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Molecular Features of the Viral and Cellular Src Kinases Involved in Interactions with the GTPase-Activating Protein

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GTPase-activating protein (GAP) enhances the rate of GTP hydrolysis by cellular Ras proteins and is implicated in mitogenic signal transduction. GAP is phosphorylated on tyrosine in cells transformed by Rous sarcoma virus and serves as an in vitro substrate of the viral Src (v-Src) kinase. Our previous studies showed that GAP complexes stably with normal cellular Src (c-Src), although its association with v-Src is less stable. To further investigate the molecular basis for interactions between GAP and the Src kinases, we examined GAP association with and phosphorylation by a series of c-Src and v-Src mutants. Analysis of GAP association with c-Src/v-Src chimeric proteins demonstrates that GAP associates stably with Src proteins possessing low kinase activity and poorly with activated Src kinases, especially those that lack the carboxy-terminal segment of c-Src containing the regulatory amino acid Tyr-527. Phosphorylated Tyr-527 is a major determinant of c-Src association with GAP, as demonstrated by c-Src point mutants in which Tyr-527 is changed to Phe. While the isolated amino-terminal half of the c-Src protein is insufficient for stable GAP association, analysis of point substitutions of highly conserved amino acid residues in the c-Src SH2 region indicate that this region also influences Src-GAP complex formation. Therefore, our results suggest that both Tyr-527 phosphorylation and the SH2 region contribute to stable association of c-Src with GAP. Analysis of in vivo phosphorylation of GAP by v-Src mutants containing deletions encompassing the SH2, SH3, and unique regions suggests that the kinase domain of v-Src contains sufficient substrate specificity for GAP phosphorylation. Even though tyrosine phosphorylation of GAP correlates to a certain extent with the transforming ability of various c-Src and v-Src mutants, our data suggest that other GAP-associated proteins may also have roles in Src-mediated oncogenic transformation. These findings provide additional evidence for the specificity of Src interactions with GAP and support the hypothesis that these interactions contribute to the biological functions of the Src kinases.

Viral Src (v-Src) and its cellular homolog, c-Src, are membrane-localized phosphoproteins that possess tyrosine kinase activity (2, 21, 25, 46). Cellular Src is expressed in most normal cells and has a tightly regulated phosphotransferase activity, while oncogenic v-Src, which is encoded in Rous sarcoma virus (RSV), is a highly activated kinase (21, 25). Studies with viral and cellular Src mutants have shown that the extent to which the kinase is activated correlates with its ability to transform cells, suggesting that v-Src transforms cells by phosphorylating critical substrates involved in cell growth control (10). Chicken c-Src and RSV v-Src differ by amino acid substitutions distributed throughout the v-Src molecule and by a substitution of 19 amino acids of c-Src with 12 novel residues at the carboxy terminus of v-Src (25, 46). One of the carboxy-terminal amino acids deleted from v-Src is Tyr-527, which has been shown to negatively regulate the kinase activity of c-Src when phosphorylated (11, 12, 23). Substitution of Tyr-527 with Phe and subsequent autophosphorylation at Tyr-416 is sufficient to activate the kinase activity of c-Src, enabling this activated c-Src protein to transform cells (8, 32, 48). In addition, various point mutations throughout v-Src compared with c-Src have been shown to contribute to Src activation (24, 26, 30).

Both c-Src and v-Src have the same general structure, which has been divided into functional domains (Fig. 1). The carboxy-terminal half of each molecule comprises the catalytic domain, while the amino-terminal half contains several distinct regions (25, 46). At the extreme amino terminus is the membrane-binding domain, containing residues required for modification with myristic acid, which localizes c-Src and v-Src to cellular membranes (14, 28). The rest of the amino-terminal half of the Src protein is termed the modulatory domain because mutations in this region of c-Src and v-Src modulate kinase and biological activity up or down (25). The modulatory domain contains a unique sequence that, unlike the remainder of the Src protein, does not show homology with other Src-like tyrosine kinases (9). The SH2 and SH3 regions, which constitute most of the modulatory domain, possess amino acid motifs that are highly conserved not only among other nonreceptor tyrosine kinases, but also among numerous functionally unrelated proteins (46, 47, 51, 56). SH2 and SH3 region-containing proteins that have been implicated in signal transduction pathways include a phospholipase C (PLC-II), the v-Crk oncoprotein, and a GTPase-activating protein (GAP) (38, 55, 58). Recent data suggest that SH2 regions interact with phosphotyrosine residues on target proteins and thereby function as sites for mediation of interactions among proteins that transduce mitogenic signals (1, 7, 37, 41).

GAP has been found to complex with and be phosphorylated by the platelet-derived growth factor receptor and the epidermal growth factor receptor (17, 29, 31, 35, 36). GAP is also phosphorylated on tyrosine in cells transformed by the oncogenic nonreceptor tyrosine kinases v-Src, v-Abl, and v-Fps (17). We have previously demonstrated that c-Src associates stably with complexes containing GAP and that,

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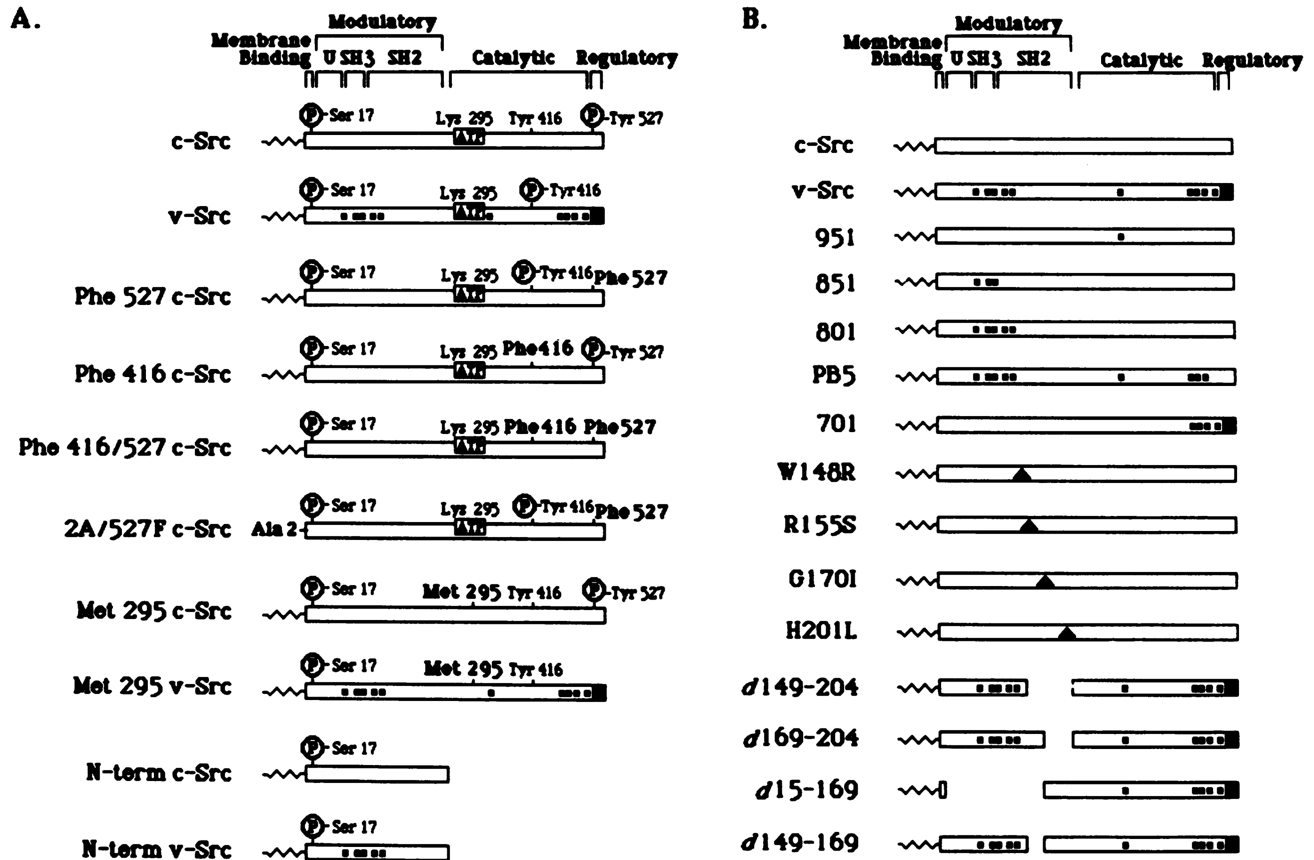

FIG. 1. (A) Structures of chicken c-Src, RSV v-Src, c-Src and v-Src point mutants, and N-term mutants. Src domain structure (U indicates the unique region), major phosphorylation sites (circled P), ATP binding site, and amino-terminal myristic acid are indicated. Black squares indicate point substitutions in v-Src (Schmidt-Ruppin A) relative to chicken c-Src, and the carboxy-terminal deletion of v-Src is denoted by the solid box. Substitutions of Phe, Ala, and Met are denoted by indication of the substituted amino acid. The tyrosine phosphorylation states of all mutants are indicated. (B) Structures of chicken c-Src, RSV v-Src, c-Src/v-Src chimeras, c-Src SH2 region point mutants, and v-Src SH2 region deletion mutants. Src domain structure and myristic acid are indicated; however, the phosphorylation state of these mutants is not shown. Mutations are indicated by a square (differences in v-Src compared with c-Src), a solid triangle (c-Src SH2 region substitution mutations), an open space for v-Src SH2 region deletions, and a solid box for the carboxy-terminal deletion of v-Src.

although v-Src association with GAP complexes is less stable, v-Src phosphorylates GAP in vitro (4). Direct evidence that phosphorylation of GAP leads to cell transformation by v-Src and other tyrosine kinases is lacking. However, the signal transduction pathways of cellular Ras have been linked to those of tyrosine kinases by studies showing that microinjection of anti-Ras antibodies into cells blocks mitogenic responses to growth factor stimulation and oncogenic tyrosine kinases (43, 53). In addition, it has been shown that in cells stimulated with growth factors or transformed by oncogenic tyrosine kinases, greater amounts of Ras protein are detected in the activated GTP-bound form (20, 52).

GAP has the crucial function of downregulating the activity of normal Ras, as it enhances the intrinsic GTPase activity of cellular Ras proteins (40, 57, 58). Suppression of v-Src transformation by GAP overexpression provides evidence that regulation of Ras activity by GAP may have a central role in v-Src oncogenesis (16, 44). Other data suggest that GAP also may act as an effector protein that transduces a signal from Ras to other molecules in the cell (22, 40). Although the effect of tyrosine phosphorylation on GAP function has not been established, it is possible that phosphorylation of tyrosine inactivates GAP GTPase-enhancing ability or, alternatively, activates its putative effector function. Another possibility is that allosteric interactions among GAP and other proteins in a complex modify GAP activity. At least two tyrosine-phosphorylated proteins of unknown function, one of 62 to 68 kDa and another of 190 kDa, have been found to associate with GAP (3, 17, 41, 42).

Analysis of Src interactions with GAP is important because it may illuminate the mechanisms by which tyrosine kinases influence Ras signal transduction pathways. We show here that stable GAP association with c-Src is dependent on the kinase activation state of c-Src and, in particular, on phosphorylation of Tyr-527. The c-Src SH2 region also contributes to this stable association, although this region is not required for in vivo phosphorylation of GAP by v-Src. In addition, we found that GAP phosphorylation in vivo by activated c-Src and v-Src kinases correlates to a certain extent with the transforming ability of the Src proteins. These results further support the hypothesis that specific interactions between Src and GAP contribute to the biological functions of the Src kinases. Moreover, our results are consistent with the possibility that additional proteins asso-

ciated with Src-GAP complexes may also play roles in Src-mediated oncogenesis.

MATERIALS AND METHODS

Cells and viruses. Primary chicken embryo fibroblasts (CEF) were prepared, maintained, and infected as described before (26). CEF were grown in F-10 medium supplemented with 5% bovine calf serum, 1% chick serum, and 10% tryptose phosphate broth (Hyclone; GIBCO). CEF were infected with wild-type RSV (Schmidt-Ruppin subgroup A) or with an RSV variant encoding normal chicken c-Src (NY5H) (33). The chimeric c-Src/v-Src recombinants 951, 851, 801, PB5, and 701 have been described previously (26, 30), as have the c-Src SH2 region point mutants W148R, R155S, G170I, and H201L (45) and the Met-295 c-Src (27) and Met-295 v-Src mutants (54). The c-Src N-term and v-Src N-term viruses were constructed by digestion of the plasmids p5H and pSR-XD2 (15, 33) with *Mlu*I, which cleaves each plasmid twice and allows excision of a 919-bp fragment. Religation of the remaining fragment results in a plasmid coding for truncated c-Src and v-Src proteins, containing c-Src and v-Src amino acid residues 1 to 259. These constructs were transfected into CEF along with recombinant RSV-based vectors, and replication-competent viruses were harvested as previously described (15). The v-Src SH2 region deletion mutants *d*15-169 (NY310), *d*149-169 (NY320), *d*149-204 (NY321), and *d*169-204 (NY322) have been described previously (6, 13, 19). 527F, 2A/527F, and c-Src-encoding RSV variants were gifts of T. Parsons (University of Virginia) (50).

Mouse NIH 3T3 cells overexpressing chicken c-Src and Phe-416, Phe-527, and Phe-416/527 mutants of chicken c-Src were gifts of D. Shalloway (Cornell University) (32). Mouse cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 5% supplemented bovine calf serum (Hyclone).

Cell lysis and protein analysis. Confluent 10-cm plates of cells were washed three times with 4°C phosphate-buffered saline containing 1 mM sodium orthovanadate and then disrupted for 15 min at 4°C in lysis buffer (49) (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EGTA [ethylene glycol tetraacetic acid], 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of α2 macroglobulin per ml, 1 μM leupeptin, 1 μM antipain, and 0.1 μM aprotinin). Lysates were clarified by centrifugation for 15 min at 4°C, the protein concentrations of supernatants were normalized, and the lysates were incubated with the designated antibody for 1 h at 4°C. Protein A-Sepharose (Pharmacia) was then added for 30 min, and immunoprecipitates were washed four times by pelleting in 4°C lysis buffer. Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, loaded on 7.5% or 10% polyacrylamide–SDS gels, and separated by polyacrylamide gel electrophoresis (PAGE).

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose, and filters were incubated overnight in blocking buffer (10 mM Tris-HCl [pH 7.4], 0.9% NaCl, 0.1% Triton X-100, 5% ovalbumin). Blocked filters were incubated with the appropriate antibodies, washed extensively, and then incubated with 10 μCi of ^{125}I-labeled sheep anti-mouse immunoglobulin G (IgG; ICN). After being washed, the filters were exposed to Kodak XAR-5 film with intensifying screens at −80°C overnight. For some experiments, the secondary antibody used was horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham), which was then detected by enhanced chemiluminescence reagents (Amersham) and subsequent exposure to Kodak XAR-5 film for 1 to 30 s.

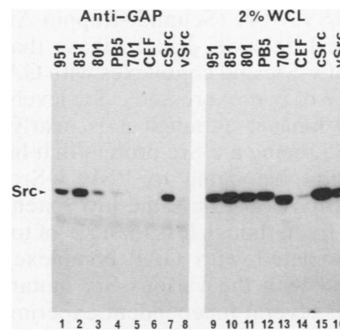

FIG. 2. Complex formation of GAP with c-Src/v-Src chimeric proteins. CEF infected with wild-type RSV or RSV variants encoding chicken c-Src or chimeric proteins (951, 851, 901, PB5, or 701) were lysed, and equivalent amounts of protein were immunoprecipitated with anti-GAP(C-term) serum, as described in Materials and Methods. Anti-GAP immunoprecipitates (lanes 1 to 8) and 2% of whole-cell lysates (WCL) (lanes 9 to 16) were subjected to electrophoresis on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with anti-Src MAb 327, followed by ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. Anti-GAP immunoprecipitates and whole-cell lysates shown in the two panels were from the same exposure of the same immunoblot for direct comparison. CEF, uninfected cells.

Antibodies. Rabbit polyclonal anti-GAP(C-term) antibodies were prepared against amino acid residues 521 to 988 of human GAP expressed in bacteria (4). Monoclonal antiphosphotyrosine antibodies were obtained from Upstate Biotechnology, Inc. The mouse anti-Src monoclonal antibody (MAb) 327 was a gift of J. Brugge (University of Pennsylvania) (34).

RESULTS

c-Src/v-Src chimera complex formation with GAP. Cellular Src forms stable complexes with GAP (4), possibly in conjunction with other associated proteins. However, although v-Src is able to phosphorylate GAP, complex formation between v-Src and GAP appears to be unstable and difficult to detect (4). We sought to determine the structural basis for these differences by analyzing GAP association with c-Src/v-Src chimeric proteins (951, 851, 801, PB5, and 701) that exhibit a range of kinase activation states (26, 30) (Fig. 1B). CEF infected with RSV variants encoding chimeric Src proteins were lysed, and GAP proteins were immunoprecipitated with rabbit anti-GAP(C-term) antiserum. Similar amounts of GAP were immunoprecipitated from cells expressing the various Src mutants (data not shown). Immunoprecipitates were resolved by SDS-PAGE, and then immunoblots were probed with monoclonal anti-Src antibodies (MAb 327) to detect the amount of each mutant Src protein coimmunoprecipitating with GAP (Fig. 2). For comparison, 2% of the total cell lysate was run in parallel with each immunoprecipitate to indicate the level of expression of each Src mutant in CEF (Fig. 2).

Mutants 951 and 851, which have weakly activated kinases, coprecipitated with GAP as efficiently as overexpressed normal c-Src. Mutant 801, which has intermediate kinase activity, associates with GAP to approximately 30% of overexpressed c-Src levels. PB5, possessing most of the

mutations of RSV v-Src (Schmidt-Ruppin A) except for the carboxy-terminal deletion, has a kinase that is at least as active as that of v-Src and complexes with GAP only to a low extent, 5 to 10% of overexpressed c-Src levels. Mutant 701 is also an active kinase, although it is nearly the structural opposite of PB5, being a c-Src protein that has the carboxy-terminal deletion belonging to RSV v-Src. This mutant associates with GAP to the same low extent as does wild-type viral Src. Even though less than 2% of total Src proteins are stably associated with GAP complexes (Fig. 2), the results obtained with the various Src mutants were highly reproducible in several independent experiments.

These results indicate that GAP complexes stably with Src proteins possessing restricted kinase activity and that as the Src kinase is activated by mutation, association with GAP is destabilized. In addition to kinase activity, the tyrosine phosphorylation state of these chimeric mutants has been extensively characterized (26). Mutants 951 and 851 are phosphorylated primarily on Tyr-527, while mutant 801 is phosphorylated approximately 20% on Tyr-527 and 80% on Tyr-416. PB5 has very low levels of phosphorylated Tyr-527, and chimera 701 lacks Tyr-527 entirely. Thus, a positive correlation also exists between phosphorylation of Tyr-527 and stable GAP association with the chimeric Src proteins.

Phosphotyrosine 527 is a key residue in GAP complex formation. The above results suggest that the phosphorylation or activation state of Src is important for stable Src-GAP complex formation. To examine further the role of tyrosine phosphorylation in stable-complex formation, we analyzed GAP association with chicken c-Src mutants containing phenylalanine substitutions for tyrosine 416, 527, or both 416 and 527 (Fig. 1A). Substitution of Phe for the regulatory Tyr-527 prevents phosphorylation at this site and activates the kinase activity of this mutant c-Src (32). The Phe-416/527 mutant has a kinase activity lower than that of the Phe-527 mutant, while the Phe-416 mutation has no activating effect on the c-Src kinase activity. These mutants, as well as normal chicken c-Src, are overexpressed at high levels in transfected NIH 3T3 cells (32).

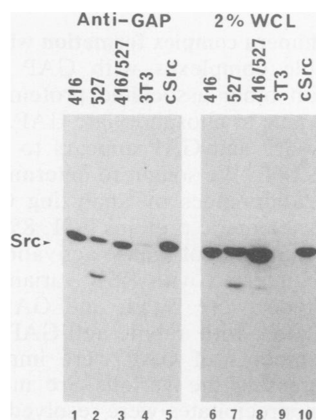

FIG. 3. Association of GAP with c-Src phosphotyrosine site point mutants. NIH 3T3 cells stably overexpressing c-Src Phe-416 (lanes 1 and 6), c-Src Phe-527 (lanes 2 and 7), c-Src Phe-416/527 (lanes 3 and 8), normal NIH 3T3 cells (lanes 4 and 9), or wild-type chicken c-Src (lanes 5 and 10) were lysed and immunoprecipitated with anti-GAP(C-term) serum. Immunoprecipitates (lanes 1 to 5) and 2% of whole-cell lysates (lanes 6 to 10) were electrophoresed on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with anti-Src MAb 327, followed by ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. Anti-GAP immunoprecipitates and whole-cell lysates shown in the two panels were from the same exposure of the same immunoblot.

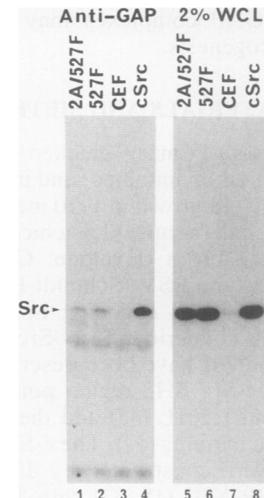

FIG. 4. GAP complex formation with myristylated and nonmyristylated c-Src phosphotyrosine site point mutants. Infected CEF overexpressing mutant c-Src with substitutions of Ala for Gly-2 and Phe for Tyr-527 (2A/527F) (lanes 1 and 5), c-Src with a substitution of Phe for Tyr-527 (527F) (lanes 2 and 6), normal CEF (lanes 3 and 7), and CEF overexpressing c-Src (lanes 4 and 8) were lysed. Anti-GAP(C-term) immunoprecipitates of lysates (lanes 1 to 4) or 2% of whole-cell lysates (WCL, lanes 5 to 8) were electrophoresed on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with anti-Src MAb 327, with ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. Anti-GAP immunoprecipitates and whole-cell lysates shown in the two panels were from the same exposure of the same immunoblot.

To determine how these point mutations affect Src-GAP interactions, we immunoprecipitated GAP from mutant-expressing cells and immunoblotted the immunoprecipitates and 2% of whole-cell lysates with anti-Src antibodies as described above (Fig. 3). We found that while GAP associates stably with c-Src Phe-416, association with mutant Phe-527 or Phe-416/527 is reduced to about 25 to 30% of overexpressed c-Src levels. As a control, we reprobed the blot shown in Fig. 3 with anti-GAP antibodies to ascertain that similar amounts of GAP were present in these immunoprecipitates (data not shown).

The importance of c-Src Tyr-527 in GAP association was further confirmed by results in CEF. Chicken cells were infected with an RSV variant encoding c-Src Phe-527 (527F) (50), and proteins from CEF lysates were immunoprecipitated with anti-GAP antibodies and immunoblotted with anti-Src antibodies. In CEF, GAP complex formation with the mutant 527F decreases to 15% of that of overexpressed c-Src (Fig. 4). CEF were also infected with an RSV variant, 2A/527F, encoding an activated c-Src that contains the change of Tyr-527 to Phe in combination with a change of the amino-terminal Gly to Ala (Fig. 1A). This produces an activated c-Src mutant that is not myristylated and does not associate with cellular membranes (50). The 2A/527F Src protein associates with GAP to the same low level as does 527F. Levels of association are not further decreased by the lack of myristylation, suggesting that myristylation and localization at cellular membranes is not necessary for c-Src association with GAP (Fig. 4). Together with the analysis of chimeric c-Src/v-Src proteins, the above data indicate that

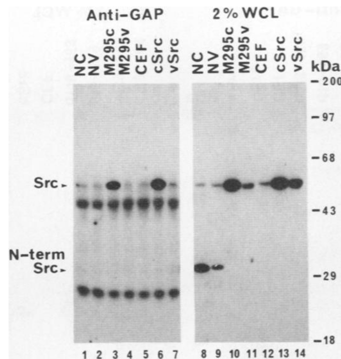

FIG. 5. Complex formation of GAP with kinase-inactive c-Src and v-Src and amino-terminal halves of c-Src and v-Src. Infected CEF overexpressing wild-type c-Src and v-Src, kinase-inactive c-Src (M295c) and v-Src (M295v), and the isolated amino-terminal halves of c-Src (NC) and v-Src (NV) were lysed and immunoprecipitated with anti-GAP(C-term) serum. Immunoprecipitates (lanes 1 to 7) and 2% of whole-cell lysates (WCL, lanes 8 to 14) were subjected to electrophoresis on a 10% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with anti-Src MAb 327, with ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. Positions of molecular mass markers are indicated. Anti-GAP immunoprecipitates and whole-cell lysates shown in the two panels were from the same exposure of the same immunoblot.

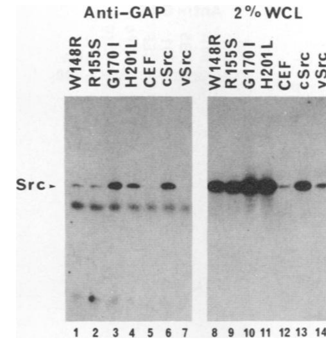

FIG. 6. GAP association with c-Src SH2 region point mutants. Lysates from CEF infected with wild-type RSV or RSV variants encoding chicken c-Src or c-Src SH2 region point substitution mutants W148R, R155S, G170I, and H201L were immunoprecipitated with anti-GAP(C-term) serum. Immunoprecipitates (lanes 1 to 7) and 2% of whole-cell lysates (WCL lanes 8 to 14) were subjected to electrophoresis on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with anti-Src MAb 327, with ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. Anti-GAP immunoprecipitates and whole-cell lysates shown in the two panels were from the same exposure of the same immunoblot.

phosphorylated Tyr-527 is important for stable association of c-Src with GAP.

Amino-terminal halves of the Src kinases do not associate stably with GAP. The SH2 and SH3 regions are thought to mediate protein-protein interactions among signal transduction molecules (7, 47). Previous studies have shown that the amino-terminal halves of the Src kinases associate with the phosphatidylinositol-3 kinase (18). We investigated whether the amino-terminal halves of the c-Src and v-Src proteins (Fig. 1A), containing the intact modulatory domain (including the unique, SH2, and SH3 sequences in isolation from the catalytic domain), could interact with GAP. Both mutants were expressed at high levels in CEF infected with mutant-encoding RSV variants (Fig. 5). After equivalent amounts of GAP protein were immunoprecipitated with anti-GAP antibodies and the immunoprecipitates were immunoblotted with anti-Src antibodies, no evidence of complex formation with GAP was detected for the c-Src N-term or v-Src N-term polypeptide (Fig. 5). Although this does not exclude the involvement of SH2 or SH3 regions in Src-GAP interactions (see below), this suggests that these regions alone in Src are not sufficient to mediate the Src-GAP interaction.

The data shown thus far suggest that a determinant in the carboxy-terminal half of Src, such as phosphorylated Tyr-527, is important in mediating interactions with GAP. To determine whether the catalytic activity of the Src kinases is essential for stable GAP association, we analyzed Src-GAP association in the same manner as described above, using two kinase-inactive Src mutants, c-Src Met-295 and v-Src Met-295 (Fig. 1A). These mutants have a Met substitution for Lys-295, which is the ATP-binding site of the Src protein and is necessary for phosphotransferase activity (27, 54). When these mutants were expressed in infected CEF, we found that c-Src Met-295 still complexes efficiently with GAP (Fig. 5). As with wild-type v-Src, however, even after prolonged exposure to X-ray film, v-Src Met-295 does not associate detectably with GAP (Fig. 5). Together, these results demonstrate that catalytic activity per se does not influence the association of v-Src or c-Src with GAP, even though stable GAP complex formation depends upon a structural determinant in the carboxy-terminal half of Src.

c-Src SH2 regions contribute to GAP association. To determine whether the SH2 region makes any contribution towards Src-GAP complex formation, GAP association with c-Src mutants carrying SH2 region point mutations was examined. These mutants have substitutions in four highly conserved residues shared by many SH2 region-containing proteins (Fig. 1B). One of these mutants, W148R, has a slightly activated kinase and is partially transforming in CEF (45). G170I and R155S are also slightly transforming, although to a lesser extent than W148R, while H201L is not transforming at all (45). All four point mutants are highly phosphorylated on Tyr-527 and show no detectable autophosphorylation on Tyr-416 (45). CEF were infected with RSV variants encoding these mutants, and lysates of CEF were immunoprecipitated with anti-GAP antiserum and immunoblotted with anti-Src antibodies as described above. We reproducibly found that association with GAP is reduced in all cases compared with the corresponding levels of Src expression (Fig. 6). Complex formation with W148R and R155S is reduced to about 25% of overexpressed c-Src levels and with G170I and H201L to about 50%. As a control, reprobing of the blot shown in Fig. 6 with anti-GAP antibodies demonstrated that similar amounts of GAP were present in the immunoprecipitates (data not shown). These data suggest that the SH2 region plays a role in Src-GAP complex formation.

GAP phosphorylation by c-Src/v-Src chimeras. An important question is whether tyrosine phosphorylation of GAP by Src kinases correlates with cell transformation. We therefore examined in vivo phosphorylation of GAP by Src mutants. CEF expressing the c-Src/v-Src chimeras described above (Fig. 1B) were lysed in buffer containing phosphatase inhibitors, and GAP proteins were immunoprecipitated with anti-GAP(C-term) antibodies. Immunoprecipitates were then electrophoresed and immunoblotted with monoclonal antiphosphotyrosine antibodies. We detected a tyrosine-

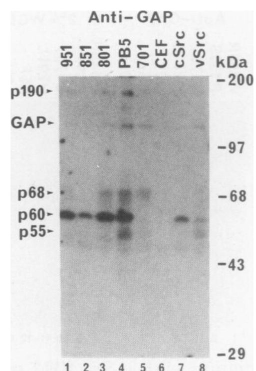

FIG. 7. Phosphorylation of GAP and associated proteins in vivo by c-Src/v-Src chimeric proteins. Infected CEF overexpressing chimeric proteins (951, 851, 801, PB5, or 701), chicken c-Src, or RSV v-Src were lysed and immunoprecipitated with anti-GAP(C-term) serum. After SDS-PAGE and subsequent transfer to nitrocellulose, immunoprecipitates were immunoblotted with monoclonal antibodies to phosphotyrosine, with ^{125}I-labeled sheep anti-mouse IgG as the secondary antibody. GAP and coimmunoprecipitated phosphoproteins p190, p68, p60, and p55 are shown by arrows. Positions of molecular mass markers are indicated.

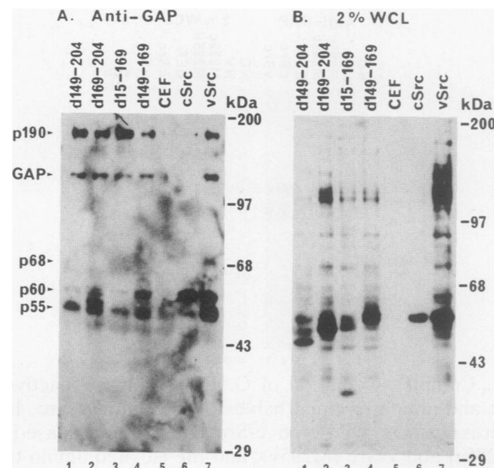

FIG. 8. (A) Phosphorylation of GAP and associated proteins in vivo by v-Src SH2 region deletion mutants. Anti-GAP(C-term) immunoprecipitates from infected CEF overexpressing v-Src SH2 region deletion mutants *d*149-204, *d*169-204, *d*15-169, and *d*149-169 or wild-type c-Src or v-Src were subjected to electrophoresis on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with monoclonal antiphosphotyrosine antibodies, with sheep anti-mouse IgG conjugated to horseradish peroxidase as the secondary antibody. Enhanced chemiluminescence reagents were used as a detection system (see Materials and Methods). GAP and coimmunoprecipitated proteins p190, p68, p60, and p55, are denoted by arrows. Positions of molecular mass markers are indicated. (B) In vivo phosphorylation of total cellular proteins by v-Src SH2 region deletion mutants. Whole-cell lysates (WCL) of infected CEF expressing v-Src SH2 region deletion mutants or wild-type c-Src and v-Src were electrophoresed on a 7.5% polyacrylamide–SDS gel, transferred to nitrocellulose, and immunoblotted with monoclonal antiphosphotyrosine antibodies, with sheep anti-mouse IgG conjugated to horseradish peroxidase as the secondary antibody. Enhanced chemiluminescence reagents were used as a detection system. Positions of molecular mass markers are indicated. Exposure times are different for panels A and B.

phosphorylated protein of 125 kDa that comigrates with GAP in immunoprecipitates from lysates of cells expressing chimeric Src proteins and RSV v-Src but not from normal CEF or CEF overexpressing c-Src (Fig. 7). GAP is relatively highly phosphorylated in immunoprecipitates from CEF infected with 701, 801, PB5, and wild-type RSV. Tyrosine phosphorylation of GAP is reduced to approximately 30% of wild-type v-Src levels in CEF infected with the more weakly transforming 851 and 951 mutant viruses. A fraction of each immunoprecipitate was immunoblotted with anti-GAP antibody to ascertain that equivalent amounts of GAP protein were present in each immunoprecipitate, and whole-cell lysates were immunoblotted with anti-Src antibody to ensure that similar amounts of each Src protein were expressed (data not shown). These results indicate that the more highly activated transforming Src mutants phosphorylate GAP in vivo more efficiently than weakly activated Src proteins.

Several tyrosine-phosphorylated proteins were observed to coprecipitate with GAP (Fig. 7). Among these are proteins of 62 to 68 kDa (p68) and 190 kDa (p190), which may be identical to the GAP-associated proteins described previously (3, 17, 41, 42). Levels of p68 phosphorylation correspond to the overall in vivo kinase activities of the various c-Src/v-Src chimeric mutants (26). p68 from anti-GAP immunoprecipitates of PB5-, 801-, and 701-infected CEF is highly phosphorylated, but phosphotyrosine levels in p68 are significantly reduced in immunoprecipitates from 951- and 851-infected CEF. The phosphorylation of coprecipitating p190 also seems to correlate with the overall kinase activity of each chimera. In addition, we detected a heavily tyrosine-phosphorylated protein of 60 kDa coimmunoprecipitating with GAP, which may be Src. Interestingly, a novel phosphoprotein of 55 kDa (p55) was also reproducibly present in anti-GAP immunoprecipitates from wild-type v-Src- and activated chimeric Src mutant-expressing CEF. This p55 protein has not been reported previously, possibly because it might not have been detected by other antiphosphotyrosine antibodies.

SH2 and SH3 regions of v-Src are not essential for GAP phosphorylation. Because the SH2 region of c-Src influences stable Src-GAP association, we determined whether the SH2 and SH3 regions of v-Src are required for phosphorylation of GAP. Mutants *d*149-169, *d*149-204, and *d*169-204 have deletions within the SH2 region of v-Src (Fig. 1B). *d*15-169 has a large deletion spanning the SH2, SH3, and unique regions of v-Src (Fig. 1B). CEF infected with *d*149-169, *d*149-204, and *d*169-204 grow in soft-agar suspension and exhibit fusiform morphologies (6, 13, 19). Mutant *d*15-169 is more defective in its ability to induce transformation of CEF (6, 13). The overall in vivo kinase activities of these mutants are shown in Fig. 8B, in which whole-cell lysates from CEF expressing these mutants were subjected to SDS-PAGE and then immunoblotted with antiphosphotyrosine antibodies. The results demonstrate that all of these mutants have an impaired kinase activity and phosphorylate cellular substrates less efficiently than wild-type v-Src.

GAP was immunoprecipitated from lysates of CEF expressing the v-Src SH2 region deletion mutants, and immunoprecipitates were immunoblotted with antiphosphotyrosine antibodies (Fig. 8A). Anti-GAP immunoblots of a fraction of these anti-GAP immunoprecipitates indicated that the same amount of GAP protein was present in each immunoprecipitate, while anti-Src immunoblots of whole-cell lysates demonstrated that similarly high levels of each Src protein were expressed (data not shown). We observed that all four mutants reproducibly phosphorylate GAP less

efficiently than wild-type v-Src, reflecting the overall decrease in kinase activity of the mutants compared with wild-type v-Src (compare Fig. 8A and B). However, as each Src mutant phosphorylates GAP to the same extent, it is significant that no correlation between GAP phosphorylation and the size or region of the mutations was observed. Although the GAP-associated phosphoprotein p68 was observed to coprecipitate with GAP in wild-type RSV-infected CEF, coprecipitating phosphorylated p68 was greatly reduced in the v-Src mutant-infected CEF (Fig. 8A). In striking contrast, phosphorylation of coprecipitating p190 was not reproducibly reduced from levels observed in wild-type v-Src-expressing CEF. Proteins of 55 to 60 kDa were also observed in these anti-GAP immunoprecipitates. The 55-kDa protein (p55) is phosphorylated in immunoprecipitates from all of the v-Src mutant-expressing CEF, although it is phosphorylated to a lesser extent by the more defective *d*15-169 mutant. A highly tyrosine-phosphorylated 60-kDa protein is also observed to coprecipitate with GAP in CEF overexpressing wild-type c-Src. Taken together, these results suggest that the v-Src SH2 region is required for efficient phosphorylation of p68 but not for phosphorylation of GAP in vivo.

DISCUSSION

Our results demonstrate that GAP associates stably with normal c-Src and c-Src/v-Src chimeras that have low levels of transforming and kinase activities. As the kinase activation state and transforming ability of the chimeric mutants increases, however, stable association with GAP decreases. Stable-complex formation with GAP thus appears to generally correlate inversely with activation of the Src kinase. Furthermore, the more highly activated and transforming Src chimeras have low levels of Tyr-527 phosphorylation or lack this residue completely, while chimeras with low kinase activity and transforming ability are phosphorylated almost entirely on Tyr-527. We therefore observe that stable Src-GAP complex formation also correlates closely with levels of Tyr-527 phosphorylation.

To determine more precisely the role of this residue in Src-GAP association, we examined GAP coimmunoprecipitation with the c-Src Phe-527 mutant. GAP association with c-Src Phe-527 was reduced substantially from that with overexpressed normal c-Src. These results demonstrate that Tyr-527 is a major determinant of c-Src association with GAP. Nevertheless, low levels of c-Src Phe-527 protein remain complexed with GAP. This may indicate that GAP has high affinity for phosphorylated Tyr-527 and surrounding residues, but retains a low affinity for this sequence even in the absence of the phosphate group.

We found that SH2 region point mutations at conserved residues in c-Src also significantly reduce levels of GAP association. These point mutants are the only c-Src variants observed that are phosphorylated exclusively on Tyr-527 (45) and yet associate poorly with GAP. In addition, all of the SH2 point mutants are phosphorylated on Tyr-527 to the same extent as normal c-Src (5). As these point mutations might be expected to create only local disturbances in Src structure, this suggests that the SH2 region directly influences Src interactions with GAP. These results are not inconsistent with our data showing that GAP cannot complex with mutants containing only the amino-terminal half of c-Src or v-Src, but rather suggest that carboxy-terminal residues (including Tyr-527) are required in conjunction with SH2 sequences to stably bind GAP.

Our studies have thus demonstrated the involvement of two crucial features of the c-Src protein—phosphorylated Tyr-527 and the SH2 region—in stable-complex formation with GAP. In contrast, we found that neither Tyr-416 autophosphorylation nor kinase activity per se influences Src-GAP interactions. Phosphotyrosine residues have been postulated to be the targets of SH2 region-mediated protein-protein interactions, and recent studies have indicated that SH2 region-containing proteins, or isolated SH2 region polypeptides, bind tyrosine-phosphorylated proteins with high affinity (7, 37, 39, 41). One possibility is that c-Src-GAP complexes are mediated by direct interaction of phosphorylated Tyr-527 with GAP SH2 sequences. However, we have also shown that c-Src SH2 regions contribute to the c-Src-GAP complex. Another possibility is that phosphorylated Tyr-527 of c-Src interacts with its own SH2 region, thereby stabilizing an overall conformation of c-Src that is recognized by GAP. It should be noted that our findings do not exclude the possibility that GAP and c-Src associate only indirectly in complexes with intermediary phosphoproteins. Further studies are needed to distinguish among these possibilities. An additional caveat is that our measurement of the extent of Src-GAP association is dependent upon the resistance of the immune complexes to dissociation during cell lysis and washing; we cannot exclude that interaction between Src proteins and GAP are more stable in intact cells.

The results presented here suggest that regions shown to be important in stable c-Src-GAP complex formation do not influence the ability of v-Src and activated c-Src to phosphorylate GAP. For instance, mutant 801 demonstrated only partially reduced association with GAP, while mutant 701 did not complex detectably with this protein. Mutant 801 has partially reduced Tyr-527 phosphorylation, whereas 701 lacks Tyr-527 completely. These differences between 801 and 701 are not reflected in their ability to phosphorylate GAP, as the levels of phosphotyrosine in GAP are similar for cells expressing either of these mutants. Furthermore, all of the v-Src SH2 region deletion mutants phosphorylate GAP to the same extent, despite large differences among mutants in the size of the deleted regions. This lack of correlation between the location or size of the deletion and GAP phosphorylation suggests that the unique, SH2, and SH3 regions are not required for v-Src phosphorylation of GAP. Thus, neither Tyr-527 nor SH2 sequences exert a large influence on the ability of the Src kinase to phosphorylate GAP. These results suggest that specificity for v-Src phosphorylation of GAP resides within the catalytic domain of the v-Src kinase.

In vivo kinase assays indicate that levels of GAP phosphorylation in CEF expressing c-Src/v-Src chimeras correspond approximately to the overall kinase activity of each mutant (26). Because the transforming abilities of the chimeras parallel their kinase activities, GAP phosphorylation correlates with cell transformation in these mutant-expressing CEF. In contrast, although GAP phosphorylation appears to reflect the overall reduction in kinase activity of the v-Src SH2 region deletion mutants, levels of GAP phosphorylation do not correspond to the range of transformation parameters exhibited by these mutants. For instance, v-Src *d*149-169 is relatively transforming in CEF, while cells expressing v-Src *d*15-169 are more defective for anchorage-independent growth and mitogenesis (6, 13). Similar levels of GAP phosphorylation are induced by either v-Src mutant. One interpretation of these findings is that GAP phosphorylation may be necessary but not sufficient for cell transfor-

mation. It is possible that for full cell transformation, additional factors, whose association or phosphorylation is dependent on the SH2 and SH3 regions of Src, must also interact with Src.

Several tyrosine-phosphorylated proteins were observed to associate with GAP in this study. A phosphoprotein with an apparent molecular mass of 62 to 68 kDa (p68) coimmunoprecipitates with GAP from cells expressing all c-Src/v-Src chimeric mutants as well as wild-type v-Src, although mutants 951 and 851 phosphorylate p68 to a very low extent. Other groups (3, 17, 41, 42) have described a similar phosphoprotein of 62 to 68 kDa that coprecipitates with GAP in cells transformed by Src and other activated tyrosine kinases. In our studies, levels of p68 phosphorylation correlate with the overall kinase activity of each chimeric Src mutant (26). Even though phosphorylated GAP is readily detectable in anti-GAP immunoprecipitates from CEF infected with v-Src SH2 region deletion mutants, levels of phosphorylated p68 are substantially reduced in CEF expressing these mutants. These findings support previous reports (3, 41) indicating that alterations in the Src SH2 region lead to reduced phosphorylation of p68 and correlate with the reduced kinase activities of these Src mutants. In addition, a tyrosine-phosphorylated protein of 190 kDa (p190), similar to one described previously (3, 17, 41, 42), coprecipitates with GAP. Phosphorylation of this protein also reflects overall c-Src/v-Src chimera kinase activity. However, p190 phosphorylation does not correlate with the kinase activity or transformation phenotype of the v-Src SH2 region deletion mutants. In CEF expressing these v-Src SH2 mutants, we observed that levels of p190 phosphorylation are not reduced from those in wild-type v-Src. We also detected a previously undescribed GAP-associated protein with an apparent molecular mass of 55 kDa (p55) that is phosphorylated on tyrosine in v-Src-transformed cells. Phosphorylation of this novel phosphoprotein appears to correlate with the kinase activity of each mutant. Further studies are needed to elucidate the roles of p55, p68, and p190 in Src oncogenesis.

Our results suggest that Src and GAP engage in multiprotein complexes, the composition of which depends upon the structure and activation state of Src. The effect of these interactions on the GTPase-activating or possible signal-transducing properties of GAP remains to be determined. Tyrosine phosphorylation of GAP may modify its activity, resulting in activation of mitogenic signaling pathways. In this case, normal c-Src may complex stably with GAP in order to sequester GAP in readiness for the transient activation that c-Src undergoes when cells are appropriately stimulated. By contrast, in cells transformed by RSV, although v-Src does not sequester GAP, it might constitutively phosphorylate GAP, leading to cellular transformation. Alternatively, allosteric interactions among proteins in a complex with GAP might alter GAP activity (42), leading to mitogenic signal transduction.

These results provide new evidence for the specificity of Src-GAP interactions. In particular, our data suggest that the specificity for stable-complex formation resides in the SH2 region and Tyr-527 phosphorylation of c-Src, while specificity for GAP phosphorylation resides in the catalytic domain of v-Src. These results further support the hypothesis that GAP plays a role in c-Src-mediated functions as well as in cell transformation by v-Src. Our findings also underscore the importance of understanding how different structural domains of Src might interact with one another and with multiprotein complexes in Src-mediated signal transduction.

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REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. Science **250**:979–982.
2. **Bishop, J. M., and H. E. Varmus.** 1984. Functions and origins of retroviral transforming genes, p. 999–1108. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Bouton, A. H., S. B. Kanner, R. Vines, H.-C. R. Wang, J. B. Gibbs, and J. T. Parsons.** 1991. Transformation by pp60^{src} or stimulation of cells with epidermal growth factor induces the stable association of tyrosine-phosphorylated cellular proteins with GTPase-activating protein. Mol. Cell. Biol. **11**:945–953.
4. **Brott, B. K., S. Decker, J. Shafer, J. B. Gibbs, and R. Jove.** 1991. GTPase-activating protein interactions with the viral and cellular Src kinases. Proc. Natl. Acad. Sci. USA **88**:755–759.
5. **Brott, B. K., and R. Jove.** Unpublished data.
6. **Calothy, G., D. Laugier, F. R. Cross, R. Jove, T. Hanafusa, and H. Hanafusa.** 1987. The membrane-binding domain and myristylation of p60$^{v\text{-}src}$ are not essential for stimulation of cell proliferation. J. Virol. **61**:1678–1681.
7. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
8. **Cartwright, C. A., W. Eckhart, S. Simon, and L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
9. **Cooper, J. A.** 1990. The *src* family of protein-tyrosine kinases, p. 85–113. *In* B. E. Kemp (ed.), Peptides and protein phosphorylation. CRC Press, Boca Raton, Fla.
10. **Cooper, J. A., and T. Hunter.** 1983. Regulation of cell growth and transformation by tyrosine-specific protein kinases: the search for important cellular substrate proteins. Curr. Top. Microbiol. Immunol. **107**:125–161.
11. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxyl terminus stimulates pp60$^{c\text{-}src}$. Mol. Cell. Biol. **6**:4467–4477.
12. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**:1471–1477.
13. **Cross, F. R., E. A. Garber, and H. Hanafusa.** 1985. N-terminal deletions in Rous sarcoma virus p60^{src}: effects on tyrosine kinase and biological activities and on recombination in tissue culture with the cellular *src* gene. Mol. Cell. Biol. **5**:2789–2795.
14. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 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.
15. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation of p60^{src} are dispensable for transformation. Cell **34**:597–607.
16. **DeClue, J. E., P. Redford, W. C. Vass, and D. R. Lowy.** 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.
17. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343**: 377–381.
18. **Fukui, Y., and H. Hanafusa.** 1991. Requirement of phosphati-

dylinositol-3 kinase modification for its association with p60^{src}. Mol. Cell. Biol. **11**:1972–1979.

19. **Fukui, Y., M. C. O'Brien, and H. Hanafusa.** 1991. Deletions in the SH2 domain of p60^{v-src} prevent association with the detergent-insoluble cellular matrix. Mol. Cell. Biol. **11**:1207–1213.
20. **Gibbs, J. B., M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, and U. S. Vogel.** 1991. Modulation of guanine nucleotides bound to Ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). J. Biol. Chem. **265**:20437–20442.
21. **Golden, A., and J. S. Brugge.** 1988. The *src* oncogene, p. 149–173. *In* E. P. Reddy, A. M. Skalka, and T. Curran (ed.), The oncogene handbook. Elsevier, New York.
22. **Hall, A.** 1990. *ras* and GAP—who's controlling whom? Cell **61**:921–923.
23. **Hunter, T.** 1987. A tail of two *src*'s: mutatis mutandis. Cell **49**:1–4.
24. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
25. **Jove, R., and H. Hanafusa.** 1987. Cell transformation by the viral *src* oncogene. Annu. Rev. Cell Biol. **3**:31–56.
26. **Jove, R., T. Hanafusa, M. Hamaguchi, and H. Hanafusa.** 1989. *In vivo* phosphorylation states and kinase activities of transforming pp60^{c-src} mutants. Oncogene Res. **5**:49–60.
27. **Jove, R., S. Kornbluth, and H. Hanafusa.** 1987. Enzymatically inactive p60^{c-src} mutant with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory domain. Cell **50**:937–943.
28. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1985. Mutation of NH_2-terminal glycine of p60^{src} prevents both myristoylation and morphological transformation. Proc. Natl. Acad. Sci. USA **82**:4625–4628.
29. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex. Cell **61**:125–133.
30. **Kato, J.-Y., T. Takeya, C. Grandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming p60^{c-src} protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
31. **Kazlauskas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1990. Binding of GAP to activated PDGF receptors. Science **247**:1578–1581.
32. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60^{c-src} transforming ability by mutation of its primary site of tyrosine phosphorylation. Cell **49**:65–73.
33. **Levy, J. B., H. Iba, and H. Hanafusa.** 1986. Activation of the transforming potential of p60^{c-src} by a single amino acid change. Proc. Natl. Acad. Sci. USA **83**:4228–4232.
34. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
35. **Malloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson.** 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **342**:711–714.
36. **Margolis, B., N. Li, A. Koch, M. Mohamaddi, D. R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson, and J. Schlessinger.** 1990. The tyrosine phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J. **9**:4375–4380.
37. **Matsuda, M., B. J. Mayer, Y. Fukui, and H. Hanafusa.** 1990. Binding of the transforming protein p47$^{gag-crk}$ to a broad range of phosphotyrosine-containing proteins. Science **248**:1537–1539.
38. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
39. **Mayer, B. J., P. K. Jackson, and D. Baltimore.** 1990. The noncatalytic *src* homology region 2 segment of *abl* tyrosine kinase binds to tyrosine-phosphorylated proteins with high affinity. Proc. Natl. Acad. Sci. USA **88**:627–631.
40. **McCormick, F.** 1989. Ras GTPase activating protein: signal transmitter and signal terminator. Cell **56**:5–8.
41. **Moran, M., C. A. Koch, D. Anderson, C. Ellis, L. England, G. S. Martin, and T. Pawson.** 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. USA **87**:8622–8626.
42. **Moran, M. F., P. Polakis, F. McCormick, T. Pawson, and C. Ellis.** 1991. Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21^{ras} GTPase-activating protein. Mol. Cell. Biol. **11**:1804–1812.
43. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for c-*ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313**:241–243.
44. **Nori, M., U. S. Vogel, J. B. Gibbs, and M. J. Weber.** 1991. Inhibition of v-*src*-induced transformation by a GTPase-activating protein. Mol. Cell. Biol. **11**:2812–2818.
45. **O'Brien, M. C., Y. Fukui, and H. Hanafusa.** 1990. Activation of the proto-oncogene p60^{c-src} by point mutations in the SH2 domain. Mol. Cell. Biol. **10**:2855–2862.
46. **Parsons, J. T., and M. J. Weber.** 1989. Genetics of *src*: structure and functional organization of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. **147**:79–127.
47. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene **3**:491–495.
48. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60^{c-src}. Cell **49**:75–82.
49. **Reynolds, A. B., S. B. Kanner, H.-C. R. Wang, and J. T. Parsons.** 1989. The stable association of activated pp60^{src} with two tyrosine-phosphorylated cellular proteins. Mol. Cell. Biol. **9**:3951–3958.
50. **Reynolds, A. B., D. J. Roesel, S. B. Kanner, and J. T. Parsons.** 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. Mol. Cell. Biol. **9**:629–638.
51. **Sadowski, I., J. C. Stone, and T. Pawson.** 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus p130$^{gag-fps}$. Mol. Cell. Biol. **6**:4396–4408.
52. **Satoh, T., E. Masami, M. Nakafuku, T. Akiyama, T. Yamamoto, and Y. Kaziro.** 1990. Accumulation of p21^{ras}-GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **87**:7926–7929.
53. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320**:540–543.
54. **Snyder, M. A., J. M. Bishop, J. R. McGrath, and A. D. Levinson.** 1985. A mutation at the ATP-binding site of pp60^{v-src} abolishes kinase activity, transformation, and tumorigenicity. Mol. Cell. Biol. **5**:1772–1779.
55. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of Src. Nature (London) **332**:269–272.
56. **Sudol, M.** Non-receptor protein tyrosine kinases. *In* R. Kumar (ed.), The molecular basis of human cancer. Futura Publishing Co., New York, in press.
57. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science **238**:542–545.
58. **Vogel, U. S., R. A. Dixon, M. D. Schaber, R. E. Diehl, M. S. Marshall, E. M. Scolnick, I. S. Sigal, and J. B. Gibbs.** 1988. Cloning of bovine GAP and its interaction with oncogenic *ras* p21. Nature (London) **335**:90–93.