MOLECULAR AND CELLULAR BIOLOGY, Sept. 1989, p. 3951–3958
0270-7306/89/093951-08$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 9, No. 9

Stable Association of Activated pp60^{src} with Two Tyrosine-Phosphorylated Cellular Proteins

ALBERT B. REYNOLDS, STEVEN B. KANNER, HWA-CHAIN R. WANG, AND J. THOMAS PARSONS*

Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 27 March 1989/Accepted 12 June 1989

We have identified two phosphotyrosine-containing cellular proteins with relative molecular masses of 130,000 (pp130) and 110,000 (pp110) daltons in chicken embryo cells that coimmunoprecipitated with pp60$^{v\text{-}src}$ and activated forms of chicken pp60$^{c\text{-}src}$ (pp60^{527F}). Most if not all of the tyrosine-phosphorylated forms of pp130 and pp110 could be immunoprecipitated from lysates with any of several *src* protein-specific monoclonal antibodies directed against at least three spatially distinct epitopes. Consequently, of the more than 15 prominent phosphoproteins detected on immunoblots with phosphotyrosine-specific antibodies, pp130 and pp110 were selectively removed by *src* protein-specific immunoprecipitation, and their presence in the immunoprecipitates appears to have been due to a direct interaction with activated *src* proteins. *src* protein variants that induce different morphological phenotypes were altered in their ability to form detergent-stable complexes with pp130 and pp110 or with pp110 alone. Mutant *src* proteins, defective for myristylation, showed increased tyrosine phosphorylation of and association with pp110. Expression of *src* variants with mutations in the A box (pp60$^{dl92/527F}$) or B box (pp60$^{dl155/527F}$) of the *src* homology region induced differences in phosphorylation of pp130 and pp110, as well as changes in their association with variant *src* proteins. Sequences within the B-box region appeared to be necessary for stable complex formation with pp130 and pp110 and may be involved in the interaction of activated *src* proteins with cellular substrates.

The transforming gene of Rous sarcoma virus (RSV), v-*src*, encodes a 60-kilodalton (kDa) protein tyrosine kinase (pp60$^{v\text{-}src}$) whose expression is both necessary and sufficient to initiate and maintain cellular transformation (1, 2, 19–21, 23, 35, 56; J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). Its normal cellular counterpart, c-*src*, encodes a similar protein (pp60$^{c\text{-}src}$) which is not oncogenic, even when expressed at high levels (22, 41, 57). Although there are numerous amino acid differences between the two proteins, the major oncogenic difference occurs at the C-terminal end, where 19 C-terminal amino acids in pp60$^{c\text{-}src}$ have been replaced with 12 unrelated amino acids in pp60$^{v\text{-}src}$ (55, 60). The kinase activity of pp60$^{c\text{-}src}$ is restricted by phosphorylation of a C-terminal tyrosine residue, Tyr-527, which is not present in pp60$^{v\text{-}src}$ (10, 11, 13, 34). Mutations within this C-terminal regulatory region, e.g., Tyr-527 to Phe or Ser or the truncation of the c-*src* protein within the C-terminal 17 residues, activate the tyrosine protein kinase activity of pp60$^{c\text{-}src}$ and concomitantly, the transforming potential of the c-*src* gene product (8, 30, 31, 47, 52).

Other structural domains within pp60^{src} have been shown to influence transforming activity. Myristylation is required for stable membrane association and transformation (6, 7, 14, 16, 54). Substitution of Ala or Glu for Gly-2 yields a soluble *src* protein with unaltered tyrosine kinase activity (5, 24, 25, 51). The phenotypes of cells expressing these nonmyristylated *src* proteins suggest that myristylation and membrane association are necessary for the phosphorylation of transformation-specific cellular proteins. Two other regions of pp60^{src}, referred to as the *src* homology region 2 (SH2) and the *src* homology region 3 (SH3), appear to be important for transformation (23, 42, 44, 53). The SH2 region, common to nonreceptor cytoplasmic protein tyrosine kinases, is defined by amino acids 140 to 274 and appears necessary for efficient fibroblast transformation by v-*src* and v-*fps* (23, 49, 53, 63). The SH3 region encompasses amino acids 84 to 139 and, though not required for transformation by pp60$^{v\text{-}src}$ (49), deletions within this region activate the transforming activity of pp60$^{c\text{-}src}$ (40; unpublished results). The SH3 region is conserved in the *src*-like proteins and in the c-*abl* protein but is absent from the c-*fps*/*fes* protein (53). Sequences within the SH2 and SH3 regions bear striking homology with several unrelated proteins which lack kinase activity, e.g., phospholipase C 148, the *crk* oncogene product; GAP, the GTPase activating protein; and α-spectrin (39, 58, 59, 62). These regions of homology are often referred to as the A box (residues 88 to 137 of SH3), the B box (residues 148 to 187 of SH2), and the C box (residues 220 to 231 of SH2) (39, 58). Cells expressing *src* proteins containing sequence alterations within these regions of pp60$^{v\text{-}src}$ exhibit a variety of phenotypic alterations, including fusiform morphology and temperature-sensitive and transformation-defective phenotypes (15, 23, 42, 64). Evidence from our laboratory has shown that pp60$^{c\text{-}src}$ bearing deletions within the A box induces a partially transformed phenotype (48; unpublished results) characterized by an unusual elongated cellular morphology. In contrast, small deletions within the B box inactivate the transforming activity of pp60$^{v\text{-}src}$ or the activated form of the c-*src* protein, pp60^{527F}, without significantly affecting the intrinsic protein tyrosine kinase activity of the variant proteins (63; unpublished results). The morphological and biochemical properties of these deletion variants indicate that the A and B boxes may modulate the interaction of pp60^{src} with cellular regulatory proteins and/or putative substrates (45, 63).

Antibodies to phosphotyrosine have been used to characterize the pattern of tyrosine-phosphorylated proteins in cells infected with RSV, activated forms of pp60$^{c\text{-}src}$ (e.g., pp60^{527F}), or transformation-defective *src* mutants (9, 18, 26,

* Corresponding author.

27, 36, 37, 51). Comparison of the patterns of phosphotyrosine-containing proteins in cells infected with RSV (or activated c-*src*) and unmyristylated *src* variants has led to the identification of transformation-specific phosphotyrosine-containing proteins (18, 26, 37, 51). One such protein, a 120-kDa protein (p120), was phosphorylated only in cells infected with RSV or activated c-*src* (c-src^{527F}) and not in cells infected with unmyristylated variants of $pp60^{src}$ (37, 51). Cell fractionation studies showed that transformation-specific p120 was associated with cellular membranes (37, 51).

During the above-mentioned studies, we observed that two tyrosine phosphoproteins, with M_rs of 130,000 and 110,000 coimmunoprecipitated with activated mutants of $pp60^{src}$. Here we demonstrate that the stable association of pp130 and pp110 with $pp60^{src}$ is significantly increased, and may be dependent upon, activation of the *src* protein kinase. We also show that coimmunoprecipitation of pp130 and/or pp110 with $pp60^{src}$ is blocked by deletion of sequences within the A and B boxes of activated *src* proteins and that the pp130 protein and, to a lesser extent, pp110, appear to be substrates for the $pp60^{src}$ kinase in vitro. These observations suggest that the association of $pp60^{src}$ with pp130 and pp110 may represent a functionally important interaction required either for *src*-mediated cell transformation or for normal *src* protein function.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Primary chicken embryo cells were prepared from group-specific^{-} negative embryos (SPAFAS, Inc.) as previously described (3, 4). Construction and transfection of pRL plasmids bearing chicken c-*src* gene mutants pRL-527F and pRL-2A/527F in a nonpermuted RSV clone were previously described (4, 52). For transfection experiments, DNA (1 to 2 μg) was applied to cells by standard calcium phosphate transfection techniques, and cell morphology was routinely monitored over a period of 4 to 21 days (4, 52). Replication of transformation-defective virus was assessed by resistance to superinfection with PrA RSV as described previously (4).

Preparation of cell extracts, metabolic labeling, immunoprecipitation, and immune complex kinase assay. Cells were labeled with 150 μCi of [^{35}S]methionine (Dupont, NEN Research Products, Boston, Mass.; 1,000 Ci/mmol) per ml for 6 h in methionine-free Dulbecco modified eagle medium supplemented with 10% dialyzed fetal calf serum and extracts were prepared as described previously (52). For clearing experiments, the detergent lysis buffer used to prepare extracts contained additional EDTA (5 mM) to ensure that phosphorylation did not occur during the incubation with monoclonal antibody (MAb) EC10. The protein concentrations of individual lysates were determined with the BCA protein assay kit (Pierce Chemical Co., Rockford, Ill.). The lysates were adjusted to 1 mg/ml, and 0.4 mg of total cell protein was reacted with 4μg of MAb EC10 (43). After 1 h at 4°C, 25 μl (packed volume) of protein A-Sepharose beads (Pharmacia, Inc., Piscataway, N.J.) was washed once in lysis buffer, added to the tubes, and incubated for 1 h. For kinase reactions, immunoprecipitates were washed twice in lysis buffer and twice in phosphate-buffered saline before initiation of the kinase reactions by the addition of 10 μl of 2× reaction buffer {40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 20 mM $MnCl_2$, 20 μCi of [γ-^{32}P]ATP (~6,000 Ci/mmol; Dupont, NEN)}. The reactions were terminated after 10 min by the addition of 20 μl of 2× Laemmli sample buffer (32). For [^{35}S]methionine and Western immunoblotting experiments, immunoprecipitates were washed four times in the detergent lysis buffer and then rapidly denatured with hot (100°C) Laemmli sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

pTyr-Abs and immunoblotting. Antibodies to phosphotyrosine (pTyr-Abs) were prepared as described previously (26, 27, 51), and their specificity has been documented (27, 29). Western immunoblotting was carried out as described by Towbin et al. (61) except that the standard transfer buffer was modified by the addition of SDS (0.075%) and sodium orthovanadate (0.5 mM), as described previously (26, 27, 51). Transfer to nitrocellulose sheets was carried out at 400 mA for 2 h at 4°C in modified transfer buffer in a standard blotter with an interelectrode distance of 7.5 cm. All remaining steps were carried out at room temperature. Nonspecific antibody-binding sites were blocked by incubation in 3% bovine serum albumin (fraction V; Boehringer Mannheim Biochemicals) for 4 to 16 h at room temperature. Affinity-purified rabbit pTyr-Abs (2 μg/ml in blocking buffer) were applied for 3 h at room temperature. The filters were then rinsed twice (~5 min per rinse) with rinsing buffer (10 mM Tris hydrochloride [pH 7.2], 0.9% NaCl, 0.01% NaN_3), once with rinsing buffer containing 0.05% Nonidet P-40 and 0.05% Tween 20, and twice again with rinsing buffer. Rabbit antibodies were detected by incubation of the rinsed filters for 1 h with 1 μCi of [^{125}I]protein A (Amersham Corp., Arlington Heights, Ill.) per ml followed by rinsing again as described above. The filters were exposed to X-RP film with an intensifying screen for 30 min to 24 h at −70°C. For detection of $pp60^{src}$, Western immunoblotting was carried out as described above, but *src* proteins were detected by using purified MAb 327 (38) radioiodinated to a specific activity of 10 μCi/μg.

Two-dimensional phosphoamino acid analysis. ^{32}P-labeled tyrosine phosphoproteins and $pp60^{src}$ proteins were subjected to 8% SDS-PAGE and identified by autoradiography of the gel. Individual polypeptide bands were isolated and the proteins were eluted from the gel slice as previously described (28). For phosphoamino acid analysis, the proteins were precipitated with trichloroacetic acid, washed with ethanol, and hydrolyzed in 100 μl of 5.7 M HCl at 110°C for 1.5 h (29). Hydrolyzed proteins were mixed with 0.3 μg each of authentic phosphotyrosine, phosphoserine, and phosphothreonine and resolved by two-dimensional thin-layer electrophoresis (12). Autoradiographic identification was compared with ninhydrin staining of authentic phosphoamino acids.

RESULTS

Activated $pp60^{src}$ is associated with two tyrosine-phosphorylated proteins. MAbs specific for $pp60^{src}$ were used to prepare immune complexes from cells infected with two activated c-*src* variants. c-src^{527F} is a transforming variant containing a single point mutation altering the site of regulatory phosphorylation, whereas c-$src^{2A/527F}$ is a nontransforming variant containing the activating mutation at residue 527 but lacking the site of amino-terminal myristylation (51). Whole-cell lysates and immune complexes isolated from these extracts were subjected to SDS-polyacrylamide gel analysis, and the presence of tyrosine-phosphorylated proteins was assessed by immunoblotting with affinity-purified antibodies to phosphotyrosine. The patterns of phosphotyrosine-containing proteins present in whole-cell lysates are

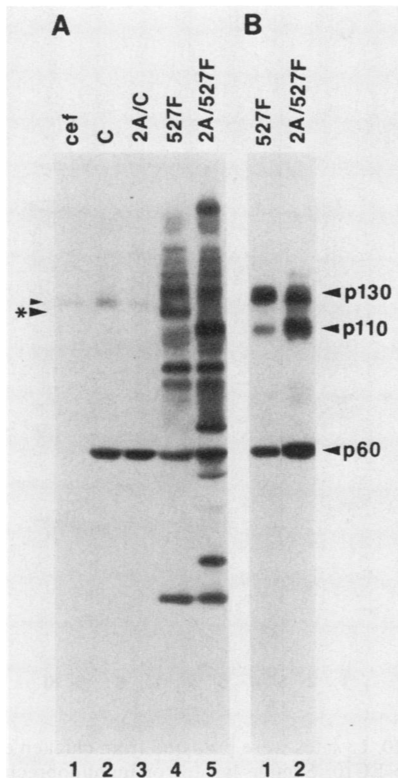

FIG. 1. Coimmunoprecipitation of pp130 and pp110 with activated c-*src* proteins. Chicken embryo cells expressing the indicated *src* variants were lysed in hot (100°C) Laemmli sample buffer (A) or in modified RIPA (29) for immunoprecipitation with the *src*-specific MAb EC10 (B). Total proteins (A) or immunoprecipitates (B) were separated by 8% SDS-PAGE and detected by immunoblotting with pTyr-Abs as described in the text. Proteins coprecipitating with activated *src* variants are designated (pp130 and pp110), the transformation-specific protein pp120 (lane 4) is indicated by an asterisk, and pp125 (lanes 1, 2, and 3) is indicated by a small arrowhead. C, c-*src*.

shown in Fig. 1A. Activation of the c-*src* protein by mutation of Tyr-527 to Phe (lane 4) resulted in an increase in the number (15 to 20) of phosphotyrosine-containing proteins. As previously reported (51), the additional mutation of the site of myristylation (Gly → Ala) led to a substantial decrease in the tyrosine-specific phosphorylation of a transformation-specific 120-kDa cellular protein (compare lanes 4 and 5). Cells expressing activated but unmyristylated pp60^{src} also contained several new phosphotyrosine-containing proteins with relative molecular masses of 39, 64, 110, and >200 kDa (lane 5; 51).

When pTyr-Abs were used to probe immunoblots containing immune complexes (prepared with the *src* protein-specific MAb EC10) from cells infected with c-*src*^{527F} and the unmyristylated variant c-*src*$^{2A/527F}$ (Fig. 1B, lanes 1 and 2), three major phosphotyrosine-containing proteins were detected, pp60^{src} and two cellular proteins with relative molecular masses of approximately 130 and 110 kDa. Comigration of pp130 and pp110 in SDS-PAGE with major phosphotyrosine-containing proteins present in whole-cell lysates of cells infected with either c-*src*^{527F} or c-*src*$^{2A/527F}$ (Fig. 1A, lanes 4 and 5) suggested that these proteins were the same.

The identity of pp130 and pp110 with two proteins in whole-cell lysates that comigrated in SDS-PAGE was confirmed by antibody-clearing experiments (Fig. 2C). Lanes 1 and 10 contained whole-cell lysates denatured at the beginning and the end of the experiment, respectively, to control for protease and phosphatase activities. pp60$^{2A/527F}$ was immunoprecipitated from 50 μg of total protein of a cell lysate with increasing amounts of MAb EC10 (lanes 2 to 5). The immune complexes (lanes 2 to 5) and the cleared lysates after immunoprecipitation (lanes 6 to 9) were then analyzed by SDS-PAGE and Western blotting with pTyr-Abs. Comparison of lanes 2 and 5 indicates that the presence of *src* protein complexes depended on the presence of MAb EC10 in the immunoprecipitation reaction mixture. In addition, comparison of lanes 6 and 9 indicates that with the appearance of pp130 and pp110 in the immunoprecipitates, a substantial proportion of these proteins was cleared from the lysates. Preclearing whole-cell extracts with protein A-Sepharose or Pansorbin prior to immunoprecipitation did not affect the subsequent recovery of pp60^{src}-pp130/pp110 complexes (data not shown).

To show that the presence of cellular phosphotyrosine-containing proteins in immune complexes was directly attributable to the immunoprecipitation of pp60^{src}, immune complexes were prepared from extracts of chicken embryo cells infected with c-*src*^{527F} and c-*src*$^{2A/527F}$, using a polyclonal rabbit anti-bacterial *src* serum (anti-bp60^{src} [17]), two *src* protein-specific MAbs, EC10 (43) and 327 (38), nonspecific rabbit serum, or nonspecific mouse MAbs (MAb 1 and MAb 2 [50]) (Fig. 2). Immune complexes were then analyzed by immunoblotting with pTyr-Abs. In addition, the patterns of whole-cell phosphotyrosine-containing proteins were examined before and after immunoprecipitation. The results of this analysis for cells infected with c-*src*$^{2A/527F}$ are shown in Fig. 2A (comparable results were obtained for c-*src*^{527F}-infected cells [data not shown]). Rabbit anti-bp60^{src} serum (lane 3) and two *src*-specific MAbs (lanes 4 and 5) readily immunoprecipitated pp60^{src} and the cellular proteins pp130 and pp110. None of the nonspecific antisera (lanes 2, 6, and 7) immunoprecipitated pp60^{src} or tyrosine-phosphorylated cellular proteins. Furthermore, the overall pattern of phosphotyrosine-containing proteins present in the extracts before and after immunoprecipitation remained essentially unchanged (lanes 1 and 9). A duplicate immunoblot probed with ^{125}I-labeled MAb 327 (Fig. 2B) confirmed the recovery of pp60^{src} in the immune complexes prepared with the individual antibodies. Immune complexes formed with *src* protein-specific MAbs EB7, EB8, and GD11, which bind epitopes distinct from those bound by MAbs EC10 and 327 (44), also contained the pp130 and pp110 proteins (data not shown). The data indicate that under the extraction conditions described above, pp130 and pp110 were specifically and tightly associated with activated *src* proteins in the immune complex.

pp130 and pp110 proteins do not form complexes with mutant pp60^{src}. To investigate the nature of the interaction of pp60^{src} and the cellular proteins, pp130 and pp110, immune complexes were prepared from cells infected with different transforming and nontransforming *src* variants. Cells infected with the variant c-*src*^{dl92} (deletion of amino acids 92 to 95) exhibited an elongated, partially transformed morphology (48). Fully activating the kinase activity of this variant by introducing the Tyr-527-to-Phe mutation resulted in an increase in in vivo phosphorylation (Fig. 3A, lanes 3 and 4). The elongated morphology was retained, but the cells became highly disorganized (data not shown). A second variant (c-*src*$^{dl155/527F}$), with a small deletion (residues 155 to 157) within a region of the SH2 region (B-box mutation) of

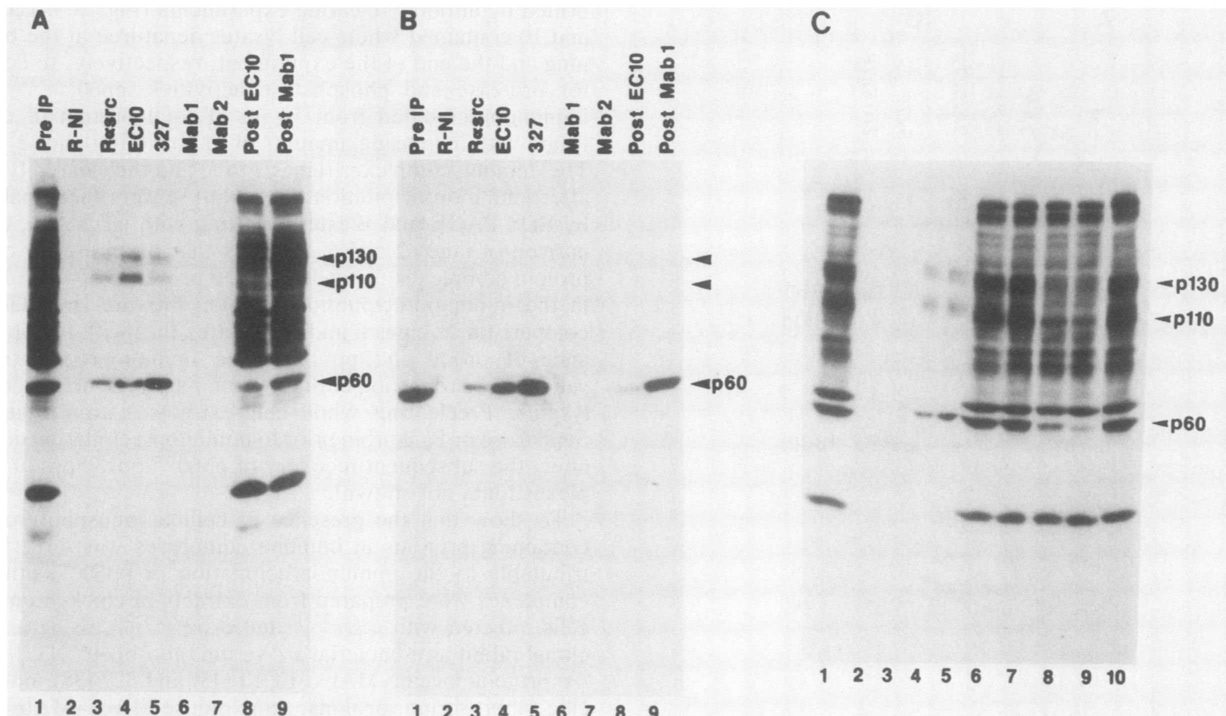

FIG. 2. Specificity of interaction between activated *src* proteins and pp130 and pp110. Lysates were prepared from chicken embryo cells expressing c-$src^{2A/527F}$ and immunoprecipitation reactions were performed with MAb EC10. Sample lysates or immunoprecipitates were divided equally, separated by electrophoresis in an 8% SDS-polyacrylamide gel, and analyzed by immunoblotting with pTyr-Abs (A) or the *src*-specific MAb 327 (B). Lanes 1, 8 and 9, Total cell lysates either prior to immunoprecipitation (lane 1) or after immunoprecipitation with MAb EC10 (lane 8) or an irrelevant MAb (50), MAb 1 (lane 9); lanes 3 to 5, immunoprecipitates prepared with *src*-specific antibodies (lane 3, rabbit anti-bp60^{src} serum; lane 4, MAb EC10; lane 5, MAb 327); lane 2, rabbit nonimmune serum; lane 6, MAb 1; lane 7, a second irrelevant MAb, MAb 2 (50). (C) pTyr blot of proteins remaining in the lysate after immunoprecipitation of $pp60^{2A/527F}$ cell lysates. Lanes 1 and 10, Total cell lysates before and after immunoprecipitation, respectively; lanes 2 to 5 immunoprecipitates obtained by using 0, 0, 0.5, and 4.0 μg, respectively, of MAb EC10. Immune complexes were collected with 100 μl (lane 2) or 30 μl (lanes 3 to 5) of Pasorbin. The cleared lysates from lanes 2 to 5 are shown in lanes 6 to 9, respectively.

activated *src* (c-src^{527F}), was defective for transformation. Figure 3A shows the patterns of phosphotyrosine-containing proteins in whole-cell extracts from uninfected cells (lane 1) and cells infected with c-*src* (lane 2), v-*src* (lane 7), c-src^{527F} (lane 5), c-$src^{2A/527F}$ (lane 6), and the deletion variants c-src^{dl92}, c-$src^{dl92/527F}$, and c-$src^{dl155/527F}$ (lanes 3, 4, and 8, respectively). In all cases, the tyrosine-specific phosphorylation of pp130 was evident, as was the phosphorylation of pp110 in the unmyristylated variant. Analysis of $pp60^{src}$ immune complexes prepared from lysates of infected cells (Fig. 3B) revealed the presence of pp130 and pp110 in immune complexes from cells infected with c-src^{527F} (lane 5), c-$src^{2A/527F}$ (lane 6), and v-*src* (lane 7). However, immune complexes from cells infected with c-$src^{dl155/527F}$ (lane 8) lacked both pp130 and pp110, while cells infected with either c-src^{dl92} or c-$src^{dl92/527F}$ (lanes 3 and 4, respectively) appeared to lack pp110. Though not evident in Fig. 3B, a minor phosphotyrosine-containing band migrating at 125 to 130 kDa was often seen in immunoprecipitates of $pp60^{c\text{-}src}$. When present, it was similar in intensity to the 125- to 130-kDa band in Fig. 3A, lane 2. The identity and potential relationship of this protein to pp130 and pp110 are unknown.

To ascertain whether the apparent lack of pp130 and pp110 in immune complexes from cells infected with c-$src^{dl155/527F}$ resulted from a decrease in the stable association of pp130 and pp110 with variant $pp60^{src}$ or from the inability of variant $pp60^{src}$ to phosphorylate associated pp110 and pp130, uninfected cells and cells infected with c-*src* and

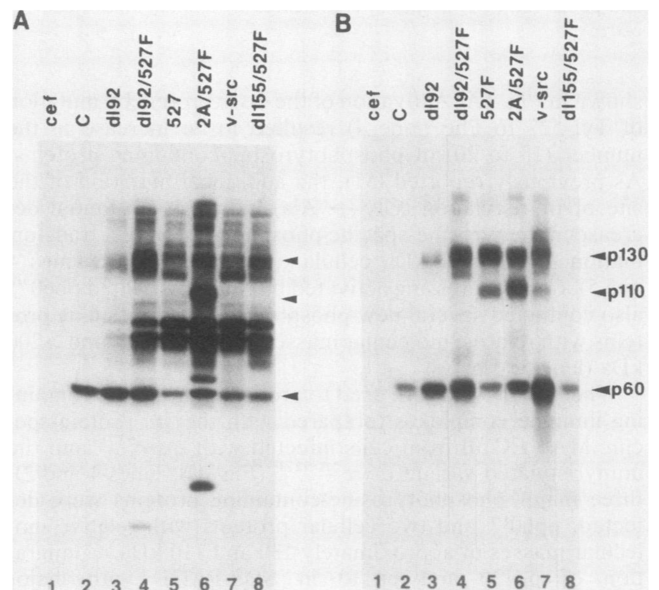

FIG. 3. Differential interaction of pp130 and pp110 with variant *src* proteins. Whole-cell lysates (A) and MAb EC10 immunoprecipitates (B) from cells expressing *src* variants were separated by 8% SDS-PAGE and analyzed by immunoblotting with pTyr-Abs. Lanes 1, *cef*; lanes 2, c-*src*; lanes 3, c-src^{dl92}; lanes 4, c-$src^{dl92/527F}$; lanes 5, c-src^{527F}; lanes 6, c-$src^{2A/527F}$; lanes 7, v-*src*; lanes 8, c-$src^{dl155/527F}$.

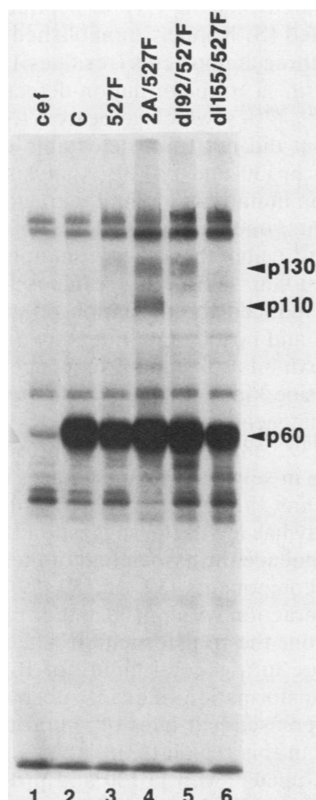

FIG. 4. Coimmunoprecipitation of pp130 and pp110 from [^{35}S] methionine-labeled cells. Immunoprecipitates were prepared with *src*-specific MAb EC10 from [^{35}S]methionine-labeled chicken embryo cells expressing the indicated variant *src* proteins, as described in the text. Proteins were separated by 8% SDS-PAGE and subjected to autoradiography for 8 days. C, c-*src*.

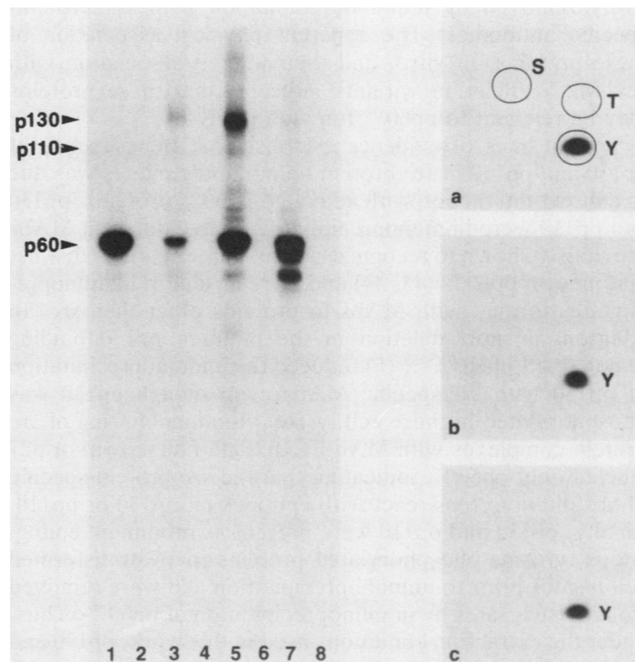

FIG. 5. In vitro phosphorylation of pp130 and pp110. (Left panel) Immune complexes obtained by using MAb EC10 (odd-numbered lanes) or a nonspecific MAb (even-numbered lanes) were prepared from cells expressing c-*src* (lanes 1 and 2), c-*src*^{527F} (lanes 3 and 4), c-*src*$^{2A/527F}$ (lanes 5 and 6), or c-*src*$^{dl155/527F}$ (lanes 7 and 8). Immune complexes were incubated with [γ-^{32}P]ATP and Mn^{2+} under standard immune complex kinase conditions as described in the text. (Right panel) Phosphoamino acid analysis of in vitro-labeled proteins pp60^{src} (a), pp130 (b), and pp110 (c).

individual transforming and nontransforming c-*src* variants were labeled with [^{35}S]methionine. Immune complexes were prepared with MAb EC10 and analyzed by SDS-PAGE. A labeled protein of approximately 130 kDa was clearly evident in immune complexes from c-*src*^{527F}-infected cells (Fig. 4, lane 3), and proteins of 130 and 110 kDa were present in c-*src*$^{2A/527F}$-infected cells (lane 4). These data confirm the association of pp130 and pp110 with activated pp60^{src} proteins, an association previously detected only by pTyr-Abs. Immune complexes from cells infected with c-*src* (lane 2) or c-*src*$^{dl155/527F}$ (lane 6) contained no detectable pp130 (or pp110), indicating that neither the tightly regulated pp60$^{c\text{-}src}$ nor the deletion variant pp60$^{dl155/527F}$ forms stable complexes with the major component of the complex, pp130. Finally, immune complexes prepared from cells infected with c-*src*$^{dl92/527F}$ contained significant amounts of pp130 but undetectable amounts of pp110, as implied by Western blotting experiments, suggesting that the structural alteration of pp60^{src} in this variant altered the association of pp60^{src} with pp110 but not with pp130. However, pp110 is difficult to detect by ^{35}S labeling, as reflected by its absence from pp60^{527F} immune complexes, in which it was consistently observed by Western blotting with pTyr-Abs.

In vitro phosphorylation of pp130 and pp110 on tyrosine. The association of pp130 and pp110 in pp60^{src} immune complexes isolated from cells infected with activated forms of the *src* gene (e.g., c-*src*^{527F} or c-*src*$^{2A/527F}$) prompted us to investigate the possibility that pp60^{src} might directly phosphorylate pp130 and/or pp110 in vitro. Immune complexes were prepared from cells infected with c-*src*, c-*src*^{527F}, c-*src*$^{2A/527F}$, and c-*src*$^{dl155/527}$. Following incubation with [γ-^{32}P]ATP and Mn^{2+} under standard immune complex kinase conditions (28, 43), the in vitro-labeled products were resolved by SDS-PAGE (Fig. 5). Immune complexes (MAb EC10 [odd-numbered lanes] or nonspecific MAb [even-numbered lanes]) containing pp60$^{c\text{-}src}$ displayed significant autophosphorylation, but little phosphorylation of pp130 and pp110 was observed. In contrast, immune complexes containing c-*src*^{527F} or c-*src*$^{2A/527F}$ exhibited both autophosphorylation activity and readily detectable phosphorylation of pp130 (lanes 3 and 5, respectively). Interestingly, pp110 appeared to be labeled less efficiently. Immune complexes containing pp60$^{dl155/527F}$ showed a significantly reduced phosphorylation of pp130 and no detectable phosphorylation of pp110. The in vitro phosphorylation of pp130/pp110 and pp60^{src} was dependent upon the presence of Mn^{2+} or Mg^{2+} and was not stimulated by the addition of Ca^{2+} (data not shown). The tyrosine-specific phosphorylation of pp60^{src}, pp130, and pp110 was confirmed by phosphoamino acid analysis of the in vitro-labeled products (Fig. 5). Further, we have previously shown that pp130 and pp110 are labeled on tyrosine, as well as on serine and threonine, in vivo (29).

DISCUSSION

We have identified two phosphotyrosine-containing cellular proteins, pp130 and pp110, that coimmunoprecipitate with pp60$^{v\text{-}src}$ and activated forms of pp60$^{c\text{-}src}$ (pp60^{527F}). Of the more than 15 prominent phosphoproteins detected on immunoblots with pTyr-Abs, pp130 and pp110 were selec-

tively removed by immunoprecipitation with *src* protein-specific antibodies. The apparent physical association of these proteins, in vitro, and their altered association with several *src* deletion variants indicates that these proteins may be relevant to pp60^{src} function in vivo.

Several lines of evidence establish that the presence of pp130 and pp110 in *src* protein immunoprecipitates was due to a direct interaction with activated pp60^{src} proteins. pp130 and pp110 were immunoprecipitated by five different MAbs previously shown to recognize at least three spatially distinct epitopes on pp60^{src} (43, 44) and were absent in immunoprecipitates formed with MAbs to proteins other than *src*. In addition, a short deletion in the putative pp130 binding region (e.g., pp60$^{dl155/527F}$) reduced the immunoprecipitation of pp130 with *src*-specific MAbs even though pp130 was phosphorylated in these cell lysates. Immunoblotting of *src* protein complexes with MAb EC10 (data not shown) or 327 labeled only pp60^{src}, indicating that the *src* protein-specific MAbs did not cross-react with epitopes on pp130 or pp110. Finally, pp130 and pp110 were present as prominent endogenous tyrosine-phosphorylated proteins in *src*-transformed cell lysates prior to immunoprecipitation and were removed from these lysates by immunoprecipitation of pp60^{src}. Thus, under the extraction conditions used in this work, pp130 and pp110 appear to be specifically and tightly associated with activated *src* proteins in immune complexes.

***src* variants exhibit altered association with pp130 and pp110.** Expression of an unmyristylated *src* variant (pp60$^{2A/527F}$) or variants with mutations in the A box (pp60^{dl92} and pp60$^{dl92/527F}$) or the B box (pp60$^{dl155/527F}$) resulted in altered phosphorylation of pp130 and pp110 and altered association of these proteins with immunoprecipitable *src* protein. Lysates from cells expressing the nonmyristylated *src* protein showed increased levels of tyrosine-phosphorylated pp110 compared with cell lysates of 527F-infected cells, in which tyrosine-phosphorylated pp110 was often at or below the limit of detection. It is possible that pp110 and unmyristylated pp60^{src} become sequestered in a functional interaction that is unresolved (or unusually stable) in the cytoplasm because of the lack of access to normal association with a key component(s) in cellular membranes. The phosphorylation of a number of other proteins (e.g., pp64 and pp39) was also selectively induced by expression of pp60$^{2A/527F}$, yet for these proteins there was no evidence of complex formation.

Tyrosine-phosphorylated pp130 present in whole-cell lysates of cells infected with A-box mutants (pp60^{dl92} or pp60$^{dl92/527F}$) migrated as a heterogeneous band on SDS-polyacrylamide gels, a characteristic that remained even after immunoprecipitation with *src*-specific MAbs. Attempts to demonstrate glycosylation of these proteins were unsuccessful (data not shown). The heterogeneity of pp130 could therefore reflect the altered phosphorylation which, in vivo, occurs on tyrosine, serine, and threonine (29). Although the amount of tyrosine-phosphorylated pp110 was reduced in immunoprecipitates with *dl*92 *src* proteins, the amount of pp130 appeared similar to that found associated with 527F *src* proteins. These results raise the possibility that altering the association of pp110 by deletion (or alteration) of its binding site could affect phosphorylation of pp130. Since immunoblotting with pTyr-Abs revealed that neither pp130 nor pp110 was associated with variant *src* proteins containing B-box mutations, formation of the pp130-pp60^{src} complex could be a prerequisite for pp110 binding and/or phosphorylation. Preliminary phosphotryptic mapping of pp110 and pp130 indicated that the tryptic peptides of these proteins are unrelated (S. Kanner, unpublished results).

A deletion of three amino acids (residues 155 to 157) in the B box results in a transformation-defective variant of pp60^{527F} (pp60$^{dl155/527F}$) which appeared to phosphorylate pp130 in vivo but did not form detectable detergent-stable complexes with pp130 and pp110. Analysis of other deletion and insertion mutations within this region revealed that two other mutants, one having a deletion of residues 161 to 163 (c-*src*$^{dl161/527F}$) and the other containing an insertion of Leu-Glu-Leu-Glu between residues 161 and 162 (c-*src*$^{is161/527F}$), reduced the formation of stable complexes between pp60^{src} and pp130 (data not shown). This region of the *src* protein exhibits substantial sequence homology with several nontyrosine kinase proteins, such as phospholipase C 148, the *crk* oncogene product, and the GTPase activating protein, GAP (39, 58, 59, 62). The presence of similar structural motifs in several tyrosine kinases and membrane-associated proteins thought to be involved in the cellular signaling pathway has led to the suggestion that these motifs may direct or influence the association of these proteins with unique cellular components (39, 45, 58, 59). Although it is possible that interaction with pp130 and/or pp110 is required for transformation, the transformation-defective phenotype could also be due to a general failure of B-box variants to interact with transformation-relevant substrates or, alternatively, to the decreased half-lives of *src* proteins with structural alterations in this region (63).

Association of pp60^{src} with pp130 and pp110 is correlated with activation of protein kinase activity. The association of pp60^{src} with pp130 and/or pp110 appeared to depend on the enzymatic activation of the *src* protein and the tyrosine phosphorylation of complexed proteins. In addition, if cell extracts were prepared in the absence of the phosphatase inhibitor orthovanadate, the amount of pp130 and pp110 associated with pp60^{src} was reduced, as determined by immunoprecipitation of *src* complexes from [^{35}S]methionine-labeled cells (data not shown). The data indicate that tyrosine phosphorylation of pp130 and pp110 or the autophosphorylation of pp60^{src} may contribute to the stable complex formation.

The extent of [^{35}S]methionine labeling of pp130 or pp110 in immune complexes with pp60^{src} suggests that only a small percentage of the *src* protein is complexed with pp130 and/or pp110. Interestingly, immunoblotting of immune complexes with pTyr-Abs generates a pp130 (and pp110) signal almost equal to that of pp60^{src}, leading us to speculate that either pp130 or pp110 is hyperphosphorylated on Tyr or that the pTyr-Abs have unusually high affinity for the pTyr epitopes present in pp130 and pp110. Further characterization of these proteins, including the relative abundance and the stoichiometry of phosphorylation, await the development of specific antibodies to detect both the tyrosine-phosphorylated and unphosphorylated forms of these proteins.

Using different antibodies directed against pp60^{src}, Lau (33, 33a) has reported that a 120-kDa protein containing phosphotyrosine can be immunoprecipitated from a variety of RSV-transformed mammalian cells but not from uninfected cells. Proteolytic peptide mapping and immunological analysis have shown that pp120 is unrelated to RSV virion proteins, vinculin, or pp60^{src} itself (33a). Furthermore, Lau has recently reported that pp120 is a potential substrate for pp60$^{v\text{-}src}$ (33a). Based on MAb EC10 immunoprecipitation and pTyr-Ab Western blotting experiments with RSV-infected field vole cells, we have concluded that the pp130

protein described here is similar, if not identical, to the pp120 protein previously described by Lau (33, 33a).

The precise role of pp130 and pp110 in the regulation of pp60^{src} activity remains to be defined. However, the apparent requirement for conserved sequences within the A and B boxes for association of pp60^{src} and pp130/pp110, along with the observation that activation of kinase activity is required for complex formation, provides an impetus to further characterize these proteins. We have recently isolated MAbs specific for pp130 and pp110 (unpublished results). These antibodies should provide reagents to determine whether the phenotypes associated with the *src* variants used in this work are directly related to their altered interactions with pp130 and pp110.

ACKNOWLEDGMENTS

We thank B. Creasy for preparation of chicken embryo cells. We acknowledge the many helpful comments and advice of M. Payne, W. Potts, A. Bouton, S. Parsons, and M. Weber. We are indebted to S. Parsons and J. Brugge for providing antibodies to pp60^{src}. We thank B. Nordin for help in preparation of the manuscript.

This investigation was supported by Public Health Service grants CA29243 and CA40042 awarded by the National Cancer Institute and grant MV-462 from the American Cancer Society. A.B.R. was supported by a fellowship from the American Cancer Society (PF2923), and S.B.K. was supported by a fellowship from the National Cancer Institute (CA08316).

LITERATURE CITED

1. **Bishop, J. M.** 1987. The molecular genetics of cancer. Science **235**:305–311.
2. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
3. **Bryant, D., and J. T. Parsons.** 1982. Site-directed mutagenesis of the *src* gene of Rous sarcoma virus: construction and characterization of a deletion mutant temperature sensitive for transformation. J. Virol. **44**:683–691.
4. **Bryant, D. L., and J. T. Parsons.** 1984. Amino acid alterations within a highly conserved region of the Rous sarcoma virus *src* gene product pp60^{src} inactivate tyrosine protein kinase activity. Mol. Cell. Biol. **4**:862–866.
5. **Buss, J. E., M. P. Kamps, K. Gould, and B. M. Sefton.** 1986. The absence of myristic acid decreases membrane binding of p60^{src} but does not affect tyrosine protein kinase activity. J. Virol. **58**:468–474.
6. **Buss, J. E., M. P. Kamps, and B. M. Sefton.** 1984. Myristic acid is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. **4**:2697–2704.
7. **Buss, J. E., and B. M. Sefton.** 1985. Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. J. Virol. **53**:7–12.
8. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
9. **Comoglio, P. M., M. F. Di Renzo, G. Tarone, F. G. Giancotti, L. Naldini, and P. C. Marchisio.** 1984. Detection of phosphotyrosine-containing proteins in the detergent-soluble fraction of RSV-transformed fibroblasts by azobenzene phosphonate antibodies. EMBO J. **3**:483–489.
10. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr^{527} is phosphorylated in pp60$^{c\text{-}src}$: implications for regulation. Science **231**:1431–1434.
11. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60$^{c\text{-}src}$. Mol. Cell. Biol. **6**:4467–4477.
12. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
13. **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.
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. **DeClue, J. E., and G. S. Martin.** 1989. Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. J. Virol. **63**:542–554.
16. **Garber, E. A., F. R. Cross, and H. Hanafusa.** 1985. Processing of p60$^{v\text{-}src}$ to its myristylated membrane-bound form. Mol. Cell. Biol. **5**:2781–2788.
17. **Gilmer, T. M., and R. L. Erikson.** 1983. Development of anti-pp60^{src} serum with antigen produced in *Escherichia coli*. J. Virol. **45**:462–465.
18. **Hamaguchi, M., C. Grandori, and H. Hanafusa.** 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. Mol. Cell. Biol. **8**:3035–3042.
19. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
20. **Hunter, T., and J. A. Cooper.** 1986. Viral oncogenes and tyrosine phosphorylation, p. 191–246. *In* P. D. Boyer and E. G. Krebs (ed.), The enzymes, vol. 17. Academic Press, Inc., Orlando, Fla.
21. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
22. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants which carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
23. **Jove, R., and H. Hanafusa.** 1987. Cell transformation by the viral *src* oncogene. Annu. Rev. Cell Biol. **3**:31–56.
24. **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.
25. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1986. Rous sarcoma virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation. Cell **45**:105–112.
26. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ros*, and v-*erb*-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2**:305–315.
27. **Kanner, S. B., T. M. Gilmer, A. B. Reynolds, and J. T. Parsons.** 1989. Novel tyrosine phosphorylations accompany the activation of pp60$^{c\text{-}src}$ during chemical carcinogenesis. Oncogene **4**:295–300.
28. **Kanner, S. B., S. J. Parsons, J. T. Parsons, and T. M. Gilmer.** 1988. Activation of pp60$^{c\text{-}src}$ tyrosine kinase specific activity in tumor-derived Syrian hamster embryo cells. Oncogene **2**:327–335.
29. **Kanner, S. B., A. B. Reynolds, and J. T. Parsons.** 1989. Immunoaffinity purification of tyrosine-phosphorylated cellular proteins. J. Immunol. Methods **120**:115–124.
30. **Kato, J., Y. Hirota, N. Nakamura, N. Nakamura, and T. Takeya.** 1987. Structural features of the carboxy terminus of p60^{src} that are required for the regulation of its intrinsic kinase activity. Jpn. J. Cancer Res. **78**:1354–1362.
31. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60$^{c\text{-}src}$ transforming ability by mutation of its primary site of tyrosine phosphorylation. Cell **49**:65–73.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
33. **Lau, A. F.** 1986. Phosphotyrosine-containing 120,000-dalton protein coimmunoprecipitated with pp60$^{v\text{-}src}$ from Rous sarcoma virus-transformed mammalian cells. Virology **151**:86–99.

33a. **Lau, A. F.** 1989. Evidence that a phosphotyrosine-containing 120,000 Da protein from Rous sarcoma virus infected cells is

phosphorylated by pp60^{v-src}. Oncogene Res. **1**:185–194.

34. **Laudano, A. P., and J. M. Buchanan.** 1986. Phosphorylation of tyrosine in the carboxyl-terminal tryptic peptide of pp60^{c-src}. Proc. Natl. Acad. Sci. USA **83**:892–896.
35. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
36. **Linder, M. E., and J. G. Burr.** 1988. Immunological characterization of proteins detected by phosphotyrosine antibodies in cells transformed by Rous sarcoma virus. J. Virol. **62**:2665–2673.
37. **Linder, M. E., and J. G. Burr.** 1988. Nonmyristoylated p60^{v-src} fails to phosphorylate proteins of 115-120 kDa in chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **85**:2608–2612.
38. **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.
39. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
40. **Nemeth, S. P., L. G. Fox, M. DeMarco, and J. S. Brugge.** 1989. Deletions within the amino-terminal half of the c-*src* gene product which alter the functional activity of the protein. Mol. Cell. Biol. **3**:1109–1119.
41. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-*src* and chicken c-*src* in rat cells demonstrates qualitative differences between pp60^{v-src} and pp60^{c-src}. Cell **37**:131–139.
42. **Parsons, J. T., and M. J. Weber.** 1989. Genetics of *src*: Structure and function of a protein tyrosine kinase. Curr. Topics Microbiol. Immunol. **147**:79–127.
43. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{src} react with enzymatically active cellular pp60^{src} of avian and mammalian origin. J. Virol. **51**:272–282.
44. **Parsons, S. J., D. J. McCarley, V. W. Raymond, and J. T. Parsons.** 1986. Localization of conserved and nonconserved epitopes within the Rous sarcoma virus-encoded *src* protein. J. Virol. **59**:755–758.
45. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene **3**:491–495.
46. **Pelham, H.** 1988. Coming in from the cold. Nature (London) **332**:776–777.
47. **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.
48. **Potts, W. M., A. B. Reynolds, T. J. Lansing, and J. T. Parsons.** 1988. Activation of pp60^{c-src} transforming potential by mutations altering the structure of an amino terminal domain containing residues 90–95. Oncogene Res. **3**:343–355.
49. **Raymond, V. W., and J. T. Parsons.** 1987. Identification of an amino terminal domain required for the transforming activity of the Rous sarcoma virus *src* protein. Virology **160**:400–410.
50. **Reynolds, A. B., and G. Oliphant.** 1986. Quantitation of the rabbit sperm acrosome stabilizing factor utilizing a sensitive immunoradiometric assay. Biol. Reprod. **35**:705–715.
51. **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.
52. **Reynolds, A. B., J. Vila, T. J. Lansing, W. M. Potts, M. J. Weber, and J. T. Parsons.** 1987. Activation of the oncogenic potential of the avian cellular *src* protein by specific structural alteration of the carboxy terminus. EMBO J. **6**:2359–2364.
53. **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.
54. **Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa.** 1985. Amino terminal myristylation of the protein kinase p60^{src}, a retroviral transforming protein. Science **227**:427–429.
55. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
56. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
57. **Shalloway, D., P. M. Coussens, and P. Yaciuk.** 1984. c-*src* and *src* homolog overexpression in mouse cells, p. 9–17. *In* G. F. Vande Woude (ed.), Cancer cells, vol. 2. Oncogenes and viral genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **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.
59. **Suh, P.-G., S. H. Ryo, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Cloning and sequence of multiple forms of phospholipase C. Cell **54**:161–169.
60. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell **32**:881–890.
61. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
62. **Vogel, U. S., R. A. F. 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: a protein that interacts with oncogenic *ras* p21. Nature (London) **335**:90–93.
63. **Wang, H.-C. R., and J. T. Parsons.** 1989. Deletions and insertions within an amino-terminal domain of pp60^{v-src} inactivate transformation and modulate membrane stability. J. Virol. **63**:291–302.
64. **Wyke, J. A., and A. W. Stoker.** 1987. Genetic analysis of the form and function of the viral *src* oncogene product. Biochim. Biophys. Acta **907**:47–69.
65. **Yonemoto, W., A. J. Filson, A. E. Queral-Lustig, J. Y. J. Wang, and J. S. Brugge.** 1987. Detection of phosphotyrosine-containing proteins in polyomavirus middle tumor antigen-transformed cells after treatment with a phosphotyrosine phosphatase inhibitor. Mol. Cell. Biol. **7**:905–913.