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The Role of the Src Homology Domains in Morphological Transformation by v-*src*

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The Src homology (SH2 and SH3) domains of v-Src are required for transformation of Rat-2 cells and for wild-type (*morph*^{r}) transformation of chicken embryo fibroblasts (CEFs). We report herein that the N-terminal domains of v-Src, when expressed *in trans*, cannot complement the transformation defect of a deletion mutant lacking the "unique," SH3, and SH2 regions. However, the same regions of Src can promote transformation when translocated to the C terminus of v-Src, although the transformation of CEFs is somewhat slower. We conclude that the SH3 and SH2 domains must be present *in cis* to the catalytic domain to promote transformation but that transformation is not dependent on the precise intramolecular location of these domains. In CEFs and in Rat-2 cells, the expression of wild-type v-Src results in tyrosine phosphorylation of proteins that bind to the v-Src SH3 and SH2 domains in vitro; mutations in the SH2 or SH3 and SH2 domains prevent the phosphorylation of these proteins. These findings are most consistent with models in which the SH3 and SH2 domains of v-Src directly or indirectly target the catalytic domain to substrates involved in transformation. However, the N-terminal domains of v-Src can promote tyrosine phosphorylation of certain proteins, in particular p130^{Cas}, even when expressed in the absence of the catalytic domain, indicating that the N-terminal domains of v-Src have effects that are independent of the catalytic domain.

INTRODUCTION

The transforming gene of Rous sarcoma virus v-*src* is a mutationally activated derivative of a cellular progenitor, c-*src* (Takeya and Hanafusa, 1983; Jove and Hanafusa, 1987). It encodes a 60-kDa protein-tyrosine kinase that is localized at the cytoplasmic face of the plasma membrane and is concentrated at sites of cell–matrix adhesion (Rohrschneider, 1980; Krueger *et al.*, 1983; Okamura and Resh, 1994; Resh, 1994). Expression of v-*src* in susceptible avian or mammalian cells leads to transformation in vitro and the acquisition of malignancy in vivo. It is generally believed that malignant transformation results from the constitutive activation of signaling pathways that are subject to physiological regulation by c-Src in normal cells, but proof of this hypothesis is lacking. One of the pathways involved in transformation is the Ras–mitogen-activated protein kinase pathway (Smith *et al.*, 1986; DeClue *et al.*, 1991; Nori *et al.*, 1991), but many other signaling pathways are activated by v-Src and appear to participate in transformation (Erpel and Courtneidge, 1995).

The structural features of Src that are necessary for transformation have been defined by extensive mutational analysis (Jove and Hanafusa, 1987; Parsons and Weber, 1989). The activity of the catalytic domain (residues 270–516) is essential for transformation. The N-terminal half of the protein contains several distinct domains that are also involved in transformation. The first 16 residues promote N-terminal myristoylation and membrane attachment, which are required for transformation (Kamps *et al.*, 1985; Resh, 1990). Also present in the N-terminal half of the molecule are the two "Src-homology" domains, SH3 and SH2, that me-

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diate protein–protein interactions in a wide variety of signaling proteins: the SH3 domain interacts with proline-rich motifs, and the SH2 domain recognizes phosphotyrosine residues within specific sequence contexts (Pawson, 1994, 1995). In c-Src the SH2 domain and the SH3 domains participate in an intramolecular interaction with a short regulatory domain at the C terminus (Cooper and Howell, 1993; Liu and Pawson, 1994). In v-Src this regulatory domain is substituted with an unrelated sequence (derived from sequences downstream of the c-*src* gene), and this intramolecular interaction is abrogated, resulting in activation of kinase activity (Takeya and Hanafusa, 1983; Cooper and Howell, 1993; Liu and Pawson, 1994). The effects of mutations in the SH3 and SH2 domains on transformation by v-*src* are complex. Avian fibroblasts or epithelial cells expressing SH2 or SH3 mutants of v-*src* may be spindly or fusiform, a phenotype first recognized by Temin in 1960 and termed by him the *morph*^{f} phenotype (Ephrussi and Temin, 1960; Temin, 1960). Mutants with alterations in the SH2 or SH3 domains may also exhibit a host-range phenotype, transforming avian but not mammalian cells or vice versa (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990a,b; Liebl *et al.*, 1992; Verderame and Varmus, 1994). Residues 17–84 of Src, lying between the membrane attachment and SH3 domains, constitute a "unique" (U) domain that is unrelated to the corresponding sequence in other members of the Src family of nonreceptor tyrosine kinases. Small deletions or insertions within the U domain do not affect morphological transformation, although a deletion encompassing the entire U domain (Δ15–81) results in partially fusiform transformation of chicken embryo fibroblasts (CEFs; Cross *et al.*, 1984).

Several hypotheses may be advanced to account for the role of the SH2 and SH3 domains in transformation. These domains might act *in cis* to promote the catalytic function of the adjacent kinase domain, for example, by a physical interaction between the Src homology domains and regulatory sites within the catalytic domain (Liebl *et al.*, 1993; Catling *et al.*, 1994; Woods and Verderame, 1994). They might also act *in cis* by targeting the adjacent kinase domain to specific intracellular locations or to specific substrates for phosphorylation; in this case the SH2 and SH3 domains would again be required *in cis* to the catalytic domain but would promote transformation through intermolecular interactions (Kanner *et al.*, 1991; Flynn *et al.*, 1993; Sakai *et al.*, 1994). Finally, the SH2 and SH3 domains might function *in trans*; that is, they might promote transformation even when not physically linked to the kinase domain, for example, by protecting phosphotyrosyl proteins from dephosphorylation (Birge *et al.*, 1992; Eide *et al.*, 1995; Scharenberg *et al.*, 1995) or by functioning as adaptors to link different signaling proteins (Pawson, 1995). To explore the role of the Src homology domains in transformation, we have expressed variants of v-Src in which the physical relationship of the U, SH2, and SH3 domains to the catalytic domain has been altered. Our results support the idea that these domains promote the targeting of v-Src to substrates that are involved in morphological transformation but also indicate that the N-terminal domains of Src can induce protein-tyrosine phosphorylation independently of the catalytic domain.

MATERIALS AND METHODS

Construction of v-src Alleles

src alleles were all derived from the v-*src* gene of the Schmidt–Ruppin strain of Rous sarcoma virus (DeLorbe *et al.*, 1980) incorporated into a *Cla*I cassette (Hughes and Kosik, 1984). All constructs were verified by sequencing. The v-*src* (R175H) allele and the linker insertion mutants SPX1 and SHX2 have been described previously (DeClue and Martin, 1989; Tian and Martin, 1996).

15C (Δ16–259). The plasmid pIRL-v-*src* (DeClue and Martin, 1989) was digested with *Ngo*MI, which cuts at a unique site within codon 15 of v-*src*, and the overlapping ends were filled in with the Klenow fragment of DNA polymerase I. The linearized DNA was then digested with *Mlu*I, which cuts at a unique site within codons 258–260 of v-*src*, and the overlapping ends were removed by digestion with mung bean nuclease. The blunt ends were then ligated to generate 15C, in which residues 16–259 are deleted.

N2 (Δ261–526). The N2 v-*src* allele was generated by introducing a stop codon after codon 260 with the polymerase chain reaction (PCR) and the plasmid pIRL-v-*src* (DeClue and Martin, 1989) as template. The 5′ primer, ATGCacgcgtGGTAGTGCGCGAGTAAAATTTAAGC, contained an *Mlu*I site (lowercase type) close to the 5′ end followed in the appropriate reading frame by a termination codon (TAG) and 20 bp of noncoding sequence (underlined) immediately 3′ to the v-*src* termination codon. The 3′ primer was a 21-bp vector sequence (CGAGTCAGTGAGCGAGGAAGC) 313 bp 3′ to the v-*src* termination codon. The PCR product was digested with *Mlu*I and *Nhe*I and this *Mlu*I-*Nhe*I fragment (which lacks the sequence of codons 261–526) was used to replace the *Mlu*I-*Nhe*I fragment in pIRL-v-*src* (DeClue and Martin, 1989). The resulting v-*src* allele N2 encodes the Src amino acid sequence from the N terminus to codon 260.

15CN. In 15CN, residues 15–259, containing the U, SH3, and SH2 domains, are transposed to the C terminus of the Src protein. The first step in the construction of 15CN used PCR, with the plasmid pIRL-15C v-*src* as template, to insert an 18-nucleotide sequence containing *Ngo*MI and *Mlu*I sites between the last codon and the stop codon (TAG) of 15C. The 5′ primer contained the sequence of codons 513–526, followed by the 18-nucleotide insert, a TAG termination codon, and 21 bp of noncoding sequence immediately 3′ to the v-*src* termination codon; the sequence of this primer was CAGGCCCAGCTGCTCCCTGCTTGTGTTCTAGAGGTCGCTGAGggccggctcgaggacgcgTAGTGCGCGAGTAAAATTTAAGCT, with the insert shown in lowercase type and the *Ngo*MI and *Mlu*I sites underlined. The 3′ primer was the same as that used for the construction of N2 (see above). The PCR product was digested with *Pvu*II, which cuts at a site within codons 515–516, and with *Nhe*I. The resulting *Pvu*II-*Nhe*I fragment was used to replace the corresponding *Pvu*II-*Nhe*I fragment in pIRL-15C v-*src*, generating the v-*src* allele 15Ct, in which the 18-bp sequence containing *Ngo*MI and *Mlu*I sites is inserted at the C terminus of 15C. In the second step, the *Ngo*M1-*Mlu*I fragment of v-*src*, which contains the U, SH3, and SH2 domains, was subcloned into the *Ngo*M1- and *Mlu*I-digested 15Ct, to yield 15CN. 15CN therefore contains, in the following order, v-*src* codons 1–15, codons 260–526, GGC (Gly), and codons

15–259. Subsequent sequencing of 15CN revealed a G → A transition resulting in a V524I substitution at codon 524 (numbered as in the wild-type sequence); this mutation was presumed to be inconsequential, as the substitution is conservative and this region is not sensitive to mutational effects on kinase activity or transformation (Parsons and Weber, 1989).

76C (Δ77–251 ins DLDM). 76C was generated from two previously constructed v-*src* mutants, SHX2 (DeClue and Martin, 1989) and *Bsp*HI-v-*src*. SHX2 contains a *Xho* linker inserted at residue 76. *Bsp*HI-v-*src* contains an insertion, introduced by PCR between codons 251 and 252, which generates the sequence CAGTCATGACC; the inserted sequence (underlined) plus the 3′ A residue forms a *Bsp*HI restriction site. The protein product encoded by *Bsp*HI-v-*src* would be expected to contain the first 251 residues of Src, followed by a serine residue encoded by the inserted TCA codon, and to be truncated by the TGA codon (boldface type). The plasmids pIRL19-SHX2 and pIRL19-*Bsp*HI-v-*src* were digested with *Xho*I and *Bsp*HI, respectively, and the resulting ends were filled-in with the Klenow fragment of DNA polymerase I. Both plasmids were then digested with *Hin*dIII, which cuts immediately 3′ to the *Cla*I cassette. The *Bsp*HI(filled)-*Hin*dIII fragment from pIRL19-*Bsp*HI-v-*src* that encodes the Src catalytic domain sequence was then ligated to the *Xho*I(filled)-*Hin*dIII fragment from pIRL19-SHX2 that contains the sequences of the vector plus the N-terminal sequence of v-*src*. This ligation yielded the deletion mutant 76C, in which the sequence GACCTCGACATG (encoding DLDM) replaces codons 77–251. Expression of this mutant in CEFs yielded an active kinase and induced fusiform transformation.

Expression of v-src Alleles

CEFs and Rat-2 cells were cultured as described previously (DeClue and Martin, 1989). v-*src* alleles were expressed in CEFs using the avian retroviral vectors RCAN (BH) or RCAS (BH) with *env* genes of subgroup A or B (Hughes *et al.*, 1987); these vectors contain the *pol* gene of the Bryan high titer (BH) strain of Rous sarcoma virus. The 15CN construct was expressed in Rat-2 cells using the murine retroviral vector fpGV-1 (Jhappan *et al.*, 1986; Robbins *et al.*, 1991), as described previously (Liebl *et al.*, 1992). To express N2 in Rat-2 cells, a *Sal*I site was inserted 6 bp 3′ to the 3′ *Cla*I site of the *src Cla*I cassette in pIRL-v-*src*, and the *Eco*RI-*Sal*I fragment containing the *src Cla*I cassette was subcloned into the murine retroviral vector pBabe-hygro (Morgenstern and Land, 1990); Rat-2 cells transfected with this construct were selected in medium containing 50 μg/ml hygromycin B (Sigma, St. Louis, MO), and a representative clone was expanded for further analysis.

Focus assays were carried out by transfecting Rat-2 cells with fpGV-1 expression constructs as described previously (Liebl *et al.*, 1992). In brief, transfected cells at a series of dilutions were transferred to two sets of plates; one set of plates was overlaid with medium containing agar to score transformed foci, the other was maintained in liquid medium containing 250 μg/ml (effective concentration) Geneticin to score G418-resistant colonies.

Immunoprecipitation

Cells were lysed in radioimmune precipitation assay (RIPA) buffer [20 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 2 mM Na_3VO_4 that was supplemented with a protease inhibitor mixture containing 174 μg/ml phenylmethylsulfonyl fluoride, 1.6 μg/ml benzamidine, 1 μg/ml phenanthroline, 0.5 μg/ml antipain, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 0.5 μg/ml chymostatin]. Lysates were clarified by centrifugation and aliquots were removed for protein determination. Aliquots (~1 mg per sample) were subjected to immunoprecipitation using anti-$p130^{Cas}$ (anti-Cas2, courtesy of Dr. H. Hirai, University of Tokyo, Tokyo, Japan; 2 μg/mg of lysate protein).

Immunoblotting

Cell lysates prepared in RIPA buffer were adjusted to SDS sample buffer (2.3% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8) by addition of 4× concentrated SDS sample buffer and boiled for 5 min. Immunoprecipitated proteins or proteins adsorbed to beads carrying glutathione *S*-transferase (GST)-SH2 fusion proteins were solubilized by boiling directly in SDS sample buffer. Proteins were resolved by SDS-PAGE and electroblotted to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Blocking buffer (3% BSA, 50 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.5% NP-40, 0.1% Tween 20) was used for all blocking, antibody incubation, and rinsing steps. Blots were probed with 2 μg/ml anti-phosphotyrosine monoclonal antibody (mAb) 4G-10 (Upstate Biotechnology, Lake Placid, NY), 2 μg/ml anti-Src mAb 217, or 1 μg/ml rabbit polyclonal anti-$p130^{Cas}$ (anti-Cas2). Antibodies were detected by incubation with horseradish peroxidase-conjugated anti-immunoglobulin antibodies (Pierce, Rockford, IL), followed by enhanced chemiluminescence detection (Amersham ECL kit, Amersham, Arlington Heights, IL). To reprobe blots with additional antibodies, the blots were first stripped by incubation for 30 min at 50°C in 2% SDS, 0.1 M 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.7), and then washed thoroughly with 0.1% Tween 20 in phosphate-buffered saline (PBS; 0.15 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Construction and Expression of a Fusion of GST with the N-Terminal Domains of Src

Fusion proteins were expressed using the vector pGEX-1λT (Pharmacia, Piscataway, NJ). Construction of a plasmid encoding a GST fusion with the SH2 domain of the R175H v-*src* allele, pGEX-SH2(175H), was described previously (Tian and Martin, 1996). This plasmid was used to generate a plasmid encoding a GST fusion with the U, SH3, and SH2 domains of v-*src* (R175H). To generate this construct, the plasmid pIRL-R175H v-*src* was digested with *Ngo*MI, which cuts between the myristoylation and U domains, at codon 14. The resulting ends were filled in with the Klenow fragment of DNA polymerase I. The DNA was then digested with *Acc*65I, which cuts within the SH2 domain sequence of v-*src* (at codons 170/171). The plasmid pGEX-SH2(175H) was digested with *Bam*HI, which cuts in the linker between the GST and v-*src* SH2 sequences, and the resulting ends were filled in with the Klenow fragment of DNA polymerase I; this DNA was then digested with *Acc*65I. *Ngo*MI(filled)-*Acc*65I fragment from pIRL-R175H v-*src* was then ligated into the *Bam*HI(filled)-*Acc*65I fragment of pGEX-SH2(175H), generating pGEX-SH3-SH2(R175H) and pGEX-U-SH3-SH2(R175H). Expression of this GST fusion protein was confirmed by immunoblotting with anti-Src mAb 327 (Lipsich *et al.*, 1983), which recognizes an epitope within the SH3 domain.

To express the fusion construct, an overnight culture of the transformed bacteria was diluted to A_{600} = 0.1 in fresh TB medium (Ausubel *et al.*, 1994) and grown for 1 h at 37°C. Isopropyl β-D-thiogalactopyranoside was then added to 0.5 mM, and the culture was incubated for an additional 3 h. The bacteria (40–80 ml) were harvested by centrifugation, washed in ice-cold PBS, and sonicated in 10 ml of PBS supplemented with 0.25% Tween 20 and the protease inhibitor mixture. NP-40 was then added to 0.75% and after 10 min at 0°C the lysate was clarified by centrifugation at 16,000 × *g* for 10 min. The NaCl concentration was adjusted to 0.5 M and the lysate was then agitated for 10 min at room temperature with 100–150 μl (packed volume) glutathione-Sepharose 4B beads (Pharmacia). The beads were pelleted, washed five times in binding buffer, and stored for short periods at 0°C in PBS supplemented with 0.25% Tween 20, 0.75% NP-40, 2 mM EDTA, 5% glycerol, and protease inhibitors.

Binding to Phosphotyrosylproteins

Glutathione-Sepharose beads bearing approximately 30 μg of GST fusion protein were incubated at 4°C for 1.5 h with 1 ml of cell lysate

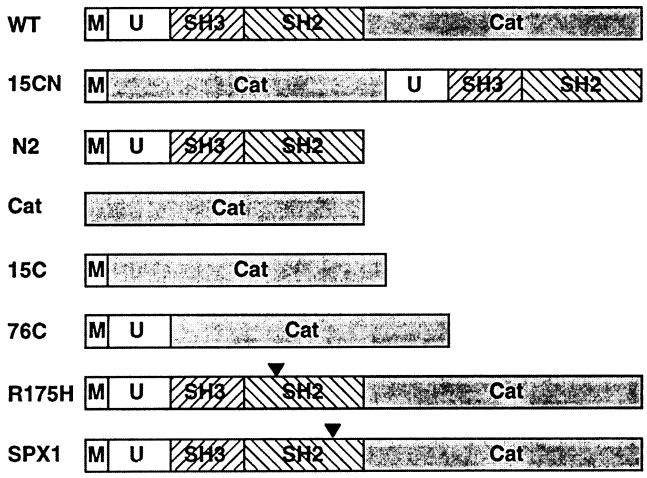

Figure 1. Domain structure of v-*src* alleles used in this study. M, myristoylation and membrane attachment domain; Cat, catalytic (protein-tyrosine kinase) domain. Construction of v-*src* alleles is described in MATERIALS AND METHODS.

in RIPA buffer (approximately 1 mg of protein). The beads were then harvested by centrifugation at 1500 × *g* for 15 s and washed three times with RIPA buffer. Bound phosphotyrosyl proteins were solubilized in SDS sample buffer and analyzed by immunoblotting.

RESULTS

Expression of the N-Terminal Domains of Src In Cis and In Trans to the Catalytic Domain

The SH2 and SH3 domains of v-Src are required for transformation of Rat-2 cells and for wild-type (*morph*^{r}) transformation of CEFs. To determine whether the location of these domains within the v-Src molecule is critical for transformation, a construct (15CN; Figure 1) was generated in which the U, SH3, and SH2 domains were translocated to the C terminus of the molecule. This construct was expressed in CEFs or Rat-2 cells using avian and mammalian expression vectors. The transforming ability of the construct was compared with that of wild-type v-Src and with that of a deletion mutant (15C, Figure 1) in which the U, SH3, and SH2 domains had been deleted.

CEFs expressing wild-type v-Src exhibited the rounded morphology typical of Src-transformed cells, and the 15C mutant exhibited the fusiform morphology characteristic of SH3 and SH2 mutants (Figure 2, B and D). CEFs expressing 15CN contained cells that resembled those transformed by wild-type v-Src (Figure 2E); however, the transformation process was slower, and even at later times, the cultures were heterogeneous, containing mixtures of rounded, epithelioid, and fusiform cells (Figure 2F). In addition the 15CN construct in a murine retroviral expression vector transformed Rat-2 cells with the same efficiency (104%) as wild-type v-Src, whereas the 15C construct did not transform Rat-2 cells. We conclude that *src* transformation is not dependent on the intramolecular location of the region of v-Src containing the U, SH3, and SH2 domains, although the translocated domain may be less efficient in promoting transformation.

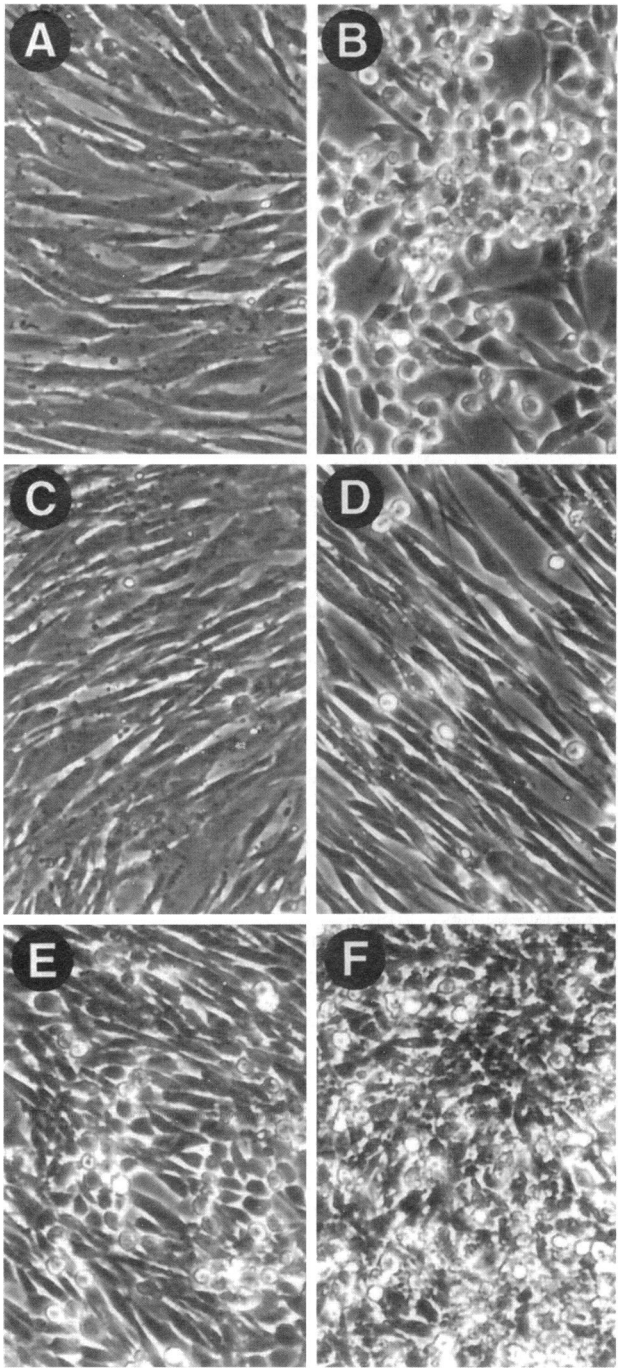

Figure 2. Morphological transformation of CEFs by RCAN vectors expressing no insert (A), wild-type v-Src (B), N2 (C), 15C (D), or 15CN (E and F). Photomicrographs were taken at 5 d (A–E) or 7 d (F) after infection.

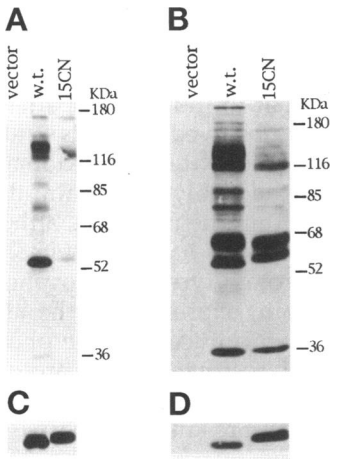

Figure 3. Effect of 15CN expression on tyrosine phosphorylation of cellular proteins. Wild-type (w.t.) v-*src* and 15CN were expressed in CEFs (A and C) and in Rat-2 cells (B and D). Cellular proteins were resolved by SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine mAb (A and B) or anti-Src mAb (C and D).

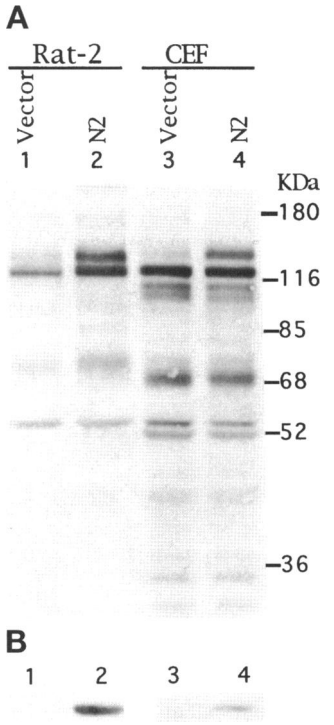

Figure 4. Effect of N2 expression on tyrosine phosphorylation of cellular proteins. N2 (lanes 2 and 4) or control vectors (lanes 1 and 3) were expressed in Rat-2 cells (lanes 1 and 2) or in CEFs (lanes 3 and 4). Cellular proteins were resolved by SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine mAb (A) or anti-Src mAb (B).

To determine whether the N-terminal domains of v-Src could promote transformation when expressed *in trans* to the kinase domain, another construct, N2, was generated (see Figure 1). This construct encodes only the N-terminal regions of v-Src, as residues 266–526 are deleted. Expression of N2 in CEFs or Rat-2 cells was verified by blotting with anti-Src mAb 2–17 (see Figure 4B). N2 had no apparent effect on the morphology or growth of CEFs (Figure 2C) or Rat-2 cells. Moreover, CEFs expressing both N2 and 15C retained the fusiform morphology of cells expressing 15C. We conclude that the SH3 and SH2 domains expressed *in trans* cannot complement the morphological transformation defect of an SH3–SH2 deletion and, thus, that these domains must be present *in cis* to the catalytic domain to promote wild-type transformation. A comparable complementation test is not possible in Rat-2 cells, because a mutant lacking the Src homology domains is catalytically inactive in these cells (DeClue and Martin, 1989; Liebl *et al.*, 1993).

Effects of the N-Terminal Domains of Src on Protein-Tyrosine Phosphorylation

These observations suggested that the SH2 and SH3 domains may target Src to substrates necessary for transformation. To determine whether transformation by these constructs was correlated with the pattern of protein-tyrosine phosphorylation, lysates of CEFs or Rat-2 cells expressing wild-type, 15CN, and N2 constructs were examined by immunoblotting with anti-phosphotyrosine antibody (Figure 3, A and B; Figure 4A); expression of the *src* constructs was verified by immunoblotting with anti-Src mAb (Figures 3, C and D; Figure 4B). The overall pattern of tyrosine phosphorylation in CEFs or Rat-2 cells expressing 15CN was similar to that in cells transformed by wild-type v-Src (Figure 3, A and B). However, in CEFs and to some extent in Rat-2 cells, the overall level of tyrosine phosphorylation was reduced, suggesting that the translocated SH2 and SH3 domains either inefficiently target the kinase domain or decrease its catalytic activity; this reduced level of tyrosine phosphorylation may account for the slower kinetics of transformation by 15CN. The subset of phosphotyrosyl proteins that appear both in cells expressing wild-type v-Src and in cells expressing 15CN (for example, the species at 110–120 kDa) may possibly represent significant targets of v-Src.

Surprisingly, N2 expression resulted in elevated tyrosine phosphorylation of a number of proteins, in particular a prominent 130-kDa protein (Figure 4A); the abundance of other phosphotyrosyl proteins with apparent molecular weights of 210 kDa and 72–75 kDa was also enhanced in cells expressing N2. The Src SH2 domain is known to bind to $p130^{Cas}$ (Kanner *et al.*, 1991; Sakai *et al.*, 1994). To determine whether the 130-kDa phosphotyrosyl protein observed in CEFs expressing N2 was $p130^{Cas}$, lysates of these cells were

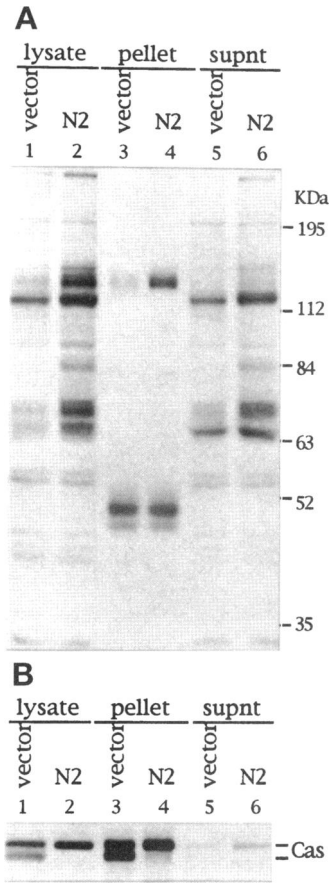

Figure 5. Immunoprecipitation of p130^{Cas} from CEFs expressing N2. P130^{Cas} was immunoprecipitated from lysates of CEFs expressing N2 (lanes 2, 4, and 6) or vector alone (lanes 1, 3, and 5). Cell lysates, immunoprecipitates, and immunodepleted supernatants were resolved by SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine mAb (A). The blot was then stripped and reprobed with anti-p130^{Cas} (B).

subjected to immunoprecipitation with anti-p130^{Cas} antibody; the precipitated proteins and the immunodepleted supernatants were probed with anti-phosphotyrosine and anti-p130^{Cas} antibodies (Figure 5, A and B). The proteins recognized by the anti-p130^{Cas} antibody migrated as a doublet, of which the upper band was recognized by anti-phosphotyrosine antibody. The anti-p130^{Cas} antibody specifically depleted the 130-kDa phosphotyrosyl protein from lysates of cells expressing N2 (Figure 5A, lanes 2, 4, and 6); the immunoprecipitated protein comigrated with the upper of the two p130^{Cas} bands. The expression of N2 caused a shift of the lower band to the upper position in the doublet, presumably because of tyrosine phosphorylation. A species that was not tyrosine-phosphorylated but migrated with the same mobility as the tyrosine-phosphorylated species was detected in cells expressing the vector alone (Figure 5B, lanes 1 and 3); this probably corresponds to the Cas-B species described by Sakai *et al.* (1994).

We conclude that to induce the pattern of tyrosine phosphorylation observed in cells transformed by wild-type v-Src, the N-terminal domains must be present *in cis* to the catalytic domain; the location of these domains within the molecule, although not critical for transformation, has some effect on the level and pattern of protein-tyrosine phosphorylation. The N-terminal domain, expressed in the absence of the kinase domain, can induce tyrosine phosphorylation, but only of a very restricted set of proteins, in particular p130^{Cas}; the tyrosine phosphorylation observed in the absence of the v-Src kinase domain is not sufficient for transformation.

Effects of the N-Terminal Domains of Src on Phosphorylation of Proteins that Bind to the Same Domains

The findings described above indicate that the N-terminal domains of Src must be *in cis* to the catalytic domain to promote wild-type transformation of CEFs and transformation of Rat-2 cells and to promote the pattern of tyrosine phosphorylation characteristic of cells expressing wild-type v-Src. These observations suggest that the N-terminal domains of Src target the catalytic activity to sites where it can phosphorylate substrates necessary for wild-type transformation. At least some of the proteins to which the Src N terminus binds are phosphotyrosyl proteins, since it has been shown previously that the Src SH2 domain binds to proteins phosphorylated by the Src kinase. This suggested that there may be a class of phosphotyrosyl proteins present in infected cells that bind specifically to the N-terminal domains of Src and that are phosphorylated only if the N-terminal domains of Src are intact.

To examine this possibility we isolated phosphotyrosyl proteins from transformed CEFs by precipitation with a GST fusion to the N-terminal domains of a mutant Src in which the Arg residue at position 175 in the conserved FLVRES motif of the SH2 domain was substituted with a His residue. The rationale for the use of this protein was as follows. We have shown previously that the R175H mutation reduces but does not eliminate the binding of the Src SH2 domain to phosphotyrosyl proteins, without affecting the ability of the mutant Src protein to transform CEFs to a wild-type (*morph*^{r}) phenotype (Tian and Martin, 1996); similar observations have been made on the R175K and R175A mutants of v-Src (Tian and Martin, 1996; Verderame, 1997). Measurements of the affinity of the mutant Src proteins to phosphotyrosyl peptides indicate that the binding of the Src SH2 domain to low-affinity phosphopeptides is not required for wild-type

transformation of CEFs (Verderame, 1997). We reasoned therefore that a fusion protein containing the R175H SH2 domain might recognize a more restricted set of proteins than the wild-type SH2 domain and that this could simplify the characterization of proteins recognized by the Src N terminus, whose phosphorylation might correlate with transformation. CEFs were infected with vectors expressing wild-type v-Src; the R175H mutant of v-Src; the 76C mutant, which contains a deletion of both SH3 and SH2 domains (Figure 1) and induces fusiform transformation of CEFs; and the host-range mutant SPX1, which contains a linker insertion within the SH2 domain (Figure 1), induces fusiform transformation of CEFs, and does not transform Rat-2 cells (DeClue and Martin, 1989). Lysates of these cells were then incubated with glutathione-Sepharose beads bearing the GST fusion protein containing the N-terminal domain of the R175H mutant, designated GST-U.SH3.SH2(H). Bound proteins were isolated by centrifugation and both bound proteins and cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. The results are shown in Figure 6.

In control CEFs, only a few phosphotyrosyl proteins were observed to bind to the GST-U.SH3.SH2(H) fusion proteins (Figure 6, A and B, lane 2). In cells transformed by wild-type or R175H Src, a variety of phosphotyrosyl proteins bound to the GST-U.SH3.SH2(H) fusion (Figure 6, A and B, lanes 5 and 6). In contrast, a reduced set of proteins bound to the GST-U.SH3.SH2(H) fusion protein from lysates of cells expressing the SH2 linker insertion mutant SPX1 (Figure 6, A and B, lane 4). In cells expressing the SH2-SH3 deletion mutant 76C, the level of phosphotyrosyl proteins that bound to the GST-U-SH3.SH2(H) fusion was only very slightly elevated over the level of phosphotyrosyl proteins recovered from cells expressing vector alone (Figure 6, A and B, lane 3). We conclude that in CEFs expressing v-Src, the phosphorylation of proteins that bind to the N-terminal domains of Src in vitro is dependent on the function of the SH2 and SH3 domains of v-Src. At longer exposures than those shown in Figure 6, a number of phosphotyrosyl proteins binding to the GST fusion were detected in lysates of control CEFs; many of these had the same mobility as the phosphotyrosyl proteins detected in lysates of *src*-transformed cells, suggesting that these proteins are also phosphorylated by cellular tyrosine kinases.

The most prominent phosphotyrosyl protein binding to the GST-U.SH3.SH2(H) fusion had an apparent molecular mass of 160 kDa; this protein was the most readily detectable phosphotyrosyl protein at lower autoradiographic exposures (Figure 6A). The 160-kDa protein was phosphorylated in cells expressing wild type or R175H Src but was phosphorylated at much lower levels in cells expressing SPX1 or 76C. Thus the tyrosine phosphorylation of this protein was dependent on the SH2 domain of Src and correlated with wild-type (*morph*^{r}) transformation. The identity of this protein is under investigation.

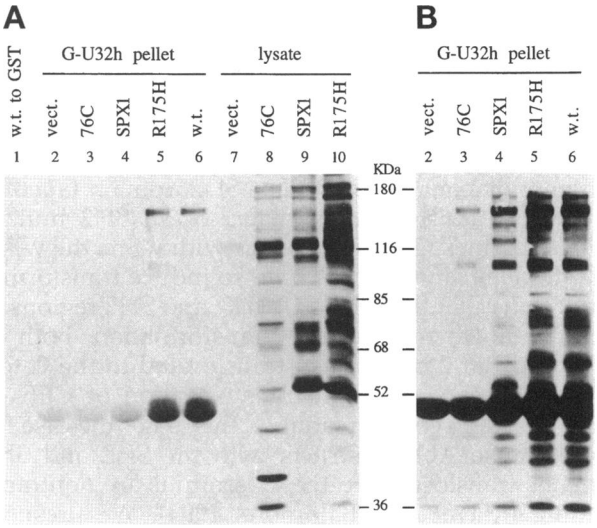

Figure 6. Binding of phosphotyrosyl proteins from CEFs expressing v-*src* mutants to GST-Src fusion proteins. CEFs were infected with vectors expressing no insert (A, lanes 2 and 7; B, lane 2), the SH3–SH2 deletion mutant 76C (A, lanes 3, 8; B, lane 3), the SH2 mutants SPX1 (A, lanes 4 and 9; B, lane 4) and R175H (A, lanes 5 and 10; B, lane 5), or wild-type v-*src* (A, lanes 1 and 6; B, lane 6). Lysates were incubated with glutathione-Sepharose beads to which was bound either GST alone (lane 1) or GST-U.SH3.SH2(175H) (GU32h pellet, lanes 2–6); the beads were isolated by centrifugation and bound proteins solubilized in SDS sample buffer. The initial cell lysates and precipitated proteins were resolved by SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine mAb (A and B; B represents a longer autoradiographic exposure of lanes 2–6 in A). Use of approximately equimolar amounts of GST fusion proteins was verified by staining with Coomassie brilliant blue. The recognition of the GST fusion proteins by anti-phosphotyrosine mAb is presumably due to the presence of undissociated phosphotyrosyl peptides.

DISCUSSION

In mammalian cells, the SH2 domain of v-Src and its catalytic activity are both required for the induction of a variety of different aspects of the transformed phenotype; in avian fibroblasts, however, the SH3 and SH2 domains are required for wild-type morphological transformation, but not for other aspects of the transformed phenotype, such as anchorage-independent growth and enhanced hexose uptake (DeClue and Martin, 1989; Verderame *et al.*, 1989; Hirai and Varmus, 1990a,b; Liebl *et al.*, 1992; Verderame and Varmus, 1994). For this reason we have focused on the role of the Src homology domains in morphological transformation. The results described above indicate that the SH2 and SH3 domains of v-Src are required *in*

cis to the catalytic domain to promote wild-type (*morph*^{r}) transformation of CEFs. This conclusion is based on the finding that the N-terminal domains of Src when expressed *in trans* cannot complement the transformation defect of a deletion mutant lacking the U, SH3, and SH2 regions. Similarly an N-terminal v-Fps polypeptide containing the Fps SH2 domain failed to rescue the transformation defect of an SH2 mutant v-Fps protein, suggesting that, as with v-Src, the v-Fps SH2 domain cannot act *in trans* to induce transformation (Koch *et al.*, 1989). The U, SH3, and SH2 regions of Src can, however, promote transformation, both of CEFs and Rat-2 cells, when translocated to the C terminus of v-Src, although transformation of CEFs is somewhat slower. Interestingly, this is not the case for Abl, because Abl constructs with the SH2 and SH3 domains translocated to the C terminus are nontransforming (Mayer and Baltimore, 1994). We presume that the translocated SH3 and/or SH2 domains are required for the transforming potential of 15CN, but additional mutational analysis will be required to define the respective roles of these domains in the translocation construct. Both in CEFs and in Rat-2 cells, the expression of wild-type v-Src results in tyrosine phosphorylation of proteins that can subsequently bind to the v-Src SH3 and SH2 domains in vitro. These phosphotyrosyl proteins are recovered at lower levels from cells expressing v-Src mutants that lack functional SH2 or SH3 and SH2 domains. We assume that this reflects alterations in the phosphorylation of these proteins rather than changes in their synthesis or degradation. It may be inferred, therefore, that mutations in the SH2 or SH3 and SH2 domains can prevent the phosphorylation of this group of proteins by v-Src. In addition, the N-terminal domains of Src can promote tyrosine phosphorylation of certain proteins, in particular p130^{Cas}, even when expressed in the absence of the catalytic domain, indicating that the N-terminal domains of v-Src have effects that are independent of the catalytic domain.

It is conceivable that the requirement for SH3 and SH2 domains *in cis* to the catalytic domain results from a regulatory interaction between the N-terminal and C-terminal domains. Although it appears somewhat unlikely that this interaction could occur when the N-terminal domains have been translocated to the C terminus, there are instances in which circularly permuted protein molecules can fold correctly (Pan and Uhlenbeck, 1993). We and others have reported observations that suggest there may be intramolecular regulatory interactions between the v-Src kinase domain and the N-terminal SH3 and SH2 domains (Liebl *et al.*, 1993; Catling *et al.*, 1994; Woods and Verderame, 1994). However, the evidence presented herein indicates that in cells expressing v-Src, the appearance of phosphotyrosyl proteins that bind to the v-Src SH3 and SH2 domains is dependent on the function of these domains in vivo. This has previously been shown to be the case for Src substrates such as p110^{AFAP} (Kanner *et al.*, 1991; Flynn *et al.*, 1993), p130^{Cas} (Kanner *et al.*, 1991; Sakai *et al.*, 1994) and Sam68 (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994; Lock *et al.*, 1996) and suggests that the role of the SH3 and SH2 domains in transformation by v-Src is dependent on their ability to promote intermolecular interactions. It is possible that the SH3 and/or SH2 domains are not required for the initial phosphorylation of certain protein substrates but that the phosphotyrosyl residues in the phosphorylated substrates bind to the v-Src SH2 domain and are thereby protected from the action of phosphatases. It has been shown previously that phosphotyrosyl proteins can be protected from the action of phosphotyrosyl-protein phosphatases through binding of SH2 domains (Birge *et al.*, 1992; Eide *et al.*, 1995; Scharenberg *et al.*, 1995). However, the failure of the N-terminal domains to complement the morphological transformation defect when expressed *in trans* to the catalytic domain suggests that this latter mechanism cannot represent the sole role of these domains in morphological transformation.

Thus, our findings and those of other groups (Kanner *et al.*, 1991; Flynn *et al.*, 1993; Fumagalli *et al.*, 1994; Sakai *et al.*, 1994; Taylor and Shalloway, 1994; Lock *et al.*, 1996) are most consistent with models in which the SH3 and SH2 domains of v-Src directly or indirectly target the catalytic domain to substrates involved in transformation. The SH2 and SH3 domains of v-Src may target the protein to multiprotein complexes or subcellular locations where specific substrates are concentrated, such as focal adhesions; these structures contain many known v-Src substrates that are also phosphorylated at tyrosine in normal cells, such as vinculin, talin, paxillin, FAK, and p130^{Cas} (Sefton *et al.*, 1981; DeClue and Martin, 1987; Kaplan *et al.*, 1994; Okamura and Resh, 1994; Schaller and Parsons, 1994; Turner, 1994). Alternatively, the Src homology domains could promote direct interactions with Src substrates such as p110^{AFAP}, P130^{Cas}, or Sam68. It must be stressed, however, that it has not been demonstrated that transformation is dependent on the tyrosine phosphorylation of p110^{AFAP}, p130^{Cas}, Sam68, or any of the unidentified phosphotyrosyl proteins described herein, although the finding that expression of a p130^{Cas} antisense RNA can reverse v-*src*-induced morphological transformation (Auvinen *et al.*, 1995) provides some support for this idea. The tyrosine phosphorylation of the 160-kDa protein described herein is dependent on the function of the SH2 domain and correlates with *morph*^{r} transformation; the identity of this protein and its possible role in transformation remain to be determined.

The finding that the N-terminal domains of v-Src can promote tyrosine phosphorylation of p130^{Cas} and

other proteins indicates that the N-terminal domains can influence tyrosine phosphorylation independently of the catalytic domain, although without inducing observable morphological transformation. One possible explanation, as discussed above, is that the N-terminal domains can protect phosphotyrosyl proteins from dephosphorylation by phosphatases (Birge *et al.*, 1992; Eide *et al.*, 1995; Scharenberg *et al.*, 1995). A second possibility, similar to that advanced for the role of v-Crk in enhancing the phosphorylation of $p130^{Cas}$ by the Abl tyrosine kinase (Mayer *et al.*, 1995), is that the N-terminal domain can function as an adaptor to bridge substrate molecules to an (unknown) tyrosine kinase; a construct similar to N2 has recently been shown to promote association of $p130^{Cas}$ with the focal adhesion kinase FAK (Schlaepfer *et al.*, 1997). A third possibility is that the expression of the N-terminal domains activates endogenous Src family kinases by competing with the intramolecular interaction between the C-terminal phosphotyrosyl residue and the N terminus (Cooper and Howell, 1993; Liu and Pawson, 1994). It is of interest that a construct similar to N2 has been shown to increase cell spreading in c-Src-deficient fibroblasts (Kaplan *et al.*, 1995); it is possible that this effect may be mediated by enhanced phosphorylation of Cas.

Finally, it should be stressed that the questions addressed herein concern the mechanism of transformation by mutationally activated v-Src and may or may not have a bearing on the normal function of c-Src. c-Src has evolved to perform cellular functions involved in growth regulation and cell–matrix signaling. Since its relatively recent transduction from the chicken genome v-Src has been subject to selection to induce transformation when expressed under the control of a retroviral promoter. The constraints that operate on c-Src and v-Src may thus be quite different. Nevertheless it seems likely that the SH2 and SH3 domains of c-Src must be *in cis* to the catalytic domain for c-Src to exert its normal cellular functions. For example, integrin-dependent signaling to Ras is in part mediated by binding of the c-Src SH2 domain to the FAK autophosphorylation site Tyr-397, resulting in tyrosine phosphorylation of Tyr-925 and the formation of a binding site for the adaptor protein Grb-2 (Schlaepfer *et al.*, 1994). The relationship between the SH3- and SH2-dependent events in transformation by v-Src and the SH3- and SH2-dependent events in signaling by c-Src remains to be determined.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994). Current Protocols in Molecular Biology, New York: Wiley-Interscience.

Auvinen, M., Paasinen-Sohns, A., Hirai, H., Andersson, L.C., and Holtta, E. (1995). Ornithine decarboxylase- and ras-induced cell transformations: reversal by protein tyrosine kinase inhibitors and role of $pp130^{cas}$. Mol. Cell. Biol. *15*, 6513–6525.

Birge, R.B., Fajardo, J.E., Myer, B.J., and Hanafusa, H. (1992). Tyrosine-phosphorylated epidermal growth factor receptor and cellular p130 provide high affinity binding substrates to analyze Crk-phosphotyrosine-dependent interactions in vitro. J. Biol. Chem. *267*, 10588–10595.

Catling, A.D., Fincham, V.J., Frame, M.C., Haefner, B., and Wyke, J.A. (1994). Mutations in v-Src SH3 and catalytic domains that jointly confer temperature-sensitive transformation with minimal temperature-dependent changes in cellular tyrosine phosphorylation. J. Virol. *68*, 4392–4399.

Cooper, J.A., and Howell, B. (1993). The when and how of Src regulation. Cell *73*, 1051–1054.

Cross, F.R., Garber, E.A., Pellman, D., and Hanafusa, H. (1984). A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. *4*, 1834–1842.

DeClue, J.E., and Martin, G.S. (1987). Phosphorylation of talin at tyrosine in Rous sarcoma virus-transformed cells. Mol. Cell. Biol. *7*, 371–378.

DeClue, J.E., and Martin, G.S. (1989). Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. J. Virol. *63*, 542–554.

DeClue, J.E., Zhang, K., Redford, P., Vass, W.C., and Lowy, D.R. (1991). Suppression of src transformation by overexpression of full-length GTPase-activating protein (GAP) or of the GAP C terminus. Mol. Cell. Biol. *11*, 2819–2825.

DeLorbe, W.J., Luciw, P.A., Goodman, H.M., Varmus, H.E., and Bishop, J.M. (1980). Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. *36*, 50–61.

Eide, B.L., Turck, C.W., and Escobedo, J.A. (1995). Identification of Tyr-397 as the primary site of tyrosine phosphorylation and $pp60^{src}$ association in the focal adhesion kinase, pp125FAK. Mol. Cell. Biol. *15*, 2819–2827.

Ephrussi, B., and Temin, H. (1960). Infection of chick iris epithelium with the Rous sarcoma virus in vitro. Virology *11*, 547–552.

Erpel, T., and Courtneidge, S.A. (1995). Src family protein tyrosine kinases and cellular signal transduction pathways. Curr. Opin. Cell Biol. *7*, 176–182.

Flynn, D.C., Leu, T.H., Reynolds, A.B., and Parsons, J.T. (1993). Identification and sequence analysis of cDNAs encoding a 110-kilodalton actin filament-associated pp60src substrate. Mol. Cell. Biol. *13*, 7892–7900.

Fumagalli, S., Totty, N.F., Hsuan, J.J., and Courtneidge, S.A. (1994). A target for Src in mitosis. Nature *368*, 871–874.

Hirai, H., and Varmus, H.E. (1990a). Mutations in *src* homology regions 2 and 3 of activated chicken c-*src* that result in preferential transformation of mouse or chicken cells. Proc. Natl. Acad. Sci. USA *87*, 8592–8596.

Hirai, H., and Varmus, H.E. (1990b). Site-directed mutagenesis of the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of $p60^{c\text{-}src}$. Mol. Cell. Biol. *10*, 1307–1318.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. *61*, 3004–3012.

Hughes, S., and Kosik, E. (1984). Mutagenesis of the region between *env* and *src* of the SR-A strain of Rous sarcoma virus for the purpose of constructing helper-independent vectors. Virology *136*, 89–99.

Jhappan, C., Vande Woude, G.F., and Robins, T.S. (1986). Transduction of host cellular sequences by a retroviral shuttle vector. J. Virol. *60*, 750–753.

Jove, R., and Hanafusa, H. (1987). Cell transformation by the viral src oncogene. Annu. Rev. Cell Biol. *3*, 31–56.

Kamps, M.P., Buss, J.E., and Sefton, B.M. (1985). Mutation of NH_2-terminal glycine of $p60^{src}$ prevents both myristoylation and morphological transformation. Proc. Natl. Acad. Sci. USA *82*, 4625–4628.

Kanner, S.B., Reynolds, A.B., Wang, H.-C.R., Vines, R.R., and Parsons, J.T. (1991). The SH2 and SH3 domains of $pp60^{src}$ direct stable association with tyrosine phosphorylated proteins p130 and p110. EMBO J. *10*, 1689–1698.

Kaplan, K.B., Bibbins, K.B., Swedlow, J.R., Arnaud, M., Morgan, D.O., and Varmus, H.E. (1994). Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. *13*, 4745–4756.

Kaplan, K.B., Swedlow, J.R., Morgan, D.O., and Varmus, H.E. (1995). c-Src enhances the spreading of src−/− fibroblasts on fibronectin by a kinase-independent mechanism. Genes Dev. *9*, 1505–1517.

Koch, C.A., Moran, M., Sadowski, I., and Pawson, T. (1989). The common src homology region 2 domain of cytoplasmic signaling proteins is a positive effector of v-fps tyrosine kinase function. Mol. Cell. Biol. *9*, 4131–4140.

Krueger, J.G., Garber, E.A., and Goldberg, A.R. (1983). Subcellular localization of $pp60^{src}$ in RSV-transformed cells. Current Topics in Microbiology and Immunology, Berlin: Springer Verlag. 51–124.

Liebl, E.C., England, L.J., DeClue, J.E., and Martin, G.S. (1992). Host range mutants of v-*src*: alterations in kinase activity and substrate interactions. J. Virol. *66*, 4315–4324.

Liebl, E.C., England, L.J., and Martin, G.S. (1993). Reactivation of host-dependent *Src* kinase activity by co-expression with a heterologous tyrosine kinase. Virology *195*, 265–267.

Lipsich, L.A., Lewis, A.J., and Brugge, J.S. (1983). Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. *48*, 352–360.

Liu, X., and Pawson, T. (1994). Biochemistry of the Src protein-tyrosine kinase: regulation by SH2 and SH3 domains. Recent Prog. Horm. Res. *49*, 149–160.

Lock, P., Fumagalli, S., Polakis, P., McCormick, F., and Courtneidge, S.A. (1996). The human p62 cDNA encodes Sam68 and not the RasGAP-associated p62 protein. Cell *84*, 23–24.

Mayer, B.J., and Baltimore, D. (1994). Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. Mol. Cell. Biol. *14*, 2883–2894.

Mayer, B.J., Hirai, H., and Sakai, R. (1995). Evidence that SH2 domains promote processive phosphorylation by protein-tyrosine kinases. Curr. Biol. *5*, 296–305.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Nori, M., Vogel, U.S., Gibbs, J.B., and Weber, M.J. (1991). Inhibition of v-src-induced transformation by a GTPase-activating protein. Mol. Cell. Biol. *11*, 2812–2818.

Okamura, H., and Resh, M.D. (1994). Differential binding of $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ to cytoskeleton is mediated by SH2 and catalytic domains. Oncogene *9*, 2293–2303.

Pan, T., and Uhlenbeck, O.C. (1993). Circularly permuted DNA, RNA and proteins—a review. Gene *125*, 111–114.

Parsons, J.T., and Weber, M.J. (1989). Genetics of *src*: structure and functional organization of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. *147*, 79–127.

Pawson, T. (1994). SH2 and SH3 domains in signal transduction. Adv. Cancer Res. *64*, 87–110.

Pawson, T. (1995). Protein modules and signalling networks. Nature *373*, 573–580.

Resh, M.D. (1990). Membrane interactions of $pp60^{v\text{-}src}$: a model for myristylated tyrosine protein kinases. Oncogene *5*, 1437–1444.

Resh, M.D. (1994). Myristylation and palmitylation of Src family members: the fats of the matter. Cell *76*, 411–413.

Robbins, J., Dilworth, S.M., Laskey, R.A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell *64*, 615–623.

Rohrschneider, L.R. (1980). Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. Proc. Natl. Acad. Sci. USA *77*, 3514–3518.

Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994). A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J. *13*, 3748–56.

Schaller, M.D., and Parsons, J.T. (1994). Focal adhesion kinase and associated proteins. Curr. Opin. Cell Biol. *6*, 705–710.

Scharenberg, A.M., Lin, S., Cuenod, B., Yamamura, H., and Kinet, J.P. (1995). Reconstitution of interactions between tyrosine kinases and the high affinity IgE receptor which are controlled by receptor clustering. EMBO J. *14*, 3385–3394.

Schlaepfer, D.D., Broome, M.A., and Hunter, T. (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, $p130^{cas}$, and Nck adaptor proteins. Mol. Cell. Biol. *17*, 1702–1713.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature *372*, 786–791.

Sefton, B.M., Hunter, T., Ball, E.H., and Singer, S.J. (1981). Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell *24*, 165–174.

Smith, M.R., DeGudicibus, S.J., and Stacey, D.W. (1986). Requirement for c-ras protein during viral oncogene transformation. Nature *320*, 540–543.

Takeya, T., and Hanafusa, H. (1983). Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell *32*, 881–890.

Taylor, S.J., and Shalloway, D. (1994). An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature *368*, 867–871.

Temin, H.M. (1960). The control of cellular morphology in embryonic cells infected with Rous sarcoma virus in vitro. Virology *10*, 182–197.

Tian, M., and Martin, G.S. (1996). Reduced phosphotyrosine binding by the v-Src SH2 domain is compatible with wild-type transformation. Oncogene *12*, 727–734.

Turner, C.E. (1994). Paxillin: a cytoskeletal target for tyrosine kinases. Bioessays *16*, 47–52.

Verderame, M.F. (1997). $pp60^{v\text{-}src}$ transformation of rat cells but not chicken cells strongly correlates with low-affinity phosphopeptide binding by the SH2 domain. Mol. Biol. Cell *8*, 843–854.

Verderame, M.F., Kaplan, J.M., and Varmus, H.E. (1989). A mutation in v-*src* that removes a single conserved residue in the SH-2 domain of $pp60^{v\text{-}src}$ restricts transformation in a host-dependent manner. J. Virol. *63*, 338–348.

Verderame, M.F., and Varmus, H.E. (1994). Highly conserved amino acids in the SH2 and catalytic domains of v-src are altered in naturally occurring, transformation-defective alleles. Oncogene *9*, 175–182.

Woods, K.M., and Verderame, M.F. (1994). Autophosphorylation is required for high kinase activity and efficient transformation ability of proteins encoded by host range alleles of v-src. J. Virol. *68*, 7267–7274.