Transformation and pp60^{v-src} Autophosphorylation Correlate with SHC-GRB2 Complex Formation in Rat and Chicken Cells Expressing Host-Range and Kinase-active, Transformation-defective Alleles of *v-src*

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The biochemical properties of several pp60^{v-src} substrates believed to participate in *src*-mediated transformation were examined in cells expressing a kinase-active, transformation-defective *v-src* allele (*v-src*-F172Δ/Y416F) and its parental allele, *v-src*-F172Δ, a host-range-dependent allele that transforms chicken cells to a fusiform morphology, but does not transform rat cells. Because pp60^{v-src} is dependent on autophosphorylation for transforming ability, these alleles provide a unique opportunity to examine the role of pp60^{v-src} autophosphorylation in regulating substrate interactions. Increased pp125^{FAK} tyrosine phosphorylation and high levels of pp60^{v-src}-associated phosphatidylinositol-3' kinase activity were detected specifically in chicken cells exhibiting round, refractile transformation but not in cells transformed to a fusiform morphology. Increased pp125^{FAK} kinase activity, but not increased pp125^{FAK} tyrosine-phosphorylation correlated with pp60^{v-src} autophosphorylation and increased anchorage-independent growth. Thus, pp125^{FAK} and PI3'K may participate in morphological transformation by *v-src*. Furthermore, association of phosphorylated SHC with the adapter GRB2 correlated with increased anchorage-independent growth (and autophosphorylation) in both rat and chicken cells independent of the morphological phenotype induced. Therefore, host-range dependence for transformation may be regulated through association of SHC with GRB2, thus implicating SHC as a crucial substrate for *src*-dependent transformation.

INTRODUCTION

The mechanism by which the protein tyrosine kinase (PTK)¹ pp60^{v-src} transforms cells into a neoplastic state has not been elucidated despite intensive studies (reviewed in Parsons and Weber, 1989). A large number of cellular proteins, both structural and enzymatic, are

phosphorylated on tyrosine in response to pp60^{v-src}, and many of these proteins have been suggested to participate in the process of transformation.

Previous studies in our laboratory identified a series of *v-src* alleles that transform both chicken and rat cells (wild-type [wt] *v-src* and *v-src*-Y416F), transform only chicken cells but not rat cells (*v-src*-F172Δ) (Var-mus *et al.*, 1981; Verderame *et al.*, 1989), or cannot transform either chicken or rat cells (*v-src*-F172Δ/Y416F) (Woods and Verderame, 1994). Unlike wt *v-src* or *v-src*-Y416F, which both transform chicken cells to a round refractile morphology, *v-src*-F172Δ transforms chicken cells to a fusiform morphology. F172, a con-

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¹ Abbreviations used: F, phenylalanine; PAGE, polyacrylamide gel electrophoresis; PI3'K, phosphatidylinositol-3' kinase; P-Tyr, phosphotyrosine; SDS, sodium dodecyl sulfate; SH2, *src* homology region 2; PTK, protein tyrosine kinase; Y, tyrosine.

served residue found in all but one known *src*-homology-2 (SH2) domain (Koch *et al.*, 1991), is predicted to be required for proper folding of the SH2 domain but not to have a direct interaction with phosphotyrosine (P-Tyr)-containing peptides (Waksman *et al.*, 1992). Each of these alleles encodes a protein that retains kinase activity, although the transformation-defective allele *v-src*-F172Δ/Y416F has significantly less activity than wt (Woods and Verderame, 1994). The ability of mutant *v-src* alleles to break correlations between phosphorylation of a particular substrate and transforming ability is a well established method of diverting attention away from irrelevant substrates and maintaining a focus on potentially relevant substrates (Parsons and Weber, 1989).

This report describes the biochemical analysis of several potentially relevant pp60^{v-src} substrates in both rat and chicken cells expressing host-range or transformation-defective *v-src* alleles. Because there is strong evidence that *src*-dependent transformation requires p21^{ras} activity (Smith *et al.*, 1986), two regulators of p21^{ras} were examined in the present study. The phosphorylation state of p42^{SHC}, p56^{SHC}, and p66^{SHC} (collectively referred to as SHC, an upstream activator of p21^{ras}) and p120^{ras}-GAP (a negative regulator of p21^{ras}) were examined; proteins that physically associate with SHC and p120^{ras}-GAP were also examined (p21^{ras} activation is reviewed in Pronk and Bos, 1994). Because many downstream effectors in signaling pathways bind stably to their upstream activators, two substrates that form stable complexes with pp60^{v-src} were also examined: pp125^{FAK} (Xing *et al.*, 1994) and phosphatidylinositol-3'-kinase (PI3'K; Fukui and Hanafusa, 1989). Finally, the P-Tyr state and subcellular localization of the F-actin binding protein cortactin was determined (Wu *et al.*, 1991). Recent observations that the GRB2/SOS complex is associated with pp125^{FAK} in *v-src*-transformed cells (Schlaepfer *et al.*, 1994), and that PI3'K activity is associated with activated pp125^{FAK} (Chen and Guan, 1994b) highlight the ongoing interest in the role these substrates serve in the regulation of proliferation.

Results presented in this report suggest host-range dependence for transformation is regulated at the level of the SHC/GRB2 interaction, indicating the importance of SHC phosphorylation in *src*-mediated transformation, and they demonstrate a correlation between anchorage-independent growth, increased p125^{FAK} kinase activity, and increased pp60^{v-src}-associated PI3'K activity. Regulation of these various biochemical responses by the pp60^{v-src} SH2 domain, and by pp60^{v-src} autophosphorylation is discussed.

MATERIALS AND METHODS

Cell Culture and *v-src* Alleles

All cell culture, DNA transfections, and viral reverse transcriptase assays were performed as previously described (Verderame *et al.*, 1989; Woods and Verderame, 1994; Verderame and Varmus, 1994). The *v-src* alleles used in this study (wt *v-src*, *v-src*-Y416F, *v-src*-F172Δ, and *v-src*-F172Δ/Y416F) were cloned into the replication competent avian retroviral vector RCAS (Hughes *et al.*, 1987). Rat cell lines Rat-1, B31, and L (expressing no *v-src*, wt *v-src*, or *v-src*-F172Δ, respectively) have been previously described (Oppermann *et al.*, 1981; Varmus *et al.*, 1981; Verderame *et al.*, 1989). Rat-1 and L have normal growth properties, whereas B31 is transformed by all parameters examined.

Immunoprecipitations

Immunoprecipitations for p120^{ras}-GAP (Pronk *et al.*, 1992), cortactin (Wu *et al.*, 1991), pp60^{v-src} (Parker *et al.*, 1984), and pp125^{FAK} (Guan and Shalloway, 1992) were performed as described previously using the following monoclonal antibodies: B4F8 for p120^{ras}-GAP (Santa Cruz Biotechnology, Santa Cruz, CA); 4F11 for cortactin (a gift of J. Thomas Parsons, University of Virginia, Charlottesville, VA); monoclonal antibody 127 for pp60^{v-src} (a gift of Joan Brugge, Ariad Pharmaceuticals, Cambridge, MA); and anti-FAK serum for pp125^{FAK} (Xing *et al.*, 1994). SHC and GRB2 were precipitated after cell lysis in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 2 mM EGTA using rabbit polyclonal antiserum #S14630 or mouse monoclonal antibody #G16720, respectively (Transduction Laboratories, Lexington, KY). Although chicken SHC proteins migrate faster than their mammalian counterparts, we have retained the p46^{SHC}, p52^{SHC}, and p66^{SHC} designations (Crowe *et al.*, 1994).

pp125^{FAK} Kinase Assays

Kinase assays were performed as described previously (Guan and Shalloway, 1992). Reaction products were separated on 7.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS). The amount of ³²P transferred to substrate was quantitated by scintillation.

Protein Blots

Proteins from whole cell lysates or from immunoprecipitations were separated on 7.5% or 10% polyacrylamide gels containing SDS and transferred to nitrocellulose. P-Tyr-containing proteins in p120^{ras}-GAP immunoprecipitations were detected by PY20 anti-P-Tyr antibody (ICN Pharmaceuticals, Costa Mesa, CA) followed by alkaline phosphatase-conjugated secondary antibodies. All other antigens were detected with horseradish peroxidase-conjugated secondary antibodies and Renaissance chemiluminescence reagents (DuPont NEN, Boston, MA).

Immunofluorescence

Indirect immunofluorescence for cortactin was performed as described previously (Wu *et al.*, 1991) using monoclonal antibody 4F11. Briefly, cells were fixed in 3% paraformaldehyde and permeabilized in 0.4% Triton X-100, then incubated with 4F11 antibody for 1 h at 25°C. The excess antibody was washed away in phosphate-buffered saline (pH 7.4), and the cells were incubated with fluorescein-conjugated goat anti-mouse IgG antibody. Subcellular localization of cortactin was visualized by fluorescent microscopy using a Nikon Labphot-2 (Melville, NY).

PI3'K Assays

pp60^{v-src} immunoprecipitations were washed as described previously (Verderame *et al.*, 1989) then subjected to PI3'K assays. Immunoprecipitates were resuspended in PI3'K assay buffer (20 mM Tris, pH 7.4; 100 mM NaCl; 10 mM MgCl₂; 0.5 mM EGTA; 120 μ M adenosine). Lipid micelles were prepared by mixing phosphatidylinositol and phosphatidylserine 1:1 (v/v) and drying them in a stream of N₂. The lipids were reconstituted in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, and 1 mM EDTA to 2 μ g/ μ l, and then sonicated for 5–10 s at 25°C using a probe sonicator. Ten micrograms of lipids were added to the immunoprecipitates and the mixture was heated to 30°C. Reactions were started by the addition of 10 μ Ci [γ -³²P]ATP and 100 nM ATP and allowed to proceed for 10 min at 30°C. The reactions were stopped by the addition of 1 N HCl. Reaction products were extracted once in 1:1 (v/v) CHCl₃:methanol, once in 1 N HCl, and were separated on silica plates as previously described (Walsh *et al.*, 1991). The amount of ³²P incorporated into phosphatidylinositol phosphate was quantitated using a Betascope 603 analyzer (Betagen, Waltham, MA). PI3'K directly immunoprecipitated from parallel lysates using rabbit polyclonal antibody #P13030 (Transduction Laboratories, Lexington, KY) was also subjected to a PI3'K assay reaction to provide a standard for phosphatidylinositol phosphorylated on the 3' position.

RESULTS

p21^{ras}-Regulators: Phosphorylation State and Associated Proteins

SHC. Because SHC is phosphorylated on tyrosine in cells transformed by pp60^{v-src} (McGlade *et al.*, 1992), and is an upstream activator of p21^{ras}, (reviewed in Pronk and Bos, 1994), the phosphorylation state of SHC was examined in chicken and rat cells expressing various mutant *v-src* alleles. In chicken cells (Figure 1A), p56^{SHC} is highly phosphorylated on tyrosine whenever a kinase-active allele of *v-src* is expressed regardless of whether the cells are transformed (wt *v-src*, *v-src*-Y416F, or *v-src*-F172Δ) or not (*v-src*-F172Δ/Y416F). Increased phosphorylation of p56^{SHC} is revealed by reduced mobility in SDS-PAGE and by anti-P-Tyr probing of the SHC immunoprecipitations. The level of tyrosine phosphorylation on p56^{SHC} is only slightly reduced in nontransformed chicken cells expressing *v-src*-F172Δ/Y416F relative to chicken cells transformed by wt *v-src* even though the specific kinase activity of pp60^{v-src}-F172Δ/Y416F is almost 20-fold lower than wt. The phosphorylation status of p66^{SHC} is less clear. Protein blots probed with anti-SHC antibodies reveal that p66^{SHC} steady state levels apparently are reduced in cells expressing a kinase active *v-src*. This apparent decrease is accompanied by the appearance of a novel band detectable by anti-SHC antibodies (indicated in Figure 1A); this novel band is highly phosphorylated on tyrosine and could represent a phosphorylated form of p66^{SHC} with substantially altered mobility. p42^{SHC} tyrosine phosphorylation is not dramatically altered in any of the cells.

Because tyrosine phosphorylation of SHC can create a binding site for GRB2 (Salcini *et al.*, 1994), the

amount of GRB2 in the SHC immunoprecipitates was determined (Figure 1A). GRB2 associated with SHC is easily detectable in cells expressing any of the transforming alleles of *v-src*, but is only barely detectable in cells expressing the transformation-defective allele *v-src*-F172Δ/Y416F. It is noteworthy that the host-range allele *v-src*-F172Δ induces SHC phosphorylation and GRB2 association indistinguishable from that induced by *v-src*-Y416F; both of these alleles have specific kinase activity about 30% of wt *v-src* (Woods and Verderame, 1994), but only *v-src*-Y416F induces full morphological transformation.

SHC was also immunoprecipitated from rat cells expressing wt *v-src* or *v-src*-F172Δ (which does not transform rat cells) and was similarly analyzed for tyrosine phosphorylation and GRB2 association. As expected, p56^{SHC} phosphorylated was readily detectable in cells expressing wt *v-src*, indicated by the decreased mobility and directly revealed by anti-P-Tyr antibodies (Figure 1B). p66^{SHC} was also phosphorylated on tyrosine in transformed rat cells. The level of P-Tyr on p46^{SHC} was not altered substantially in either rat or chicken cells expressing any *v-src* allele. SHC immunoprecipitates from rat cells expressing host-range *v-src*-F172Δ (which does not transform rat cells) had greatly reduced levels of GRB2 relative to SHC immunoprecipitates from rat cells transformed by wt *v-src*.

To confirm these results, GRB2 immunoprecipitates from rat cells were probed with anti-P-Tyr and anti-SHC antibodies (Figure 2). Tyrosine-phosphorylated p56^{SHC} and p66^{SHC} are specifically detectable in the GRB2 immunoprecipitates from rat cells transformed by wt *v-src*. In contrast, tyrosine-phosphorylated p42^{SHC} is present in GRB2 immunoprecipitates from all rat cell lines.

In summary, the level of GRB2 association with tyrosine-phosphorylated SHC correlated with *v-src*-dependent transformation in both chicken and rat cells, but the level of SHC tyrosine phosphorylation did not; the host-range allele *v-src*-F172Δ is particularly revealing in this regard.

p120^{ras-GAP}. Because p120^{ras-GAP} is an in vitro substrate of pp60^{v-src} and may be involved in pp60^{v-src} transformation (Pronk and Bos, 1994), the P-Tyr content of p120^{ras-GAP} in the presence of transforming and nontransforming *v-src* alleles was also studied. Protein blots of p120^{ras-GAP}-immunoprecipitates from chicken cells (Figure 3A) and rat cells (Figure 3B) expressing various *v-src* mutants were probed with anti-P-Tyr antibodies. The P-Tyr content of the precipitated proteins was compared with the steady-state amount of p120^{ras-GAP}. (We have consistently detected increased steady state levels of p120^{ras-GAP} in chicken cells expressing any *v-src* gene compared with vector-only control cells. p120^{ras-GAP} immunoprecipitations

Figure 1. Tyrosine phosphorylation of SHC proteins. SHC proteins were immunoprecipitated from whole cell lysates of chicken cells (A) or rat cells (B) expressing various *v-src* alleles; proteins from whole cell lysates or from anti-SHC immunoprecipitates were separated on polyacrylamide gels containing SDS and blotted to nitrocellulose. Proteins were detected by anti-SHC, anti-P-Tyr, or anti-GRB2 antibodies as indicated. RCAS, chicken cells infected with vector only; "—", control rat cells.

were performed in antigen excess so that each immunoprecipitation had equal amounts of p120^{ras-GAP}. Thus, the P-Tyr content could be compared directly). In chicken cells, no difference in the P-Tyr content of

p120^{ras-GAP} was detected between cells expressing nontransforming *v-src*-F172Δ/Y416F compared with cells expressing transforming alleles *v-src*-Y416F or *v-src*-F172Δ; the P-Tyr content of p120^{ras-GAP} from cells

Figure 2. Detection of SHC in GRB2 immunoprecipitates. GRB2 was immunoprecipitated from whole cell lysates of chicken cells expressing various *v-src* alleles; proteins from whole cell lysates or from anti-GRB2 immunoprecipitates were separated on polyacrylamide gels containing SDS and blotted to nitrocellulose. Proteins were detected by anti-SHC, anti-P-Tyr, or anti-GRB2 antibodies as indicated. RCAS, chicken cells infected with vector only.

expressing wt *v-src* was slightly higher. The identity of the P-Tyr-containing protein migrating more slowly than p120^{ras-GAP} was confirmed to be p120^{ras-GAP}-associated p190 by reprobing the anti-P-Tyr blot with an anti-p190 antibody; as expected (given the results of Moran *et al.*, 1991), p190 was co-precipitated only when p120^{ras-GAP} was tyrosine phosphorylated. Like p120^{ras-GAP}, p190 tyrosine phosphorylation did not correlate with transformation by *v-src*. Anti-p62 antibodies were not available to determine the amount of this protein associated with p120^{ras-GAP} in chicken cells, however, p62 phosphorylation levels were similar in cells expressing kinase-active *v-src* alleles, regardless of the transformed phenotype. When p120^{ras-GAP} was immunoprecipitated from rat cells expressing *v-src*-F172Δ, P-Tyr was undetectable. Also, p62 and p190 phosphorylation levels were greatly reduced relative to proteins from rat cells transformed by wt *v-src*. Thus, tyrosine phosphorylation of p120^{ras-GAP}, p190, or p62 is not sufficient for *v-src*-mediated transformation.

Phosphorylation and Kinase Activity of pp125^{FAK}

pp125^{FAK} tyrosine kinase activity is increased in response to a variety of stimuli including transformation by *v-src* (Guan and Shalloway, 1992). Recently it has been shown that autophosphorylation of pp125^{FAK} creates a high affinity binding site for the pp60^{src} SH2 domain, and that pp60^{src} interacts with pp125^{FAK} via

this site (Schaller *et al.*, 1994). These data suggest that pp60^{src} and pp125^{FAK} may cooperate in *v-src* transformation. Accordingly, both pp125^{FAK} P-Tyr content and kinase activity were examined. Because pp125^{FAK} P-Tyr content and kinase activity increase in response to cells binding to fibronectin (Guan and Shalloway, 1992), these properties were studied in both attached and suspended cells. pp125^{FAK} P-Tyr increased in cells expressing wt *v-src* or *v-src*-Y416F compared with vector-only controls. Concomitantly, a slower migrating form of pp125^{FAK} appeared (Figure 4A). Compared with vector-only controls, pp125^{FAK} tyrosine phosphorylation was not increased in cells expressing either *v-src*-F172Δ or *v-src*-F172Δ/Y416F.

Surprisingly, compared with control cells a reproducible threefold increase in pp125^{FAK} kinase activity was detected in cells transformed to a fusiform morphology by *v-src*-F172Δ (Figure 4B; values representing the average of three experiments are given in Table 1) in the absence of a detectable increase in tyrosine phosphorylation of pp125^{FAK} (Figure 4A). Cells transformed to a round morphology by wt *v-src* or *v-src*-Y416F had a twofold increase in kinase activity concomitant with increased p125^{FAK} phosphorylation. All increases in kinase activity were observed in both attached and suspended cells. In contrast, pp125^{FAK} kinase activity from cells expressing transformation-defective *v-src*-F172Δ/Y416F was indistinguishable from that found in vector-only control cells.

In summary, increased pp125^{FAK} kinase activity can be dissociated from increased pp125^{FAK} tyrosine phosphorylation. Furthermore, increased p125^{FAK} kinase activity correlates with anchorage-independent growth, whereas increased tyrosine phosphorylation of pp125^{FAK} correlates with round, refractile morphological transformation.

Phosphorylation and Subcellular Localization of Cortactin

Given the intriguing results with the focal adhesion kinase, the phosphorylation state of the pp60^{v-src} substrate cortactin was examined. Cortactin is physically associated with the actin cytoskeleton (Wu and Parsons, 1993) and is phosphorylated primarily on serine and threonine residues in normal cells but becomes phosphorylated on tyrosine in cells transformed by pp60^{c-src}-Y527F (Wu *et al.*, 1991). The level of tyrosine phosphorylation on cortactin was high in chicken cells expressing wt *v-src* and *v-src*-Y416F, significantly reduced in cells expressing *v-src*-F172Δ, and virtually undetectable in nontransformed cells expressing *v-src*-F172Δ/Y416F (Figure 5A). In rat cells, cortactin was tyrosine phosphorylated in cells expressing wt *v-src*, and not in nontransformed cells expressing *v-src*-F172Δ.

Figure 3. Tyrosine phosphorylation state of p120^{ras-GAP} and p120^{ras-GAP}-associated proteins. GAP was immunoprecipitated from whole cell lysates of chicken cells (A) or rat cells (B) expressing various *v-src* alleles using B4F8 anti-p120^{ras-GAP} antibody. Proteins from whole cell lysates or from anti-p120^{ras-GAP} immunoprecipitates, as indicated, were separated on 7.5% polyacrylamide gels containing SDS and blotted to nitrocellulose. Proteins were detected by anti-p120^{ras-GAP}, anti-p190, or anti-P-Tyr antibodies as indicated. Representative blots are shown. The protein indicated by the question mark is currently unidentified. RCAS, chicken cells infected with vector only; “—”, control rat cells.

In addition to tyrosine phosphorylation, subcellular localization of cortactin may be relevant to its role in transformation by *v-src*. Cortactin is normally associated with actin filaments but in cells transformed by wt *v-src*, it is relocalized to large “podosome-like” patches (Wu *et al.*, 1991). As expected, cortactin was localized to large patches in chicken cells expressing any of the transforming *v-src* alleles (wt *v-src*, *v-src*-F172Δ, or *v-src*-Y416F; Figure 6). In contrast, localization of cortactin in cells expressing the nontransforming allele *v-src*-F172Δ/Y416F was identical to its localization in mock or vector alone-transfected cells. In nontransformed rat cells expressing *v-src*-F172Δ, cortactin localization was indistinguishable from control Rat-1 cells, whereas cortactin in rat cells transformed by wt *v-src* relocalized to large patches. Thus expression of *v-src*-F172Δ reveals a correlation between cortactin relocalization, cortactin phosphorylation on tyrosine, and *v-src* transformation.

pp60^{v-src}-associated PI3'K Activity

All transforming alleles of *v-src* encode proteins that associate with PI3'K activity (Fukui and Hanafusa, 1989, 1991). Because studies using inhibitors of pp60^{v-src} activity imply pp60^{v-src}-associated PI3'K activity may be essential for *v-src* transformation (Fukui and Hanafusa, 1989; Hamaguchi *et al.*, 1993),

levels of pp60^{v-src}-associated PI3'K activity were determined (Figure 7, values for the averages of three experiments are given in Table 1). Approximately six- to ninefold more PI3'K activity was associated with immunoprecipitated pp60^{v-src} from chicken cells transformed to a round, refractile morphology (wt pp60^{v-src} and pp60^{v-src}-Y416F) than with pp60^{v-src} from cells transformed to a fusiform morphology (pp60^{v-src}-F172Δ), or nontransformed cells (pp60^{v-src}-F172Δ/Y416F). Thus round, refractile morphological transformation correlates with high levels of pp60^{v-src}-associated PI3'K activity. Despite repeated efforts, PI3'K activity could not be reliably detected in association with wt pp60^{v-src} immunoprecipitated from rat cells (Verderame, unpublished results).

DISCUSSION

Since the discovery that pp60^{v-src} phosphorylates cellular proteins on tyrosine, the driving hypothesis has been that pp60^{v-src} transforms cells by directly phosphorylating a critical cellular substrate(s), thereby initiating a signal transduction cascade that eventually alters the growth regulatory apparatus of the cell. Despite extensive searches, this critical cellular substrate(s) has been elusive. Biochemical analysis of candidate substrates in cells transformed by mutant *v-src* alleles that induce partial transformed phenotypes, or in cells

Figure 4. (A) Tyrosine phosphorylation of pp125^{FAK}. Chicken cells expressing various *v-src* alleles were lysed after removal from the culture dish by trypsin treatment (suspended) or while still attached to the culture plate (attached). pp125^{FAK} was immunoprecipitated from cell lysates using anti-FAK serum. Immunoprecipitated proteins were separated on 7.5% polyacrylamide gels containing SDS and blotted to nitrocellulose. Proteins were detected by anti-FAK serum or anti-P-Tyr antibodies. The experiment was performed three times with similar results; a representative experiment is shown. (B) pp125^{FAK} kinase activity. Chicken cells expressing various *v-src* alleles were lysed after removal from the culture dish by trypsin treatment (suspended) or while still attached to the culture plate (attached). pp125^{FAK} was immunoprecipitated from these lysates and immune complex kinase assays were performed using E₄Y as the substrate. Reaction products were separated on 7.5% polyacrylamide gels containing SDS and the amount of ³²P incorporation was quantitated by scintillation counting. The experiment was performed three times; bars indicate standard error. The kinase activity of pp125^{FAK} from cells transformed by pp60^{v-src-F172Δ} is significantly different from control uninfected cells ($p < 0.05$, Student's *t*-test). RCAS, chicken cells infected with vector only.

transformed by kinase-active but transformation-defective alleles of *v-src* has eliminated several potentially critical substrates from consideration (Parsons and Weber, 1989). The series of wt-transforming, host-range, and transformation-defective *v-src* alleles used in this study provides a unique opportunity to explore the relevance of several putative pp60^{v-src} substrates to *src*-mediated transformation because the SH2 domain mutant *v-src*-F172Δ (unlike wt pp60^{v-src}) transforms chicken but not rat cells. Additionally, transformation by this allele is dependent on autophosphorylation, thus providing an opportunity to examine the interplay of the SH2 domain, autophosphorylation, and substrate interactions. Results from this study are summarized in Table 1.

The p21^{ras} Pathway

One biochemical pathway crucial for *src*-dependent transformation includes p21^{ras}; microinjection of neutralizing anti-p21^{ras} antibodies into cells transformed by pp60^{v-src} reverses the transformed phenotype (Smith *et al.*, 1986), which indicates p21^{ras} acts downstream of pp60^{v-src}. Both positive-acting upstream activators (SHC), and downstream negative regulators (the *ras*-GTPase activating protein p120^{ras-GAP} (reviewed in Pronk and Bos, 1994) are phosphorylated in *v-src*-transformed cells. SHC tyrosine phosphoryla-

tion recruits the SH2-containing adapter protein GRB2 into a complex with SHC (Rozakis-Adcock *et al.*, 1992). GRB2 is complexed to the guanine nucleotide exchange factor SOS, the immediate upstream activator of p21^{ras} (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Thus SHC phosphorylation indirectly activates p21^{ras}.

Results of this study are consistent with the important role SHC-phosphorylation serves in activating p21^{ras}. GRB2 was abundantly associated with phosphorylated SHC only in cells transformed by *v-src* (independent of the morphological change induced or the level of SHC phosphorylation). Results with *v-src*-F172Δ particularly strengthen this correlation; GRB2 abundantly associates with SHC in chicken cells transformed by *v-src*-F172Δ, but it is only barely detectable in rat cells that are not transformed by the same allele. SHC-dependent activation of p21^{ras} requires GRB2 association (Rozakis-Adcock *et al.*, 1992), and p21^{ras} is required for *v-src*-dependent transformation (Smith *et al.*, 1986). Accordingly, results presented here are sufficient to explain host-dependent transformation by *v-src*-F172Δ, and identify SHC as a critical substrate for *v-src*-dependent transformation.

Figure 5. Tyrosine phosphorylation of cortactin. Cortactin was immunoprecipitated from whole cell lysates of chicken cells (A) or rat cells (B) expressing various *v-src* alleles using 4F11 anti-cortactin antibody. Proteins were separated on 7.5% polyacrylamide gels containing SDS and blotted to nitrocellulose. Proteins were detected by anti-cortactin or anti-P-Tyr antibodies as indicated. Representative blots are shown. RCAS, chicken cells infected with vector only; “—”, control rat cells.

The biochemical basis for the differing SHC-GRB2 interaction between species is not clear. In particular, SHC was phosphorylated in chicken cells expressing the nontransforming allele *v-src*-F172Δ/Y416F. SHC is known to be phosphorylated on multiple tyrosines in response to epidermal growth factor stimulation (Salcini *et al.*, 1994), and presumably in *v-src*-transformed cells as well. pp60^{*v-src*}-F172Δ/Y416F apparently cannot mediate the stable phosphorylation of the GRB2 binding site on SHC (presumably the Y317 homologue; Salcini *et al.*, 1994). Phosphorylation of the GRB2 binding site in chicken SHC requires either pp60^{*v-src*} autophosphorylation or an intact pp60^{*v-src*} SH2 domain; *v-src* alleles that retain one of these two features (*v-src*-F172Δ or *v-src*-Y416F, respectively) are still transforming. This hypothesis is consistent with the observation that rat cells are not transformed by the SH2 domain mutant pp60^{*v-src*}-F172Δ, which is not autophosphorylated in rat cells (Oppermann *et al.*, 1981; Verderame *et al.*, 1989). A requirement for autophosphorylation or an intact SH2 domain could reflect an altered substrate specificity of autophosphorylated pp60^{*v-src*}, decreased protection of the relevant SHC P-Tyr from phosphatase activity by the

src SH2 domain, or a combination of these effects. An alternative possibility is that the SH2 domain and the autophosphorylation site participate in recruiting relevant proteins for phosphorylation by pp60^{*v-src*}. These possibilities are obviously not mutually exclusive (see below). We have recently cloned the chicken homologue of SHC (Verderame, unpublished results) to address these questions.

p21^{*ras*} is negatively regulated by p120^{*ras*-GAP} (Trahey and McCormick, 1987; Vogel *et al.*, 1988; reviewed in Pronk and Bos, 1994). p120^{*ras*-GAP} is weakly bound to and phosphorylated by pp60^{*v-src*} (Brott *et al.*, 1991a,b). The role of tyrosine phosphorylation of p120^{*ras*-GAP} in neoplastic transformation is controversial (Pronk and Bos, 1994). Results presented here indicate that p120^{*ras*-GAP} tyrosine phosphorylation does not correlate with *v-src* transformation. Given recent evidence that p120^{*ras*-GAP} is not the primary effector of p21^{*ras*} (Stone *et al.*, 1993), p120^{*ras*-GAP} may simply be an “innocent bystander,” subject to phosphorylation along with many other cellular proteins in the presence of pp60^{*v-src*}. If so, then phosphorylation of p120^{*ras*-GAP} would be irrelevant for *src*-mediated transformation.

Figure 6. Indirect immunofluorescence of cortactin. Chicken and rat cells expressing *v-src* alleles were fixed in 3% paraformaldehyde and permeabilized with 0.4% Triton X-100. Cortactin was detected by indirect immunofluorescence with 4F11 anti-cortactin antibody. Cells representing the cortactin localization in the majority of cells in the given population are shown. The reason for brighter punctate staining in rat cells expressing *v-src*-F172Δ compared with control rat cells is unknown. RCAS, chicken cells infected with vector only.

pp125^{FAK}

A second protein implicated in *src*-mediated signal transduction is *pp125^{FAK}*, a nonreceptor-PTK localized in focal adhesions (Hanks *et al.*, 1992; Schaller *et al.*, 1992), where wt *pp60^{v-src}* also concentrates (Rohrschneider, 1980). *pp125^{FAK}* tyrosine phosphorylation increases upon transformation by several PTKs (e.g. *pp60^{v-src}* [Guan and Shalloway, 1992] and *pp90^{gag-yes}* [Kanner *et al.*, 1990]), upon cellular attachment to extracellular matrix components (e.g. laminin, collagen type IV, vitronectin [Burridge *et al.*, 1992; Hanks *et al.*, 1992], or fibronectin [Guan *et al.*, 1991; Kornberg *et al.*, 1991; Guan and Shalloway, 1992; Hanks *et al.*, 1992]), or by treatment with a wide variety of hormones including bradykinin or bombesin (Leeb-Lundberg and Song, 1993), vasopressin or endothelin (Zachary *et*

al., 1992), and cholecystokinin-B/gastrin (Taniguchi *et al.*, 1994). These results suggest that *pp125^{FAK}* is a point of convergence for a variety of extracellular signals (Zachary and Rozengurt, 1992). In normal cells, *pp125^{FAK}* is believed to serve an intracellular signaling role as well as participating in the organization of new focal adhesions (Burridge *et al.*, 1992). Several *pp125^{FAK}* targets have been identified. GRB2 (Schlaepfer *et al.*, 1994) and PI3'K (Chen and Guan, 1994a,b) physically associate with *pp125^{FAK}* after activation, whereas paxillin (Schaller and Parsons, 1995) and probably tensin (Bockholt and Burridge, 1993) are directly phosphorylated by *pp125^{FAK}*. The functional significance of these interactions is currently unknown, however. In *v-src*-transformed cells, *pp125^{FAK}*-associated kinase activity is increased

Table 1. Summary of biochemical properties of chicken and rat cells expressing wt and mutant *v-src* alleles

	AIG ^a	Morph ^b	SHC ^c		<i>ras</i> -GAP ^d	pp125 ^{fak e}		Cortactin ^f		P13'KA ^g
			P-tyr	GRB2		P-tyr	KA	P-tyr	IIF	
Transformed ^h										
Chick										
<i>v-src</i>	+	round	+	+	+	+	2.6 (.7)	++	patchy	1.00
<i>v-src</i> -Y416F	+	round	+	+	+	+	2.9 (.8)	++	patchy	.97 (.22)
<i>v-src</i> -F172Δ	+	fusiform	+	+	+	–	3.0 (.8)	+	patchy	.16 (.02)
Rat										
<i>v-src</i>	+	round	+	+	+	nd ⁱ	nd	++	patchy	nd
Nontransformed ^j										
Chick										
<i>v-src</i> -F172Δ/Y416F	–	flat	+	–	+	–	1.1 (.1)	–	diffuse	.11 (.02)
Rat										
<i>v-src</i> -F172Δ	–	flat	–	–	–	nd	nd	–	diffuse	nd

^a Anchorage-independent growth ability (Verderame *et al.*, 1989; Woods and Verderame, 1994).

^b Morphology of cells expressing indicated *v-src* allele (Verderame *et al.*, 1989; Woods and Verderame, 1994).

^c Analysis of tyrosine phosphorylation state of SHC (P-tyr) or association of GRB2 with SHC (GRB2).

^d Tyrosine phosphorylation state of p120^{ras-GAP}.

^e Analysis of tyrosine phosphorylation state (P-tyr) and pp125^{FAK}-associated kinase activity (KA). Kinase activity is expressed relative to control uninfected chicken cells. Kinase activity is expressed relative to control (vector only) cells (average of three experiments; value in parentheses is standard error).

^f Tyrosine phosphorylation state (P-tyr) and subcellular distribution determined by indirect immunofluorescence (IIF) of cortactin.

^g pp60^{v-src}-associated P13K activity. Kinase activity is expressed relative to wt pp60^{v-src} (average of three experiments; value in parentheses is standard error).

^h Cells transformed as a result of expression by the indicated *v-src* allele.

ⁱ Not determined.

^j Cells not transformed by the expression of the indicated *v-src* allele.

(Schaller *et al.*, 1992). This increase could be due to either increased pp125^{FAK} kinase activity, or associated pp60^{v-src} kinase activity.

The data presented here indicate that increased pp125^{FAK} kinase activity correlates with *v-src*-mediated transformation even though tyrosine-phosphorylation of pp125^{FAK} was not concomitantly increased in all cases. Apparently pp60^{v-src} can activate pp125^{FAK} by a means other than detectably increasing pp125^{FAK} tyrosine phosphorylation. Increased P-Tyr of pp125^{FAK} in cells transformed by wt pp60^{v-src} is due predominantly to autophosphorylation of Y397 on pp125^{FAK} (Cobb *et al.*, 1994; Schaller *et al.*, 1994). Other pp125^{FAK} tyrosines can be phosphorylated *in vitro* by pp60^{v-src} and are phosphorylated *in vivo* in cells expressing pp60^{v-src} (Calalb *et al.*, 1995) resulting in a twofold increase in pp125^{FAK} kinase activity. What are the roles of these phosphorylated tyrosines in *src*-mediated transformation? Phosphorylation of Y397 on pp125^{FAK} creates a high affinity binding site for the pp60^{v-src} SH2 domain (phospho-YAEI) (Schaller *et al.*, 1994) that is the first reported naturally occurring high affinity binding site identified for the SH2 domain of pp60^{v-src} (Songyang *et al.*, 1993). Wt pp60^{v-src} is known to bind autophosphorylated pp125^{FAK} via the pp60^{v-src} SH2 domain (Cobb *et al.*, 1994). The SH2 domain of

pp60^{v-src}-F172Δ cannot bind phosphorylated low affinity phosphopeptides (Woods and Verderame, 1994) or high affinity phosphopeptides (Verderame, unpublished results) and thus is predicted to be unable to bind autophosphorylated pp125^{FAK}.

The loss of the pp60^{v-src}-pp125^{FAK} interaction could provide an explanation for the lack of increased pp125^{FAK} phosphorylation in cells transformed by the host-range mutant *v-src*-F172Δ. In these cells, decreased levels of P-Tyr may be a consequence of the failure of the pp60^{v-src}-F172Δ to protect phosphorylated Y397 from the action of cellular P-Tyr phosphatases; the pp60^{v-src} SH2 domain can protect phosphorylated Y397 from dephosphorylation *in vitro* (Cobb *et al.*, 1994). Failure of pp60^{v-src} to bind to pp125^{FAK} may have secondary consequences as well. Sites phosphorylated directly by pp60^{v-src} (Calalb *et al.*, 1995) may not be phosphorylated efficiently if pp60^{v-src} does not bind pp125^{FAK}. In addition to increasing pp125^{FAK} kinase activity, these P-tyr may allow other signaling molecules to interact with pp125^{FAK} (e.g. GRB2 [Schlaepfer *et al.*, 1994] or PI3'K [Chen and Guan, 1994a,b]).

A second function of the pp60^{v-src}-pp125^{FAK} interaction may be the precise localization of membrane-bound pp60^{v-src} to focal adhesions. Support for this idea derives from the observation that pp60^{v-src}-F172Δ,

Figure 7. pp60^{v-src}-associated PI3'K activity. pp60^{v-src} was immunoprecipitated from chicken cell lysates with monoclonal antibody 127. After washing, the immunoprecipitates were divided in half; one half was analyzed for pp60^{v-src} levels by protein blot, and the other half was subjected to PI3'K assay as described in MATERIALS AND METHODS. Extracted lipids were separated on silica plates in the presence of boric acid. Phosphorylated lipids were visualized by autoradiography. The amount of ³²P incorporation into the lipids was quantitated with the Betascope 603 analyzer. This experiment was repeated three times with similar results; a representative experiment is shown. RCAS, chicken cells infected with vector only.

whose SH2 domain cannot bind P-Tyr (Woods and Verderame, 1994), is not concentrated in focal adhesions (Rohrschneider and Reynolds, 1985). Of the transforming pp60^{v-src} proteins whose subcellular localization has been determined by indirect immunofluorescence, five different mutant alleles, including *v-src*-F172Δ, encode proteins that do not concentrate in focal adhesions (Rohrschneider and Reynolds, 1985) and each of these five alleles induces a fusiform morphology. Many fusiform mutants (like *v-src*-F172Δ) map to SH2. Targeting of pp60^{v-src} to focal adhesions may be required for phosphorylation of focal adhesion or cytoskeletal components that regulate cellular morphology; however, targeting of pp60^{v-src} to focal adhesions may not be sufficient for full morphological transformation (Liebl and Martin, 1992).

Cortactin

Signal transduction molecules are not the only targets of pp60^{v-src}. Actin cytoskeletal organization is usually disrupted in neoplastically transformed cells, and a number of cytoskeleton-associated proteins are phosphorylated on tyrosine in pp60^{v-src}-transformed cells (Kellie *et al.*, 1991). Cortactin is an actin-associated cytoskeletal protein (Wu and Parsons, 1993) that is phosphorylated primarily on serine and threonine residues in normal cells but becomes phosphorylated on tyrosine in cells expressing transforming variants of

c-src ("activated" *c-src*) (Wu *et al.*, 1991). Tyrosine phosphorylation of cortactin is apparently not sufficient to mediate *v-src* transformation, however, because cortactin is phosphorylated on tyrosine in cells expressing nonmyristylated, activated pp60^{c-src} (Wu *et al.*, 1991). In cells transformed by activated pp60^{c-src}, cortactin relocates from along F-actin filaments to podosomes (Wu *et al.*, 1991; Wu and Parsons, 1993).

Results presented here show that cortactin phosphorylation is reduced in chicken cells transformed to a fusiform morphology, and virtually undetectable in rat or chicken cells expressing nontransforming alleles of *v-src*. In all cells expressing transforming alleles of *v-src*, including chicken cells with reduced tyrosine phosphorylation and fusiform morphology, cortactin is relocalized to focal adhesion-like structures ("podosomes"; Wu *et al.*, 1991; Wu and Parsons, 1993). Both cortactin relocalization (this study) and actin cytoskeletal disruption (Verderame *et al.*, 1989; Woods and Verderame, 1994) correlate with *v-src*-mediated transformation. Further studies will be required to determine whether cortactin relocalization is a cause or a consequence of the disrupted actin cytoskeleton seen in *v-src*-transformed cells.

PI3'K

Cells transformed by *v-src* or *src*-related kinases (*v-yes* and *v-fps*) contain elevated levels of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-trisphosphate (Fukui *et al.*, 1991), suggesting that these PTKs alter the regulation of PI3'K activity (see Fry, 1994; Kapeller and Cantley, 1994 for a review of PI3'K). Phosphatidylinositol-3,4-bisphosphate can serve as a potent activator of protein kinase C-δ, -ε, and -η (Toker *et al.*, 1994), which can provide additional downstream signals. Activation of PI3'K occurs by stabilization of an altered conformation induced by upstream signaling proteins binding to the regulatory protein p85 (Panayotou *et al.*, 1992; Pleiman *et al.*, 1994). All 10 of the transforming *src* variants tested by Fukui and Hanafusa associated with PI3'K (Fukui and Hanafusa, 1989). This interaction is not sufficient for transformation, however, because PI3'K has been shown to associate with two transformation-defective proteins: nonmyristylated pp60^{v-src} and kinase-inactive pp60^{v-src} (Fukui and Hanafusa, 1989). Inhibition of *v-src* transformation by herbimycin A inhibited pp60^{v-src} ability to associate with PI3'K (Hamaguchi *et al.*, 1993). Collectively, these data are consistent with the hypothesis that a molecular interaction between pp60^{v-src} and PI3'K is necessary but not sufficient for *v-src* transformation.

The amount of pp60^{v-src}-associated PI3'K activity was decreased approximately sixfold in cells transformed by pp60^{v-src}-F172Δ compared with the wt pp60^{v-src} control, and this value was not further

decreased by blocking pp60^{v-src} autophosphorylation and abolishing transformation. Although the SH3 domain is the primary site of interaction between pp60^{v-src} and the PI3'K p85 regulatory subunit (Liu *et al.*, 1993), the SH2 domain contributes to the formation of the complex (Fukui and Hanafusa, 1991; Liu *et al.*, 1993). The results presented here confirm that observation, because pp60^{v-src}-associated PI3'K activity is reduced but not eliminated in cells transformed by the SH2 mutant allele *v-src-F172Δ*. Clearly the regulation of PI3'K activity by pp60^{v-src} is complex; the recent report suggesting the potential for indirect regulation of PI3'K by pp60^{v-src} (via *ras*) adds to that complexity (Rodriguez-Viciana *et al.*, 1994).

Four other *v-src* alleles inducing fusiform morphology have been examined previously for associated PI3'K activity (Fukui and Hanafusa, 1989, 1991). Like pp60^{v-src-F172Δ}, each of the four proteins associates with less PI3'K activity than wt pp60^{v-src}. One of these mutations effects only amino acids in the SH3 domain, but the other three effect residues exclusively within SH2, again emphasizing the cooperative nature of SH2 and SH3 in binding the regulatory subunit of PI3'K. These results raise the possibility that high levels of associated PI3'K activity are required for full morphological transformation. Although fusiform morphological transformation has been correlated with decreased PI3'K activity (Fukui and Hanafusa, 1989, 1991) and separately with localization of pp60^{v-src} outside of focal adhesions (Rohrschneider and Reynolds, 1985), *v-src-F172Δ* is the first allele whose encoded protein has had both properties measured. Given that localization of pp60^{v-src} to focal adhesions (perhaps via pp125^{FAK} interaction; see above) and high levels of PI3'K-associated activity both correlate with full morphological transformation when tested with a single allele, one can now envision attempts to separately restore one or the other property and determine the effects on morphological transformation. The details of PI3'K activation in *v-src*-transformed cells is further complicated because PI3'K activity is also associated with activated pp125^{FAK} (Chen and Guan, 1994a,b).

In summary, increased association of GRB2 with tyrosine phosphorylated SHC, and not pp60^{v-src} kinase activity, SHC phosphorylation level, or pp60^{v-src}-associated PI3'K activity correlated with anchorage-independent growth in both rat and chicken cells. In chicken cells, the SHC/GRB2 interaction requires either an intact SH2 domain, or autophosphorylation of pp60^{v-src}. Additionally, increased pp125^{FAK} and increased pp60^{v-src}-associated PI3'K activity specifically correlated with round refractile morphological transformation in chicken cells, and requires an intact SH2 domain, but not autophosphorylation.

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