172Δ and L186F mutations. Previous experiments have revealed that SH2-F172Δ and SH2-L186F are unable to bind low-affinity peptides (Woods and Verderame, 1994).

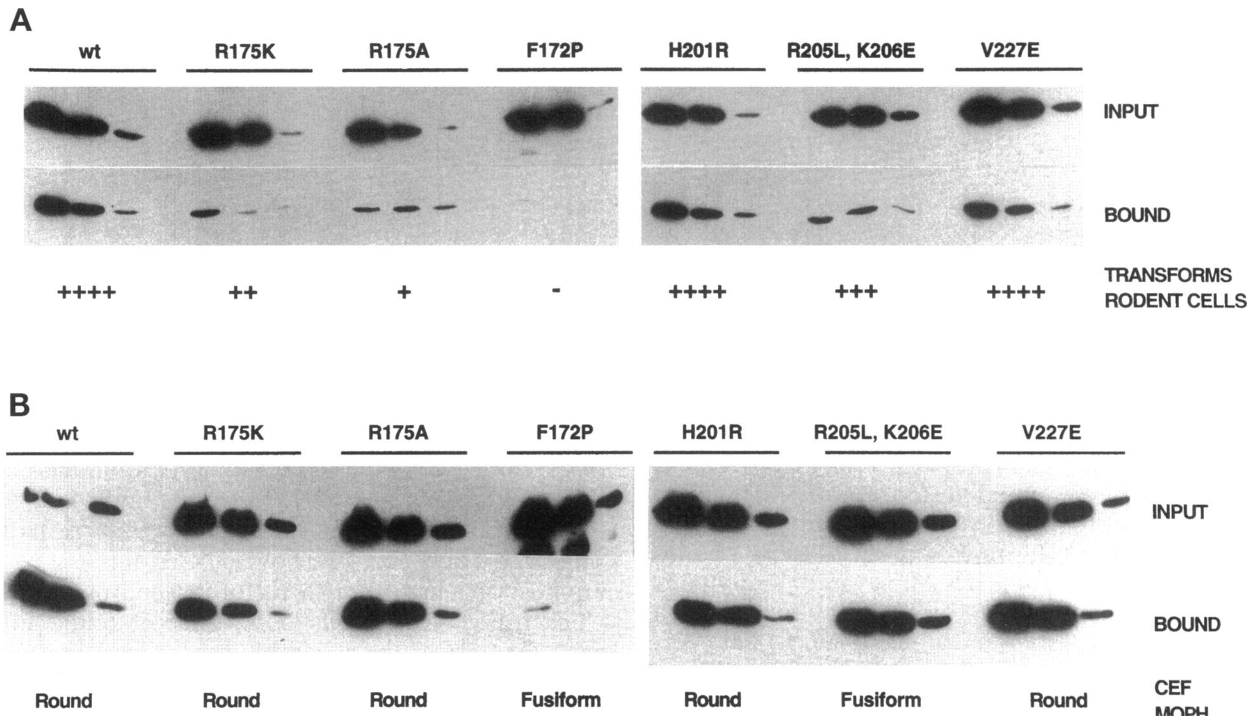

Figure 9. SH2–phosphopeptide interactions using purified recombinant fusion proteins. (A) A constant amount of Sepharose beads coupled to a low-affinity phosphopeptide was incubated with 1, 0.5, or 0.1 μg of purified SH2 fusion protein as indicated at 4°C for 30 min. Unbound proteins were washed away and bound proteins were analyzed by protein blotting after SDS-PAGE. The low constant binding of some of the mutant SH2 domains is a reproducible result; because it is concentration independent, it appears to be nonspecific binding. The cause of this nonspecific binding is unknown. (B) Identical experiments were performed by substituting Sepharose beads coupled to high-affinity phosphopeptide. Recombinant SH2 fusion proteins could not be reliably detected at levels below 0.1 μg (input) with the available reagents, precluding additional studies at lower protein concentrations.

growth. These results are reminiscent of results with v-*src*-F172Δ, which transforms rat cells only when highly overexpressed (Verderame *et al.*, 1989).

The six alleles with reduced low-affinity P-Tyr binding span a wide range of transforming potential on rat cells from virtually none [F172Δ (Verderame *et al.*, 1989) and F172P (Hirai and Varmus, 1990b)]), low [L186F (Verderame and Varmus, 1994) and R175A (this report)], moderate [R175K (this report)], to only slightly decreased [R205L/K606E (Hirai and Varmus, 1990b)]. Because total loss of P-Tyr binding results in total loss of transforming ability on rat cells (Woods and Verderame, 1994) and wt P-Tyr binding yields wt transformation (by definition), one might expect a direct relationship between transforming potential and P-Tyr binding, which was not observed. Several points should be considered in understanding this observation. 1) The autophosphorylation site was chosen as a convenient low-affinity peptide; the fact that autophosphorylation is not required for transformation by wt pp60$^{v\text{-}src}$ (Snyder *et al.*, 1983) implies it is not the relevant P-Tyr in vivo. Binding by the true in vivo target protein may differ in subtle but important ways. 2) P-Tyr binding by full-length pp60$^{v\text{-}src}$ carrying these mutations may be different from the isolated recombinant SH2 domain. Indeed, the *src* homology domain 3 domain can make important contributions to SH2 P-Tyr binding (Bibbins *et al.*, 1993; Murphy *et al.*, 1993; Okada *et al.*, 1993; Superti-Furga *et al.*, 1993; Kaplan *et al.*, 1994; Panchamoorthy *et al.*, 1994). 3) The SH2 domains were derived from two different genetic backgrounds: two of the domains with reduced low-affinity P-Tyr binding (F172P, R205L/K206E) were derived from chicken c-*src* (Hirai and Varmus, 1990a), whereas F172Δ, L186F, R175K, and R175A were derived from the Bratislava 77 strain of v-*src*. There are seven amino acid differences between these two SH2 domains, including amino acid position 180 (generically BC3; threonine in pp60$^{c\text{-}src}$ and alanine in pp60$^{v\text{-}src}$) that participates in a hydrogen bond network with the P-Tyr phosphate. This difference may decrease the affinity of the v-*src* SH2 compared with the c-*src* SH2 domain, all other things being equal. Despite these caveats, the data presented herein clearly demonstrate that decreased P-Tyr binding of low-affinity peptides correlates with decreased rat cell transformation.

While these experiments were being completed, Tian and Martin (1996) reported results from a similar

Table 1. Summary of biological and biochemical properties of v-*src* mutants

	Phosphopeptide binding		Transformation[a]		
Mutation	High affinity	Low affinity	Rodent cells	Chicken cells	
None	++++	++++	++++	++++	
F172Δ	−	−[b]	−	++++	Fusiform
L186F	−	−[b]	−	++++	Fusiform
R175K	++++	+	++	++++	
R175A	++++	+	+	++++	
R175L	++[c]	−[c]	−	++++	
F172P	+/−	+/−	−	++++	Fusiform
H210R	++++	++++	++++	++	
R205L,K206E	++++	+	+++	++++	Fusiform
V227E	++++	++++	++++[d]	++++	

[a]Data are from the following sources: F172Δ (Verderame *et al.*, 1989); L186F (Verderame and Varmus, 1994); R175A and R175K (this report); R175L, F172P, H201R, and R205L/K206E (Hirai and Varmus, 1990b).

[b]Data are from Woods and Verderame (1994).

[c]Data are from Bibbins *et al.* (1993).

[d]My unpublished observations.

set of mutants. The major difference between the two studies is that they saw wt levels of focus formation on RAT-2 cells with v-*src*-R175K, but focus formation by R175K was significantly reduced in the present study. Results in this present study reveal an increased threshold for transformation by R175K, providing a simple explanation for the different results reported. Lower levels of v-*src* expression from the MX2122 vector used in the present studies (G418 resistance induced by MX2122 requires RNA splicing that removes the v-*src* coding region; thus only a fraction of the primary transcripts are available to be translated into pp60$^{v\text{-}src}$) undoubtedly accounts for the difference.

In contrast to this relatively minor difference, other results of Tian and Martin (1996) are similar to results reported herein. In particular, decreased binding to a subset of P-Tyr-containing proteins of unknown affinity was detected. In a second relevant study, Boeuf *et al.* (1995) previously reported a general correlation between P-Tyr binding and transformation of mouse cells, but again, no attempt was made to distinguish low- versus high-affinity binding proteins. Nonetheless, the present study is in general agreement with both previous studies but extends those results by providing the first demonstration of a dramatic defect in low-affinity interactions (Figure 9). This result may explain the past difficulty in identifying crucial *src* targets. Most studies, with little underlying biological rational, have examined highly phosphorylated proteins or proteins that associate tightly with pp60$^{v\text{-}src}$. The genetic and biochemical results presented herein suggest that low-affinity interactions with pp60$^{v\text{-}src}$ may be crucial for rat cell transformation.

What then is the target for the SH2 domain in rat cells? Two obvious possibilities are that either the SH2 domain must interact with P-Tyr on pp60$^{v\text{-}src}$ itself or it must interact with P-Tyr on a cellular protein. In the former case, such an intramolecular interaction with the autophosphorylation site of pp60$^{v\text{-}src}$ (which is a low-affinity site) might result in a protein that stably assumes a conformation allowing efficient phosphorylation of critical targets (either by increasing the kinase activity or altering the substrate specificity). Such a mechanism seems unlikely given that autophosphorylation is not required for transformation by wt pp60$^{v\text{-}src}$ (Snyder *et al.*, 1983).

A more attractive hypothesis is that the SH2 domain is responsible for interactions with a cellular protein critical for transformation by pp60$^{v\text{-}src}$. The consequence of this interaction could relocalize pp60$^{v\text{-}src}$ providing access to novel substrates (as seen for CSK; Howell and Cooper, 1994; Sabe *et al.*, 1994; Clouthier *et al.*, 1995) or could result in the protection of the phosphorylation site from the action of cellular phosphates, resulting in an increased steady-state level of P-Tyr on the target protein (as can occur for the pp60$^{v\text{-}src}$–pp125^{FAK} interaction; Cobb *et al.*, 1994). However, this rat cell target is unlikely to be any of the known pp60$^{v\text{-}src}$ interacting proteins, because most of them stably associate with pp60$^{v\text{-}src}$ (e.g., Reynolds *et al.*, 1989; Fumagalli *et al.*, 1994; Sakai *et al.*, 1994; Taylor and Shalloway, 1994); results presented herein suggest it is low-affinity interactions that are crucial, at least for rat cells. A search for proteins that interact with the *src* SH2 domain with a low affinity is underway.

The requirement for low-affinity P-Tyr interactions for transformation of rat cells stands in remarkable

contrast to chicken cells, which are transformed (albeit to a fusiform morphology) even in the complete absence of an SH2 domain (e.g., *dl*5; Iswashita *et al.*, 1983). In fact, most host-dependent alleles induce fusiform morphological transformation in chicken cells (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990b; Verderame and Varmus, 1994). R175A and R175K are notable exceptions to this generalization. Transformation to a fusiform morphology is not simply a quantitative defect: reduced expression of wt v-*src* in chicken cells does not result in fusiform morphology, and increased expression of v-*src*-F172Δ (a host-dependent allele inducing fusiform morphology in chicken cells; Verderame *et al.*, 1989) does not result in round morphological transformation (my unpublished results). Interestingly, all of the mutant alleles studied herein that induce round morphological transformation in chicken cells bind high-affinity phosphopeptides as well as wt, whereas three of the four mutant alleles studied herein (F172Δ, L186F, and F172P) that induce fusiform morphology in chicken cells are unable to bind high-affinity P-Tyr peptides; R205L/K206E is the exception. The electrostatic potential of R205 (otherwise known as βD′1) stabilizes the glutamate at position +2 in the model high-affinity P-Tyr P-EEI (Waksman *et al.*, 1993). Because SH2-R205L/K206E still binds well to the model peptide P-EEI, R205 may be less important to binding P-EEI than the crystal structure would suggest (Waksman *et al.*, 1993); binding of the true substrate that controls morphological transformation in vivo may not require R205. The breakdown of the correlation between high-affinity binding and morphological transformation may simply be a reflection of the use of artificial substrates (i.e., P-EEI) and again highlights the need to identify true substrates.

Overall, what can be gleaned from this data? It has long been suggested that multiple pathways are activated by $pp60^{v\text{-}src}$ in the process of neoplastic transformation (Wyke and Stoker, 1987). The working hypothesis suggested by the accumulated data is that at least two pathways must be activated by $pp60^{v\text{-}src}$ to achieve full transformation: a "growth regulatory pathway" dependent on $p21^{ras}$ (Smith *et al.*, 1986; Stacey *et al.*, 1991; for which SHC may be the relevant target; McGlade *et al.*, 1992; Verderame *et al.*, 1995) and a "morphological pathway." In rat cells, both pathways require a functional SH2 capable of binding low-affinity peptides (i.e., an SH2 domain that functions reasonably well). In contrast, chicken cells do not require SH2 to activate the growth pathway, and the morphological pathway requires an SH2 that binds at least high-affinity peptides (i.e., an SH2 domain that retains at least some function). It is plausible that host-dependent mutants detect the evolutionary distance between chicken and rat; because v-*src* is ultimately derived from chicken c-*src*, mutant versions may lose the ability to interact with rat substrates more quickly than they lose the ability to interact with chicken substrates. In support of the idea that two pathways may be triggered by $pp60^{v\text{-}src}$, two v-*src* alleles incapable of transformation individually can transform rat cells when coexpressed (my unpublished observations).

The ability of the SH2 domain to bind low- and high-affinity phosphopeptides predicts the ability to transform rat cells and the morphology of transformed chicken cells, respectively. The challenge ahead is to use this information to focus searches for the relevant substrates of $pp60^{v\text{-}src}$.

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