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Multiple SH2-Mediated Interactions In v-*src*-Transformed Cells

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The Src homology 2 (SH2) domain is a noncatalytic region which is conserved among a number of signaling and transforming proteins, including cytoplasmic protein-tyrosine kinases and Ras GTPase-activating protein (GAP). Genetic and biochemical data indicate that the SH2 domain of the p60$^{v\text{-}src}$ (v-Src) protein-tyrosine kinase is required for full v-*src* transforming activity and may direct the association of v-Src with specific tyrosine-phosphorylated proteins. To test the ability of the v-Src SH2 domain to mediate protein-protein interactions, v-Src polypeptides were expressed as fusion proteins in *Escherichia coli*. The bacterial v-Src SH2 domain bound a series of tyrosine-phosphorylated proteins in a lysate of v-*src*-transformed Rat-2 cells, including prominent species of 130 and 62 kDa (p130 and p62). The p130 and p62 tyrosine-phosphorylated proteins that complexed v-Src SH2 in vitro also associated with v-Src in v-*src*-transformed Rat-2 cells; this in vivo binding was dependent on the v-Src SH2 domain. In addition to binding soluble p62 and p130, the SH2 domains of v-Src, GAP, and v-Crk directly recognized these phosphotyrosine-containing proteins which had been previously denatured and immobilized on a filter. In addition, the SH2 domains of GAP and v-Crk bound to the GAP-associated protein p190 immobilized on a nitrocellulose membrane. These results show that SH2 domains bind directly to tyrosine-phosphorylated proteins and that the Src SH2 domain can bind phosphorylated targets of the v-Src kinase domain. In v-*src*-transformed cells, p62 is a prominent tyrosine-phosphorylated SH2-binding protein that is found in independent complexes with v-Src and GAP. The transformation-related assembly of these complexes suggests a network of SH2-mediated interactions involving the SH2 domains of signaling proteins and their tyrosine-phosphorylated ligands, which are important for v-Src transforming activity and regulation of GAP function.

The Src homology 2 (SH2) domain of cytoplasmic protein-tyrosine kinases, such as those encoded by the *src*, *fps*, and *abl* genes, is a noncatalytic element located immediately N terminal to the kinase domain (26, 42). In activated variants of the Fps or Src tyrosine kinases, the SH2 domain is required for the efficient induction of cellular transformation. Deletions, substitutions, or insertions within the v-Src and v-Fps SH2 domains frequently impair or abrogate catalytic and transforming activities (6, 24, 25, 40, 42, 50). Furthermore, v-Fps or v-Src mutant proteins with structural alterations in the SH2 domains can induce temperature-sensitive or host-dependent transformed phenotypes (8, 9, 18, 19, 48).

Genetic and biochemical data have raised the possibility that the Src and Fps SH2 domains are involved in protein-protein interactions which regulate the association of the activated tyrosine kinases with certain cellular proteins. For example, a number of v-Src and v-Fps mutant proteins with alterations in their SH2 domains are defective in the phosphorylation of specific cellular proteins, notably a 62-kDa protein (p62), thereby implicating the SH2 domain in the recognition of cellular substrates (2, 25, 35, 52). In cells transformed by wild-type v-*src* and v-*fps*, tyrosine-phosphorylated p62 becomes physically associated with the Ras GTPase-activating protein (GAP) (2, 11, 35, 36); GAP itself is a substrate of these kinases and becomes tyrosine phosphorylated in v-*src*- and v-*fps*-transformed cells (2, 11, 36). In addition, v-Src physically associates in vivo with two tyrosine-phosphorylated proteins of 130 and 110 kDa (p130 and p110) (21, 41). Analysis of *src* deletion mutants has suggested that the v-Src SH2 domain provides a binding site for p130 following its phosphorylation by the kinase domain, whereas p110 may interact primarily with the more N-terminal SH3 domain (21). Activated Src also associates with phosphatidylinositol (PI) 3′-kinase activity (14–16) and can be found in a complex with GAP (3).

SH2 domains closely related in sequence to those first identified in the cytoplasmic tyrosine kinases have been identified in a number of cytoplasmic signaling proteins, including GAP, phospholipase C-γ, the p85 subunit of PI 3′-kinase, and the PTP1C tyrosine phosphatase (12, 13, 37, 43, 44, 46, 47, 49). SH2 domains have also been identified in two oncoproteins, v-Crk and Vav (22, 23, 26, 30), which do not contain obvious catalytic domains, and in the cytoskeletal protein tensin (7). The SH2 domains of these signaling and transforming proteins associate both in vivo and in vitro with a number of tyrosine-phosphorylated proteins (4, 26); a prototype for this interaction involves the binding of SH2 domains of signaling proteins, including Src, to autophosphorylated growth factor receptors (1). However, in addition to their propensity to associate with activated receptors, it is apparent that SH2 domains of cytoplasmic proteins are involved in a more complex series of protein-protein interactions. For example, the N-terminal SH2 domain of GAP is sufficient to bind tyrosine-phosphorylated p62 (10, 35), and the v-Crk SH2 domain associates with a number of phosphotyrosine-containing proteins from v-*crk*-transformed cells (28–30, 32, 33).

To directly test the proposition that the Src SH2 domain is

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involved in complex formation, and to investigate the extent of SH2-mediated interactions in v-*src*-transformed cells, we have examined the ability of bacterial fusion proteins containing the v-Src SH2 domain to bind cellular proteins in vitro.

MATERIALS AND METHODS

Cell culture and antibodies. Rat-2 and v-*src*-transformed Rat-2 (Rat-2 v-*src*) cell lines were maintained in Dulbecco's modified Eagle medium containing 50 U of penicillin and 50 μg of streptomycin per ml supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2. Rat-2 cells expressing the v-*src* XD6 deletion (residues 149 to 174) were a gift of Steven Martin (University of California, Berkeley) (8). Polyclonal rabbit antibodies against human GAP residues 171 to 448 have been described (11). Rabbit antiphosphotyrosine (anti-P.Tyr) antibodies were raised and affinity purified as described by Kamps and Sefton (20, 25). Anti-TrpE rabbit antiserum was raised against the N-terminal 323 residues of *Escherichia coli* TrpE protein. The anti-TrpE and anti-Src 327 mouse monoclonal antibodies were obtained from Oncogene Science Inc. (Manhasset, N.Y.). The anti-Src EB7 mouse monoclonal antibody was provided by Sally Parsons (University of Virginia, Charlottesville).

Immunoprecipitation. Approximately 5×10^6 cells were lysed in 1 ml of ice-cold PLC lysis buffer (PLC-LB) (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate [Na_3VO_4], 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin at 10 μg/ml each). Lysates were centrifuged at $10,000 \times g$ for 20 min at 4°C; 10 μl of anti-GAP antiserum or 1 μg of affinity-purified anti-P.Tyr antibodies or anti-Src (327) monoclonal antibodies were incubated with 1 ml of supernatant, and 100 μl of 10% protein A-Sepharose or 100 μl of 10% anti-mouse immunoglobulin agarose beads was added. These immune complexes were incubated for 90 min at 4°C, washed three times with HNTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4), and heated for 3 min in sodium dodecyl sulfate (SDS) sample buffer at 100°C.

Immunoblotting. Immunoprecipitates and bacterial complexes were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose in a semi-dry blotting apparatus at 0.8 mA/cm^2 for 60 min. Blots were blocked and then probed with affinity-purified rabbit anti-P.Tyr antibodies as described previously (25). Anti-P.Tyr blots were probed with 5 μCi of high-specific-activity ^{125}I-labeled protein A (35 μCi/μg; Amersham) and exposed to Kodak XAR film at −75°C with an intensifying screen. Alternatively, nitrocellulose filters immunoblotted with anti-P.Tyr or with anti-Src (EB7) antibodies (at 1 μg/ml) were further incubated with goat anti-rabbit or anti-mouse immunoglobulin antibody–alkaline phosphatase conjugates (Sigma) (25). These antibody complexes were visualized by incubating blots for 5 to 30 min in 100 mM Tris hydrochloride (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$ containing 6.6 μl of nitroblue tetrazolium per ml (50 μg/ml in 70% dimethylformamide) and 3.3 μl of 5-bromo-4-chloro-3-indolyl phosphate per ml (50 μg/ml in 100% dimethylformamide) (Sigma).

Complex formation with fusion proteins. The subcloning of restriction fragments from human GAP, v-*crk*, or v-*src* (encoding v-Src residues 87 to 291, Src-SH3/2) cDNAs into pATH bacterial TrpE expression vectors was performed by using both natural and engineered restriction sites and has been previously described (1). The Src-SH3 coding sequence (for amino acid residues 77 to 168) was excised from v-*src* plasmid DNA by using *Nci*I restriction endonuclease. Following extension with the large fragment of DNA polymerase, the fragment was inserted into the *Sma*I site in the polylinker of the pATH-2 *trpE* expression vector. In addition to Src-SH3 sequences, the TrpE-Src-SH3 fusion contains 25 C-terminal bacterially derived amino acids. To construct the Src-SH2 fusions, the coding sequence for v-Src residues 148 to 251 was amplified by polymerase chain reaction and inserted between the *Bam*HI and *Eco*RI sites of pGEX-2T or the *Bam*HI and *Sal*I sites of pATH-3. Cultures of *E. coli* RR1 with pATH expression plasmids were grown, induced with indoleacrylic acid, and lysed with ice-cold PLC-LB as described previously (1, 35). The TrpE fusion proteins were recovered from the supernatants by immunoprecipitation with polyclonal anti-TrpE antiserum immobilized on protein A-Sepharose beads. Immune complexes were washed, aliquoted, flash-frozen, and stored at −70°C until mixed with clarified mammalian cell lysate derived from approximately 5×10^6 cells. Mammalian cells were lysed on ice in 1 ml of PLC-LB and clarified as previously described (1, 35). Mixing was performed by using gentle inversion for 90 min at 4°C. Complexes were then washed three times with HNTG buffer, resuspended in 50 μl of SDS sample buffer, heated at 100°C for 3 min, and analyzed by immunoblotting with affinity-purified rabbit anti-P.Tyr antibodies. To verify that the different TrpE fusion proteins were present in equivalent amounts in the immune complexes, duplicate blots were probed with anti-TrpE rabbit antiserum. The inclusion of 1 mM dithiothreitol to reduce any adventitiously formed disulfide bonds in the bacterial SH2 domains had no effect on the binding specificities of the TrpE fusion proteins.

A similar approach was taken to isolate glutathione *S*-transferase (GST) fusion proteins, except that these were induced with isopropyl-β-D-thiogalactopyranoside as previously described and immobilized on glutathione-agarose beads (45). To ensure that the different fusion proteins were present in equivalent amount in these mixes, duplicate samples separated by SDS-PAGE were stained with Coomassie blue.

In vitro kinase reactions. Immunoprecipitates or bacterial fusion protein complexes (see above) were washed with HNTG buffer three times, resuspended in 15 μl of kinase reaction buffer (50 mM HEPES [pH 7.0], 10 mM $MnCl_2$) containing 2.5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and incubated for 10 min at 37°C. Reactions were terminated by the addition of 15 μl of 2× SDS sample buffer followed by heating at 100°C for 3 min. SDS-gels containing separated phosphoproteins were treated with 1 M KOH at 55°C for 2 h (5) prior to autoradiography.

Blotting with TrpE fusion proteins. Immunoprecipitates or whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose filters. Replica blots were blocked with 50 mM Tris (pH 8.0)–150 mM NaCl–0.02% NaN_3–3% Carnation skim mild powder–0.05% Tween 20 and then probed with an induced bacterial culture expressing TrpE or the TrpE fusion proteins, at a concentration of TrpE proteins of approximately 1 to 5 μg/ml. After a 60-min incubation at 25°C with gentle rotation, the blots were washed with blocking solution and probed with polyclonal anti-TrpE antiserum (1:200 dilution) or with 2 μg of monoclonal anti-TrpE antibody per ml for 60 min at the same temperature. Following washing, blots were either incubated with ^{125}I-

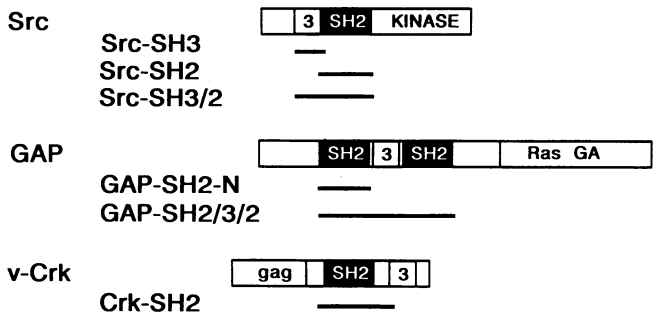

FIG. 1. Locations of the SH2 and SH3 domains in signaling and transforming proteins. The solid bars beneath the proteins indicate the regions expressed as bacterial TrpE or GST fusions used in the in vitro binding experiments. These fusions possess the following residues: TrpE-Src-SH3, v-Src 77 to 168; TrpE-Src-SH2 or GST-Src-SH2, v-Src 148 to 251; TrpE-Src-SH3/2, v-Src 87 to 291; TrpE-GAP SH2-N, human GAP 178 to 277; TrpE-GAP-SH2/3/2, GAP 178 to 444; TrpE-Crk-SH2, $P47^{gag\text{-}crk}$ 205 to 386. 3, SH3 domain; GA, GTPase-activating region of GAP; kinase, Src tyrosine kinase domain.

labeled protein A and exposed to film or incubated with alkaline phosphatase-conjugated antibodies followed by color reaction substrates.

Vanadate treatment of cell lysates. Approximately 5×10^6 cells were lysed with 250 μl of ice-cold PLC-LB with or without 100 μM Na_3VO_4. Cell lysates were clarified and left at 4°C for 24 h. Aliquots of these samples were quantified for protein content by a modified Lowry method (39). Each sample received 250 μl of 2× SDS sample buffer and was heated at 100°C for 3 min prior to SDS-PAGE.

Tryptic phosphopeptide mapping. Phosphopeptide mapping was performed essentially as described by Luo et al. (27). For mapping of p62, Rat-2 v-*src* cells were metabolically labeled with $^{32}P_i$ as described previously (36). Lysates were prepared and incubated with immobilized SH2-containing bacterial TrpE fusion proteins as described above. For p130 mapping, proteins associated with the bacterial fusion proteins or immunoprecipitates from Rat-2 v-*src* lysates were labeled with [γ-^{32}P]ATP in vitro. Phosphorylated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. These proteins were localized by autoradiography and excised from the nitrocellulose. Proteins were digested directly on nitrocellulose membranes with 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington) in 175 μl of 50 mM NH_4HCO_3 at 37°C for 2 h, at which time 10 μg of fresh trypsin was added and the incubation was continued for another 2 h. Following digestion, the supernatants were dried under vacuum and washed three times with water. The tryptic peptides were oxidized with performic acid and analyzed by electrophoresis at pH 2.1 (first dimension) and by ascending chromatography (second dimension) on thin-layer cellulose (TLC) plates as described previously (51).

RESULTS

The Src SH2 domain binds tyrosine-phosphorylated proteins from v-*src*-transformed cells. To test the ability of the v-Src SH2 and SH3 domains to bind tyrosine-phosphorylated proteins in lysates of v-*src*-transformed cells, sequences from the noncatalytic N-terminal region of v-Src were expressed in *E. coli* as TrpE fusion proteins (Fig. 1). We synthesized three bacterial proteins, of which one, Src-SH3/2, contains both the SH2 and SH3 domains. Src-SH3 contains the v-Src SH3 motif as well as 21 N-terminal amino acids of the SH2 domain, whereas Src-SH2 is composed of the SH2 domain alone. These bacterial proteins were isolated by immunoprecipitation from induced bacterial lysates, using anti-TrpE antibodies attached to Sepharose beads. The immobilized bacterial fusion proteins were then incubated with lysates of normal Rat-2 fibroblasts or Rat-2 v-*src* cells. P.Tyr-containing proteins which complexed with the bacterial fusion polypeptides were identified by immunoblotting with anti-P.Tyr antibodies. In a lysate of Rat-2 v-*src* cells, the TrpE Src-SH3/2 protein formed stable complexes with P.Tyr-containing proteins of 62, 70, and 130 kDa (p62, p70, and p130) (Fig. 2A, lane 3). In some experiments, additional minor P.Tyr-containing proteins could be identified in association with TrpE-Src-SH3/2 (Fig. 2B). The P.Tyr-containing proteins that bound with high affinity to TrpE-Src-SH3/2 appeared to represent a subset of the total tyrosine-phosphorylated proteins in v-*src*-transformed cells (Fig. 2A). A more detailed analysis employing fractionated P.Tyr-containing proteins from Rat-2 v-*src* cells has confirmed that bacterially expressed SH2 domains bind only a limited group of such phosphoproteins with high affinity in vitro (34a). The TrpE-Src-SH2 bacterial protein also bound to tyrosine-phosphorylated proteins in a lysate of Rat-2 v-*src* cells, although with lower efficiency than did the Src-SH3/2 fusion (Fig. 2B). In contrast, these phosphoproteins did not bind stably to Src-SH3 or to the parental TrpE protein (Fig. 2A, lanes 1 and 2), nor were they detected following the incubation of Src-SH3/2 with a normal Rat-2 cell lysate (data not shown). These data indicate that the Src SH2 domain binds stably to specific tyrosine-phosphorylated proteins in v-*src*-transformed cells.

The possible influence of the linked TrpE sequences on the binding activity of the Src SH2 domain was investigated by constructing a Src-SH2 bacterial fusion protein containing an N-terminal GST sequence in place of TrpE. The GST-Src-SH2 fusion protein associated with a range of P.Tyr-containing proteins similar in mobility to those bound by the Src-SH3/2 and Src-SH2 TrpE fusion proteins, notably p62, p70, and p130 (data not shown). The ability of the Src-SH2 domain to complex with tyrosine-phosphorylated proteins is therefore independent of the covalently linked, bacterially encoded polypeptide.

Similar Src-SH2 complexes are formed in vitro and in vivo. The v-Src tyrosine kinase is known to associate with several tyrosine-phosphorylated proteins in v-*src*-transformed cells (21, 41). To investigate the extent to which the complexes formed by the Src-SH2 domain in vitro mimic the interactions between activated v-Src and its substrates in vivo, we compared the proteins associated with v-Src from Rat-2 v-*src* cells with those bound by Src-SH2 in vitro. Anti-Src immunoprecipitates were separated by gel electrophoresis and analyzed by immunoblotting with anti-P.Tyr antibodies (Fig. 2C). As previously described, the most prominent v-Src-associated species is a 130-kDa phosphoprotein (p130) (21, 41). However, we also detected several more minor coprecipitating tyrosine-phosphorylated proteins, including proteins of 62 and 70 kDa (Fig. 2C, lanes 4 and 6). These v-Src-associated proteins comigrated with the p130, p62, and p70 tyrosine-phosphorylated polypeptides that bound to the bacterial fusion protein containing Src SH3 and SH2 domains in vitro (Fig. 2A).

If these phosphoproteins were indeed complexed with the Src SH2 domain in vivo, then a v-*src* mutant with a deletion

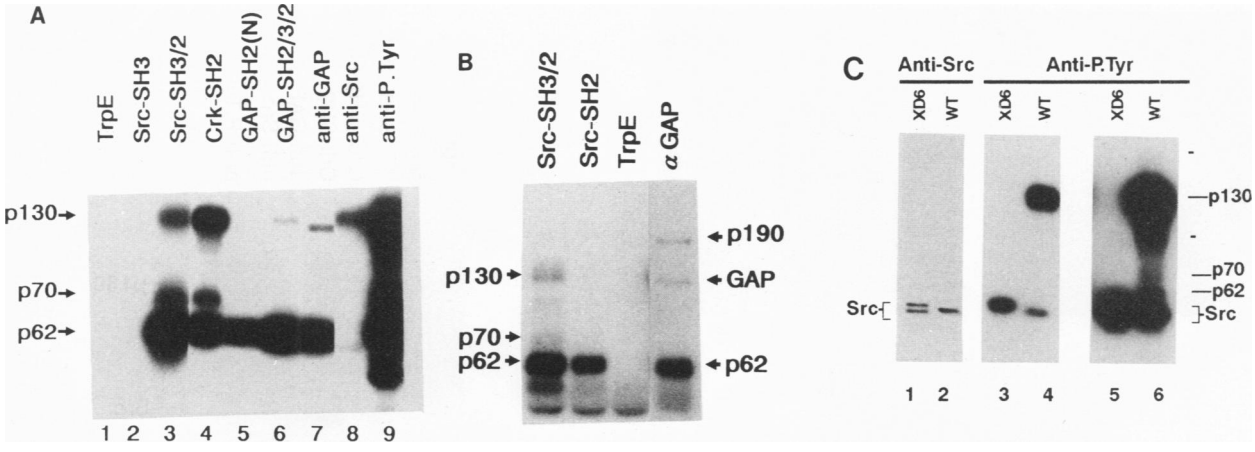

FIG. 2. In vitro and in vivo association of the Src SH2 domain with tyrosine-phosphorylated proteins from Rat-2 v-*src* cells. (A) TrpE alone or the Src-SH3, Src-SH3/2, Crk-SH2, GAP SH2-N, or GAP-SH2/3/2 bacterial TrpE fusion proteins were immobilized with anti-TrpE antibodies and mixed with a Rat-2 v-*src* cell lysate (lanes 1 to 7). Complexes were washed, resolved on 7.5% SDS-polyacrylamide gels, and analyzed for associated proteins by anti-P.Tyr immunoblotting. Complexed proteins of 62, 70, and 130 kDa are indicated. For comparison, anti-GAP, anti-Src (327), and anti-P.Tyr immunoprecipitates from the same cell lysate were analyzed by immunoblotting with anti-P.Tyr antibodies (lanes 7 to 9). Tyrosine-phosphorylated GAP and v-Src are not arrowed but can be seen migrating below p130 and p62 in lanes 7 and 8, respectively. Immunoblotting was followed by ^{125}I-protein A incubation and autoradiography for 16 h. (B) Immobilized TrpE-Src-SH3/2, TrpE-Src-SH2, or parental TrpE bacterial proteins were incubated with a lysate of Rat-2 v-*src* cells, as indicated. Associated P.Tyr-containing proteins were identified by immunoblotting with anti-P.Tyr antibodies, followed by incubation with ^{125}I-protein A. For comparison, an anti-GAP immunoprecipitate from the same lysate (αGAP) was coelectrophoresed and immunoblotted with anti-P.Tyr antibodies. The migration of Src SH2-associated proteins is shown at the left; GAP and GAP-associated proteins are indicated at the right. (C) Anti-Src (327) immunoprecipitates from Rat-2 cells expressing the XD6 v-*src* SH2 deletion (XD6) or wild type v-*src* (WT) were immunoblotted with anti-Src (EB7) antibody (lanes 1 and 2). Src proteins were detected by using goat anti-mouse immunoglobulin antibody–alkaline phosphatase conjugate and color reaction substrates. A duplicate nitrocellulose filter was analyzed for Src-associated proteins by immunoblotting with anti-P.Tyr antibodies, followed by ^{125}I-protein A incubation and then exposure to film for 16 h (lanes 3 and 4) or 96 h (lanes 5 and 6).

in SH2 should be defective in complex formation. Previous work has indicated that the ability of v-Src to bind p130 is dependent on an intact SH2 domain (41). The XD6 v-Src mutant, which encodes a protein that lacks residues 149 to 174, corresponding to the most highly conserved region of the SH2 domain, was used to test this possibility. The XD6 Src protein is stable and retains enzymatic activity in Rat-2 cells but is poorly transforming and is defective in the phosphorylation of p62 and GAP (8, 35).

Probing of anti-Src immunoprecipitates with an anti-Src monoclonal antibody showed that comparable amounts of wild-type and XD6 mutant Src proteins were recovered in the immunoprecipitates (Fig. 2C, lanes 1 and 2). Curiously, the XD6 mutant v-Src protein resolved into two species. The faster-migrating form (relative to wild-type v-Src) is an expected consequence of the 25-amino-acid deletion in XD6. The more slowly migrating band corresponds to a highly tyrosine phosphorylated form of XD6 protein (Fig. 2C; compare lanes 1 and 3); this apparent increase in tyrosine phosphorylation may account for the decreased electrophoretic mobility of one form of XD6 v-Src. Unlike wild-type v-Src, the XD6 mutant v-Src tyrosine kinase isolated from Rat-2 cells did not coprecipitate with any tyrosine-phosphorylated proteins (Fig. 2C, lanes 3 and 5). These results indicate that the XD6 deletion destroys the ability of v-Src to complex P.Tyr-containing proteins in vivo. The association of a bacterially expressed Src SH2 domain with specific tyrosine-phosphorylated proteins in a Rat-2 v-*src* cell lysate therefore represents an in vitro reconstruction of interactions that normally occur in v-*src*-transformed cells.

p62 binds to both v-Src and GAP SH2 domains in vitro and in vivo. The identities of the tyrosine-phosphorylated proteins that associate with the Src SH2 domain are unknown. To investigate their possible significance, we compared them with the phosphoproteins that bind to other SH2-containing proteins, such as GAP and v-Crk. We have previously identified a 62-kDa protein (p62) that becomes stably complexed with GAP in v-*src*-transformed or epidermal growth factor-stimulated cells (11). This protein binds to the N-terminal GAP SH2 domain in vitro (35). The tyrosine-phosphorylated 62-kDa protein that bound to the Src SH2 domain in vitro and in vivo was identical in mobility to p62, which associated with GAP in vivo and which complexed with a bacterial TrpE-GAP SH2 polypeptide in vitro (Fig. 2A, lanes 3, 5, 6, and 7). These results are consistent with the notion that p62 interacts with both the Src SH2 and GAP SH2 domains in v-*src*-transformed cells. The amount of tyrosine-phosphorylated p62 which is complexed with v-Src in vivo is rather minor compared with that associated with GAP, raising the possibility that p62 interacts only transiently or with low efficiency with v-Src but enters a more stable complex with GAP.

Src, Crk, and GAP SH2 domains bind a related set of tyrosine-phosphorylated proteins in vitro. The p47$^{gag\text{-}crk}$ oncoprotein forms complexes with several tyrosine-phosphorylated proteins in v-*crk*-transformed cells and associates with a tyrosine kinase activity (30, 31, 33). These same proteins bind to v-Crk in vitro (28, 29, 33). As with v-Src, the binding of tyrosine-phosphorylated proteins to v-Crk and v-*crk* transforming activity both require an intact SH2 domain (29, 32). To compare their in vitro binding activities, the TrpE-Src-SH3/2 and TrpE-Crk-SH2 bacterial fusion proteins were incubated with lysates of Rat-2 v-*src* cells. The same group of tyrosine-phosphorylated proteins (p62, p70,

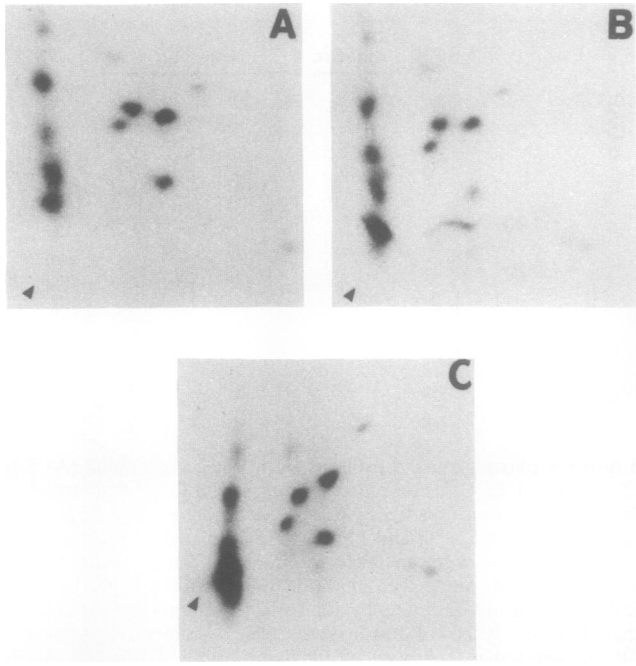

FIG. 3. Tryptic phosphopeptide analysis of p62 phosphoproteins associated with Src and GAP SH2 domains. ^{32}P-labeled p62 was isolated from metabolically labeled Rat-2 v-*src* cells by binding to either TrpE-GAP SH2-N or TrpE-Src SH3/2. SH2-associated p62 was digested with trypsin, and the resulting phosphopeptides were separated in two dimensions by electrophoresis and chromatography on TLC plates. Tryptic phosphopeptides were visualized by autoradiography. (A) Tryptic phosphopeptide map of p62 bound to TrpE-GAP SH2-N (700 cpm); (B) map of p62 bound to TrpE-Src-SH3/2 (700 cpm); (C) map of a mix of the two p62 preparations (700 cpm each). Maps were exposed to X-ray film at −70°C with intensifying screens for 2 days. The origin is indicated by an arrowhead.

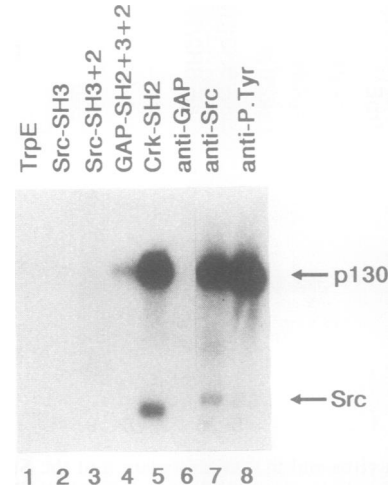

FIG. 4. GAP and Crk SH2 fusion proteins associate with tyrosine kinase activity in Rat-2 v-*src* cell lysates. TrpE alone (lane 1) or the Src-SH3 (lane 2), Src-SH3/2 (lane 3), GAP-SH2/3/2 (lane 4) or Crk-SH2 (lane 5) fusion proteins immobilized with anti-TrpE antibodies were mixed with Rat-2 v-*src* cell lysates, washed, and incubated in an in vitro kinase reaction with [γ-^{32}P]ATP. Anti-GAP (lane 6), anti-Src (327) (lane 7), and anti-P.Tyr (lane 8) immunoprecipitates were from the same cell lysate and were treated identically. Reaction products were separated on 8.25% SDS-polyacrylamide gels, followed by KOH treatment to increase the detection of labeled P.Tyr. The gel was dried at 80°C under vacuum, and autoradiography was for 20 min at −75°C with an intensifying screen.

and p130) was recognized by the v-Src and v-Crk SH2 domains (Fig. 2A, lanes 3 and 4), although the Crk SH2 domain bound more efficiently. As previously noted, a bacterial fusion protein containing the N-terminal GAP SH2 domain (GAP SH2-N) bound preferentially to p62, while a fusion protein containing both N-terminal and C-terminal GAP SH2 domains and the intervening SH3 domain (GAP-SH2/3/2) also complexed with p130 (Fig. 2A, lanes 5 and 6) (35). We have not detected in vitro binding of GAP SH2 domains to p70.

We compared the 62-kDa P.Tyr-containing proteins that bound to the GAP and v-Src SH2 domains by tryptic phosphopeptide analysis. Rat-2 v-*src* cells were metabolically labeled with $^{32}P_i$, lysed, and incubated with immobilized TrpE-GAP SH2-N or TrpE-Src-SH3/2 bacterial fusion proteins. The ^{32}P-labeled p62 proteins that associated in vitro with the GAP SH2-N and Src SH2 domains were isolated and subjected to tryptic phosphopeptide mapping (Fig. 3). The two p62 phosphopeptide maps were identical to one another, suggesting that the same p62 protein associates with both the GAP and Src SH2 domains. The tryptic phosphopeptide map of p62 was quite different from that of $p60^{v\text{-}src}$ (data not shown).

To compare the p130 SH2-associated protein, we made use of the observation that p130 can be readily phosphorylated in vitro. Incubation of an anti-v-Src immunoprecipitate with [γ-^{32}P]ATP induced the phosphorylation of both v-Src and p130 (Fig. 4, lane 7). Incubation of the fusion protein containing both GAP SH2 domains (TrpE-GAP-SH2/3/2) or the TrpE-Crk-SH2 protein with a Rat-2 v-*src* lysate followed by an in vitro kinase reaction also resulted in the specific phosphorylation of p130 on tyrosine (Fig. 4, lanes 4 and 5). p130 complexed with Src-SH3/2 in vitro did not become phosphorylated in a kinase reaction (Fig. 4, lane 3). Two-dimensional tryptic phosphopeptide maps of the p130 protein phosphorylated in vitro in Src immunoprecipitates and in TrpE-Crk-SH2 and TrpE-GAP-SH2/3/2 complexes were identical (Fig. 5). These results imply that the same 130-kDa protein associates with the Src SH2 domain in vivo as is recognized by the TrpE-SH2 bacterial proteins in vitro. In addition, the Crk and GAP SH2 domains complex with tyrosine kinase activity for which p130 is a preferential substrate, although this kinase apparently does not bind Src SH2. The kinase activity associated with the Crk and GAP SH2 domains could be due to a low level of associated v-Src.

Src, Crk, and GAP SH2 domains binds directly to tyrosine-phosphorylated proteins. A bacterially expressed Abl SH2 domain can bind directly to tyrosine-phosphorylated proteins from *abl*-transformed cells which have been immobilized on a filter (34). This procedure was adapted to examine the ability of the Src, GAP, and Crk SH2 domains to bind to proteins from Rat-2 v-*src* cells. Proteins in anti-P.Tyr immunoprecipitates from Rat-2 cells or Rat-2 v-*src* cells were denatured, resolved by SDS-PAGE, and transferred to nitrocellulose filters. Replica nitrocellulose filters were prepared and blotted with induced bacterial lysates containing the TrpE-Src-SH3/2, TrpE-GAP-SH2/3/2, or TrpE-Crk-SH2 fusion proteins. The interactions of these fusion proteins with the immobilized proteins were detected by subsequent

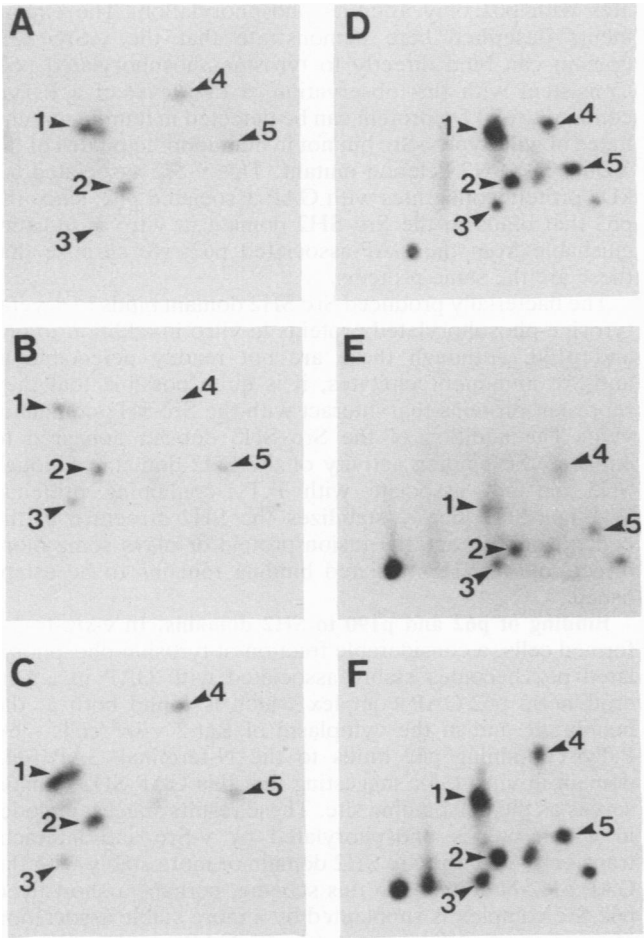

FIG. 5. Comparative tryptic phosphopeptide mapping of the 130-kDa protein associated with Crk and GAP SH2 domains and with v-Src. Crk-SH2 or GAP-SH2/3/2 fusion proteins complexed with proteins from Rat-2 v-*src* lysates and anti-Src (327) immunoprecipitates from the same cell lysate were incubated in a kinase reaction. ^{32}P-labeled phosphoproteins of 130 kDa were digested with trypsin and analyzed by two-dimensional separation on TLC plates. Tryptic phosphopeptide analysis of the 130-kDa phosphoprotein associated with Crk-SH2 (A), anti-Src (327) immune complex (B and E), or GAP-SH2/3/2 (D) was performed. To compare these phosphopeptides, samples from panels A and B and from panels D and E were mixed (C and F, respectively). Phosphopeptide maps from panels A to C and from panels D to F represent results from two separate experiments. The single samples had 500 cpm, with the exception of 200 cpm in sample E, and the mixtures had 500 cpm of each component. Autoradiographs were exposed for 7 days (A to C), 10 days (D and E), or 5 days (F) at −75°C with an intensifying screen. The five major tryptic phosphopeptides (1 through 5) are indicated with arrowheads.

incubation of the filters with anti-TrpE antibodies and ^{125}I-protein A. All three SH2-containing fusion proteins recognized proteins of 62 and 130 kDa on the filter (Fig. 6). In the case of Crk-SH2, an additional interaction with p70 was noted. No association with these proteins was observed following incubation with TrpE alone (Fig. 6, lanes 1 to 4) or with fusion proteins containing only the SH3 domains of v-Src or v-Crk (data not shown). Specific binding to the same set of proteins was also observed when a whole Rat-2 v-*src* lysate was probed directly with a TrpE-Crk-SH2 bacterial

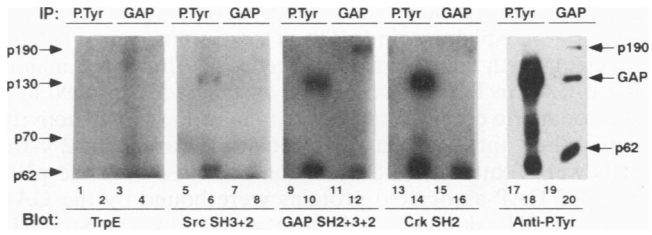

FIG. 6. Binding of bacterial SH2 fusion proteins directly to denatured tyrosine-phosphorylated proteins. Sets of anti-P.Tyr and anti-GAP immunoprecipitates from Rat-2 or Rat-2 v-*src* cells were separated by SDS-PAGE and transferred to nitrocellulose filters. Replica blots were incubated first with bacterial TrpE fusion proteins (as shown) at approximately 1 μg/ml and then with anti-TrpE antibodies, followed by ^{125}I-protein A incubation and autoradiography for 16 h. Primary incubation was with TrpE (lanes 1 to 4), TrpE-Src-SH3/2 (lanes 5 to 8), TrpE-GAP-SH2/3/2 (lanes 9 to 12), and TrpE-Crk-SH2 (lanes 13 to 16). In lanes 17 to 20, a filter identical to those described above was instead analyzed by immunoblotting with anti-P.Tyr antibodies and ^{125}I-protein A. Autoradiography was for 6 h (lanes 17 to 19) or 16 h (lane 20).

fusion protein, without prior immunoprecipitation with anti-P.Tyr antibodies (Fig. 7). In this case, SH2 binding was markedly increased by incubation of the cell lysate with the tyrosine phosphatase inhibitor Na_3VO_4 (Fig. 7, lane 7), consistent with the suggestion that tyrosine phosphorylation is crucial for SH2-protein interactions (28, 34, 35).

These results demonstrate that SH2 domains of distinct signaling proteins can bind directly to a similar group of P.Tyr-containing proteins under denaturing conditions. Furthermore, the bacterial SH2 domains generally complexed with P.Tyr-containing proteins of the same mobility whether the tyrosine-phosphorylated proteins were in solution or immobilized on nitrocellulose filters.

SH2 domains bind p190 in vitro following denaturation of

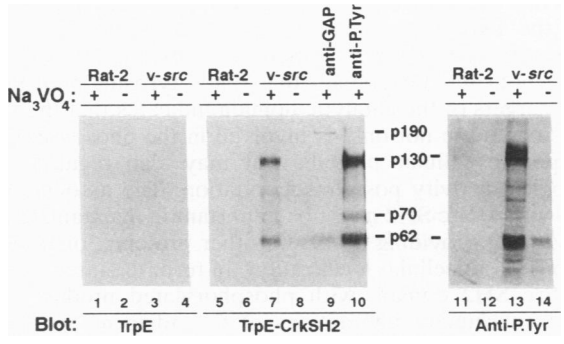

FIG. 7. Evidence that Na_3VO_4 treatment of cell lysates enhances the ability of TrpE-CrkSH2 to bind proteins from Rat-2 v-*src* lysates. Rat-2 or Rat-2 v-*src* cells were lysed with or without Na_3VO_4 as described in Materials and Methods. Total protein (25 μg) from these lysates was loaded as indicated (lanes 1 to 8 and 11 to 14), resolved by 7.5% SDS-PAGE, and transferred to nitrocellulose filters. Anti-GAP and anti-P.Tyr immunoprecipitates from Rat-2 v-*src* lysates (lanes 9 and 10) were used as a comparison for cellular proteins bound in this assay. Blots were incubated first with TrpE (lanes 1 to 4) or TrpE-CrkSH2 (lanes 5 to 10) proteins at approximately 5 μg/ml and then with monoclonal anti-TrpE antibodies. To identify P.Tyr-containing proteins, Western immunoblot blot analysis using anti-P.Tyr antibodies was used (lanes 11 to 14). Antibody complexes were visualized by using alkaline phosphatase-conjugated antibodies and color reaction substrates.

the cell lysate. In addition to p62, GAP associates with a 190-kDa phosphoprotein (p190) in v-*src*-transformed or epidermal growth factor-stimulated cells (11). Indeed, a majority of GAP in Rat-2 v-*src* cells is associated with p190 in a heterodimeric complex that appears to repress GAP activity (36). When anti-GAP immunoprecipitates from Rat-2 v-*src* cells were blotted with SH2 fusion proteins, both the p190 and p62 GAP-associated proteins were bound by the GAP and Crk SH2 domains and more weakly by the Src SH2 domain (Fig. 6, lanes 8, 12, and 16). The inability to detect SH2 binding of soluble tyrosine-phosphorylated p190 in a Rat-2 v-*src* cell lysate may indicate that p190 is all sequestered in complexes with GAP or other SH2-containing proteins and that its binding activity is revealed only following denaturation, as previously suggested (10), or may be due to a low stoichiometry of p190 tyrosine phosphorylation.

DISCUSSION

Src SH2 binds several tyrosine-phosphorylated proteins in vitro and in vivo. v-Src mutant proteins with deletions or substitutions in the SH2 domain fail to interact with a number of cellular proteins. They do not bind p130 (21), are deficient in inducing the tyrosine phosphorylation of p62 and GAP (2, 35), fail to bind effectively with PI 3′-kinase activity (15), and are not appropriately localized to the cytoskeleton (17). These data imply that the v-Src SH2 domain participates in a series of interactions with cellular proteins. We have addressed this issue directly by investigating the ability of bacterially expressed Src SH2 domain to bind tyrosine-phosphorylated proteins in vitro.

These binding experiments indicate that the Src SH2 domain forms complexes in vitro with specific P.Tyr-containing proteins from v-*src*-transformed Rat-2 fibroblasts and that at least some of these complexes mimic interactions that occur in vivo. The most prominent SH2-binding phosphoproteins identified in these incubations are p62, p70, and p130. The 130-kDa protein that associates with the Src SH2 domain in vitro comigrates with the tyrosine-phosphorylated p130 that becomes stably complexed with v-Src in vivo (41), and the two have similar tryptic phosphopeptide maps, suggesting that they are identical. The observation that v-Src has a noncatalytic domain that binds tyrosine-phosphorylated targets of the catalytic domain indicates that the v-Src tyrosine kinase not only is involved in the phosphorylation of specific cellular proteins but may also regulate their biological activity postphosphorylation. The association of p130 with v-Src SH2 might be important in maintaining p130 activity, in providing access to other proteins, or in determining its subcellular localization. In turn, the interaction of the Src SH2 domain with phosphorylated products may affect subsequent interactions of the adjacent Src kinase domain, for example, by promoting the phosphorylation of additional substrates. In this context, the apparent increase in autophosphorylation seen in the XD6 v-Src protein might be a consequence of decreased intermolecular interaction of the mutant protein's kinase domain with exogenous substrates.

We have provided indirect evidence that the Src SH2 domain might interact with p62, since the accumulation of high levels of tyrosine-phosphorylated p62 in Rat-2 v-*src* cells requires the v-Src SH2 domain (35). Because p62 can presently be identified only in its tyrosine-phosphorylated state, it is unclear whether the v-Src SH2 domain contributes to the recognition of unphosphorylated p62 and its phosphorylation by the kinase domain or whether v-Src SH2 associates with p62 only after its phosphorylation. The experiments described here demonstrate that the v-Src SH2 domain can bind directly to tyrosine-phosphorylated p62. Consistent with this observation, a low level of a P.Tyr-containing 62-kDa protein can be detected in immunoprecipitates of wild-type v-Src but not in immunoprecipitates of the XD6 v-Src SH2 deletion mutant. This v-Src-associated 62-kDa protein comigrates with GAP-associated p62; since the p62 that binds to the Src SH2 domain in vitro is indistinguishable from the GAP-associated p62, we surmise that these are the same proteins.

The bacterially produced Src SH2 domain binds to several tyrosine-phosphorylated proteins in vitro in addition to p62 and p130. Although these are not readily detectable in anti-Src immunoprecipitates, it is quite possible that they represent proteins that interact with the Src SH2 domain in vivo. The addition of the Src SH3 domain appeared to enhance the binding activity of the SH2 domain, although SH3 did not associate with P.Tyr-containing proteins. Whether SH3 simply stabilizes the SH2 structure in the context of the bacterial fusion protein or plays some more direct role in SH2-mediated binding remains to be established.

Binding of p62 and p190 to SH2 domains. In v-*src*-transformed cells, a considerable fraction of tyrosine-phosphorylated p62 becomes stably associated with GAP in a heterodimeric p62-GAP complex which is found both at the membrane and in the cytoplasm of Rat-2 v-*src* cells (36). P.Tyr-containing p62 binds to the N-terminal GAP SH2 domain in vitro (35), suggesting that this GAP SH2 domain serves as the p62 binding site. These results suggest a model in which p62 is phosphorylated by v-Src and interacts transiently with the Src SH2 domain or more stably with the GAP SH2-N domain. In this scheme, perhaps a short-lived p62-Src complex is supplanted by a more stable association of tyrosine-phosphorylated p62 with GAP.

Bacterially produced Src, GAP, and v-Crk SH2 domains bind to a similar set of tyrosine-phosphorylated proteins regardless of whether these are in solution or immobilized on a filter. The ability of these SH2 domains to bind to P.Tyr proteins which have been previously denatured by boiling in SDS is consistent with the proposition that SH2 domains recognize short peptide sequences and that tyrosine phosphorylation is a key regulator of SH2-binding (26).

The majority of GAP in v-*src*-transformed cells is associated with p190, in a complex which seems to reduce the ability of GAP to stimulate Ras GTPase activity (36). The SH2 domains of GAP and v-Crk complexed directly with nitrocellulose-bound p190 from a lysate that had been resolved on an SDS-polyacrylamide gel (Fig. 6, lanes 8, 12, and 16). Src SH2 binding to p190 immobilized on a membrane was not as efficient, and is not evident in Fig. 6, but was detectable after prolonged autoradiography (data not shown). In Fig. 6, it is apparent that GAP (and v-Crk) SH2 domains bind to p190 recovered from Rat-2 v-*src* lysates by immunoprecipitation with anti-GAP but not anti-P.Tyr antibodies (compare lanes 10 and 12). This result shows that p190 is not a major P.Tyr-containing protein, consistent with our previous results showing p190 to contain predominantly phosphoserine (36). The finding that GAP SH2 domains can bind p190 in vitro raises the possibility that under some circumstances, serine/threonine phosphorylation can also regulate SH2 binding. This is consistent with our previous demonstration of the sensitivity of the GAP-p190 complex to phosphatase treatment (36) and the recent demonstration of P.Tyr-independent SH2 binding in the Bcr-Abl protein (38).

However, the relative contribution of serine/threonine and tyrosine phosphorylation of p190 to its association with GAP remains to be established.

A network of SH2-mediated interactions in v-src-transformed cells. These data suggest a network of interactions in which a single tyrosine-phosphorylated ligand such as p62 can interact in vivo with multiple SH2-containing proteins. Conversely, the SH2 domains of v-Src and GAP can each form separate complexes with several distinct phosphoproteins. This system could provide a rapid means of communication between different signal transduction pathways. The biological significance of these SH2-ligand interactions in v-*src*-transformed cells is suggested by two observations. One is that the v-Src SH2 deletion which prevents complex formation also impairs transforming activity in Rat-2 cells. In addition, the inhibitory effect of p190 on GAP activity indicates that this SH2-mediated association may be important in controlling Ras activity.

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