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Cell Biology

A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein

(*src*/sodium arsenite/neoplastic transformation/protein kinase)

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ABSTRACT **A single viral protein (pp60^{src}) mediates neoplastic transformation of cells infected with Rous sarcoma virus. Immunoprecipitation of pp60^{src} has revealed two cellular proteins (M_r 50,000 and 89,000) that appear to associate with pp60^{src} in a specific manner. Neither of the cellular proteins has been well characterized, but it is thought that both may participate in the function of pp60^{src}. Treatment of avian cells with unphysiological temperature or certain chemical agents amplifies the production of several proteins in the manner of the "heat shock" response earlier described for *Drosophila*. We report here that one of these proteins, with a molecular weight of 89,000, is identical to the 89-kilodalton protein found associated with pp60^{src}. The 89-kilodalton protein is a major constituent of both uninfected and infected cells, even in the absence of inducing agents, but only a small fraction of this protein appears to associate with pp60^{src} in cells transformed by Rous sarcoma virus. The complex containing pp60^{src} and the 89-kilodalton protein can be precipitated by an immune reaction involving pp60^{src} alone. The complexed form of the 89-kilodalton protein did not react directly with antibodies but regained its reactivity subsequent to release from the complex. We conclude that the 89-kilodalton protein is bound to pp60^{src} in a relatively stable complex. We suggest that the 89-kilodalton protein may have overlapping roles in viral oncogenesis and the heat shock response, and that evidence on the function of the protein in either setting may illuminate its function in the other. In addition, it may prove profitable to search for other overlaps between the cellular response to heat shock and the neoplastic transformation of cells by pp60^{src}.**

Neoplastic transformation of fibroblasts infected with Rous sarcoma virus (RSV) is mediated by a 60,000-dalton [60-kilodalton (60-kDal)] phosphoprotein (pp60^{src}) (1–3) that is encoded in the viral gene *src* (4) and displays the enzymatic activity of a protein kinase (2, 5). Both crude (6) and purified (7, 8) preparations of pp60^{src} phosphorylate tyrosine exclusively in protein substrates. Moreover, neoplastic transformation of cells by *src* causes an increase in the amount of phosphotyrosine in cellular proteins (6, 9). These findings have prompted the hypothesis that oncogenesis by RSV is attributable to inordinate or inappropriate phosphorylation of tyrosine in proteins whose function is essential to the maintenance of normal cell structure and growth. Pursuit of this hypothesis will require identification and characterization of the cellular proteins with which pp60^{src} interacts. As a potentially useful step towards these ends, we report here that a protein known to be associated with pp60^{src} in extracts of infected cells is one of a small number of cellular proteins whose synthesis is substantially augmented during the response of eukaryotic cells to noxious agents, such as chelating compounds (10, 11), transition metals, sulfhydryl reagents (12, 13), and heat (14). This finding may provide new experimental access to the interaction of pp60^{src} with host proteins.

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METHODS

The sources of most of our chemical and biological reagents have been described (2). We used [^{32}P]orthophosphate from ICN and [^{35}S]methionine (*ca.* 1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) from Amersham. Cells were propagated and labeled according to our published procedures (15). Heat shock of cells was performed by incubating cultures at 45°C for 3 hr prior to metabolic labeling with radioactive precursors. For induction with arsenite, cells were exposed to growth medium containing the ion (50 μM) for 3 hr prior to labeling. Extracts for immunoprecipitation were prepared by dissolving previously washed cells in 0.15 M NaCl/0.02 M sodium phosphate, pH 7.2 /1 mg of bovine serum albumin per ml/1% Nonidet P40. Sera containing antibodies against pp60^{src}, viral structural proteins, or both were prepared as described (2, 15). When necessary, sera were adsorbed by the prior addition of purified RSV that had been disrupted with nonionic detergent (2). Rabbit antibodies against the 95-kDal chicken heat shock protein were kindly provided by Milton Schlesinger (16). The 95-kDal protein in his nomenclature corresponds to the 89-kDal protein in our work. Immunoprecipitates were harvested by adsorption to formalin-fixed *Staphylococcus aureus* (17, 18) and analyzed by electrophoresis in gels of polyacrylamide (19). Solid-phase immunoadsorbents were prepared by linking antisera to Sepharose and then used as described (8). The composition of individual proteins was analyzed by partial hydrolysis with proteases (18, 20) and by two-dimensional separation of tryptic peptides, as described (13, 15).

RESULTS

Proteins Precipitated in Conjunction with pp60^{src}. The transforming protein pp60^{src} can be isolated from extracts of RSV-infected cells by immunoprecipitation with antisera taken from rabbits bearing tumors induced by RSV ("rabbit tumor antisera") (1). In order to simplify the analysis of immunoprecipitates, we generally adsorb the tumor antisera with disrupted RSV in order to remove antibodies directed against structural proteins of the virus (2). We and others (6, *) have found that precipitation of pp60^{src} with adsorbed antisera also precipitates two other phosphoproteins with molecular weights of 50,000 and 89,000 (Fig. 1*A*, lane 4). [The experiment illustrated in Fig. 1 was performed with [^{35}S]methionine, which does not label the

Abbreviations: RSV, Rous sarcoma virus; SR-RSV, Schmidt–Ruppin strain of RSV; *src*, the gene responsible for oncogenesis by RSV; pp60^{src}, the phosphoprotein encoded in *src*; Pr76^{gag}, a polyprotein precursor of RSV structural proteins; pp50^{cell}, a 50,000-dalton phosphoprotein of the host cell for RSV; pp89^{cell}, an 89,000-dalton phosphoprotein of the host cell for RSV; kDal, kilodalton.

* J. S. Brugge, personal communication.

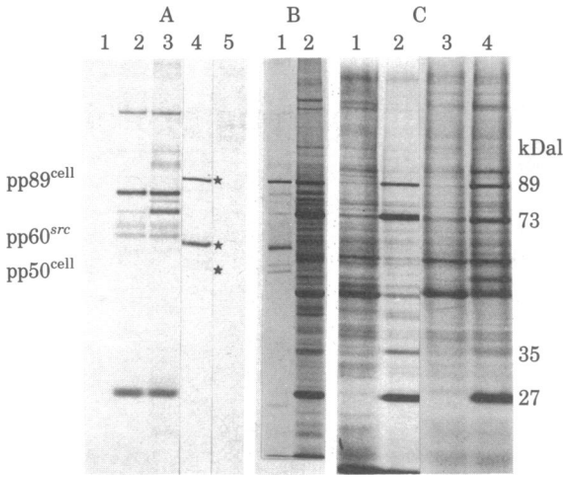

FIG. 1. Comparison of immunoprecipitated proteins to proteins induced by sodium arsenite or heat. (*A*) Immunoprecipitated proteins. Immunoprecipitates were prepared from cells that had been labeled with [^{35}S]methionine for 3 hr at 41°C. Immunoprecipitated proteins were fractionated by electrophoresis in a gel of 9% polyacrylamide. Lanes 1–4 depict results obtained with chicken fibroblasts transformed by SR-RSV; lane 5, results obtained with uninfected chicken fibroblasts. Immunoprecipitates were prepared with normal rabbit serum (lane 1), antiserum directed against p27 of RSV (lane 2), antiserum raised against disrupted RSV (lane 3), and rabbit tumor antiserum that had been preadsorbed with disrupted RSV (lanes 4 and 5). The stars along lane 4 mark the location of the three proteins whose names are given at the left. (*B*) Comparison of immunoprecipitated proteins and proteins induced with sodium arsenite. Chicken fibroblasts transformed by SR-RSV were labeled and analyzed by immunoprecipitation with rabbit tumor antiserum, as in *A*. Uninfected cells were incubated with sodium arsenite, labeled, and analyzed as in *C* (lane 2). The immunoprecipitated and arsenite-induced samples were analyzed in parallel lanes of the same gels. Lane 1, immunoprecipitate from RSV-transformed cells; lane 2, sample from arsenite-induced uninfected cells. (*C*) Proteins induced by sodium arsenite or heat. Uninfected chicken fibroblasts were incubated in experimental conditions for a total of 5 hr, labeled during the last 2 hr with [^{35}S]methionine, and analyzed directly by electrophoresis in gels of 9% polyacrylamide. Lane 1, normal growth medium at 37°C; lane 2, growth medium containing sodium arsenite at 50 μmol at 37°C; lane 3, normal growth medium at 37°C; lane 4, normal growth medium at 45°C.

50,000-kDal protein well; the protein is more apparent in immunoprecipitates prepared from cells labeled with ^{32}P (see Fig. 4*B*).] Because all available evidence indicates that these are cellular rather than viral proteins, we have adopted the tentative designations of pp50^{cell} and pp89^{cell}. Coprecipitation of pp60^{src}, pp50^{cell}, and pp89^{cell} is most readily perceptible with cells transformed by the Schmidt–Ruppin strain of RSV (SR-RSV) and variants derived from it when sera induced by the Schmidt–Ruppin strain are used†; we have used this strain of RSV throughout the work described here.

The immunoprecipitation of pp50^{cell} and pp89^{cell} is specific: these proteins are not recovered from RSV-transformed cells with either normal rabbit serum (Fig. 1*A*, lane 1) or sera directed against structural proteins of RSV (Fig. 1*A*, lanes 2 and 3), and neither protein can be recovered from uninfected normal or spontaneously transformed cells by immunoprecipitation with RSV tumor serum (Fig. 1*A*, lane 5, and unpublished data). We conclude that the immunoprecipitation of pp50^{cell} and pp89^{cell} by RSV tumor antisera requires the coincident precipitation of pp60^{src}. In work to be reported elsewhere, we† and Brugge* have suggested that the two cellular proteins are precipitated because they are bound to pp60^{src} in a relatively stable complex. Further data supporting this suggestion will be presented below. These findings implicate pp50^{cell} and pp89^{cell} in the function of *src* but provide no indication as to the nature of the two proteins. An informative finding emerged serendipitously, however, from our analyses of proteins whose synthesis is augmented by the application of certain chemical agents (or unphysiological temperatures) to eukaryotic cells.

Identification of pp89^{cell} as a Heat Shock Protein. Treatment of uninfected chicken embryo fibroblasts with sodium arsenite greatly amplifies the abundance of proteins with molecular weights of 89,000, 73,000, 35,000, and 27,000 (12, 13). An example of this phenomenon is illustrated in Fig. 1*C*, lane 2. [We have shown elsewhere that the induction of these proteins is due to increases in the rates of their synthesis (13).] An overlapping (but not identical) set of proteins is also induced by incubating the cells at the relatively high temperature of 45°C (Fig. 1*C*, lane 4). One of the induced proteins has the same electrophoretic mobility as the pp89^{cell} recovered from RSV-transformed cells by immunoprecipitation (Fig. 1*B*, lanes 1 and 2). Both the immunoprecipitated and the induced forms of pp89^{cell} could be labeled with ^{32}P (see below). By contrast, none of the proteins induced by arsenite or heat appeared similar to the immunoprecipitated pp50^{cell} (Fig. 1 and unpublished data).

An 89-kDal protein can be detected in uninduced and uninfected cells without benefit of immunoprecipitation (see Fig. 1*C*, lanes 1 and 3, and ref. 13); we have found that this protein has the same peptide map as its induced cognate.† It therefore appears that pp89^{cell} is indigenous to normal cells and that its synthesis is augmented by arsenite or heat. By contrast, the transformation of cells by RSV had no obvious effect on the constitutive amounts of pp89^{cell} (data not illustrated). We have also shown that pp50^{cell} is a cellular—rather than a viral—protein,† but we currently have no evidence that it is present in uninfected cells, and it remains possible that production of the protein is induced (or at least augmented) by transformation.

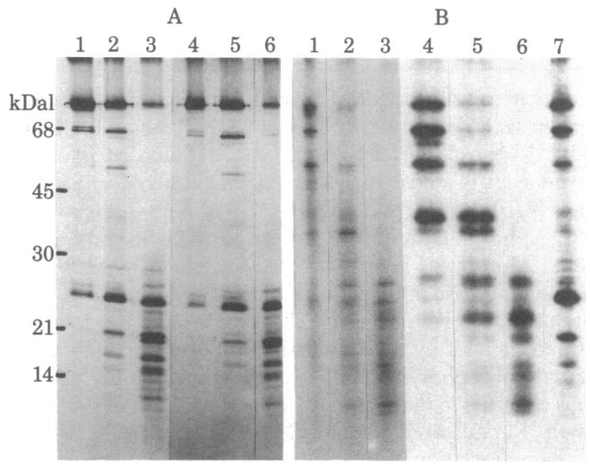

FIG. 2. Analysis of proteins by partial hydrolysis with proteases. Materials were prepared as described for Fig. 1. Appropriate regions of polyacrylamide gels were excised and used for further analysis by partial hydrolysis with the V8 protease of *S. aureus* (20). (*A*) Proteins labeled with [^{35}S]methionine. Preparations of pp89^{cell} isolated from heat-shocked chicken fibroblasts (lanes 1–3) and immunoprecipitated extracts of chicken cells transformed by SR-RSV (lanes 4–6) were treated with various amounts of V8 protease and the products of hydrolysis were fractionated by electrophoresis in gels of 14% polyacrylamide. The amounts of protease were as follows: 20 ng, lanes 1 and 4; 100 ng, lanes 2 and 5; and 500 ng, lanes 3 and 6. (*B*) Proteins labeled with ^{32}P. Lanes 1–6 are arranged as in *A*. Lane 7, [^{35}S]methionine-labeled pp89^{cell} with 100 ng of protease for direct comparison.

† H. Oppermann, A. D. Levinson, L. Levintow, H. E. Varmus, J. M. Bishop, and S. Kawai, unpublished data.

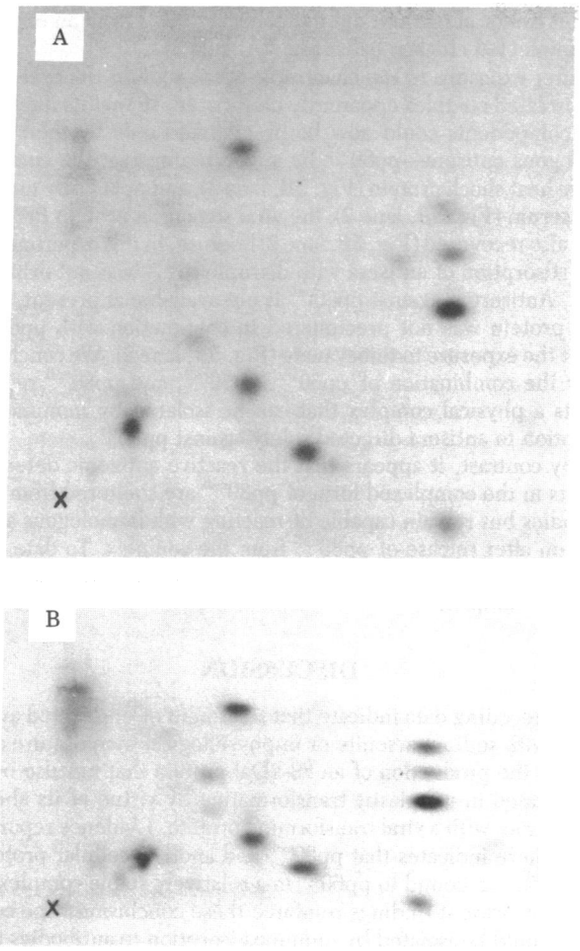

FIG. 3. Tryptic peptides of pp89^{cell} from immunoprecipitates and from arsenite-induced uninfected cells. Proteins labeled with [^{35}S]methionine were prepared as in Fig. 1. Proteins were recovered from appropriate regions of polyacrylamide gels and used for analysis of tryptic peptides (13). The peptides were separated by electrophoresis (horizontal dimension) and ascending chromatography (vertical dimension). The origins for electrophoresis are located in the lower left corners of the panels (x). (*A*) pp89^{cell} recovered by immunoprecipitation with tumor-bearing rabbit serum. (*B*) pp89^{cell} recovered from arsenite-induced uninfected chicken fibroblasts. Chromatography was for 3 hr in *A* but only for 1 hr in *B*.

In order to explore the apparent resemblance between the immunoprecipitated and induced forms of pp89^{cell}, we examined their chemical composition and molecular topography. First, we found that partial hydrolysis of the proteins with the V8 protease of *S. aureus* produced identical patterns of fragments from the two forms of [^{35}S]methionine-labeled pp89^{cell} (Fig. 2*A*). Moreover, the distributions of phosphate among the products were similar in the two cases (Fig. 2*B*). Second, we analyzed the tryptic peptides of the two forms of pp89^{cell} and again found identical patterns (Fig. 3). We conclude that the immunoprecipitated and induced forms of pp89^{cell} have similar, if not identical, compositions and topographies.

The Complex Is Precipitated by Antibodies Directed Against pp60^{src}. The complex between pp60^{src}, pp50^{cell}, and pp89^{cell} was first identified by the use of rabbit tumor antisera. We used immunocompetition to demonstrate that precipitation of the two cellular proteins in this manner was attributable to an immune reaction with pp60^{src} alone. As expected, the rabbit tumor antiserum precipitated pp89^{cell}, pp60^{src}, and pp50^{cell}

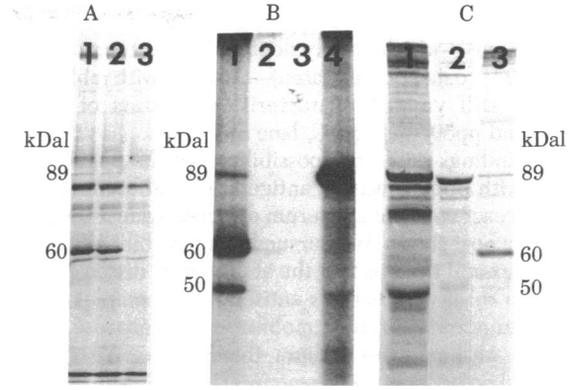

FIG. 4. Demonstration that pp60^{src} is joined to two cellular proteins in an immunoprecipitable complex. Cells were labeled with either [^{35}S]methionine or [^{32}P]phosphate and extracts were immunoprecipitated with various antisera, all as described for Fig. 1. Immunoprecipitates were analyzed by electrophoresis in gels of 9% polyacrylamide. (*A*) The immunoprecipitation of pp89^{cell} can be blocked by pp60^{src}. An extract of chicken cells, transformed by SR-RSV and labeled with [^{35}S]methionine, was immunoprecipitated with rabbit tumor antiserum in the absence of competitor (lane 1), in the presence of extract of uninfected hamster cells (lane 2), and in the presence of extract of hamster cells transformed by SR-RSV (lane 3). (*B*) Rabbit tumor antisera cannot precipitate pp89^{cell} directly. An extract of chicken cells, transformed by SR-RSV and labeled with ^{32}P, was exposed three times to *S. aureus* saturated with IgG of rabbit tumor antiserum (lanes 1, 2, and 3), and once thereafter to antiserum against pp89^{cell} (lane 4). (*C*) Antiserum directed against pp89^{cell} does not precipitate pp60^{src}. An extract of chicken cells, transformed by SR-RSV and labeled with [^{35}S]methionine, was allowed to react twice in succession with IgG against pp89^{cell} bound to *S. aureus* (lanes 1 and 2) and once thereafter with rabbit tumor antiserum (lane 3).

(Fig. 4*A*, lane 1; pp50^{cell} is not visible in the reproduction). Addition of an extract of uninfected hamster cells (BHK) had no effect on the immunoprecipitation (Fig. 4*A*, lane 2), whereas an extract of hamster cells transformed by SR-RSV (and therefore containing pp60^{src}) blocked the precipitation of all three proteins (Fig. 4*A*, lane 3). We conclude that pp50^{cell} and pp89^{cell} are joined with pp60^{src} in a complex that can be precipitated by an immune reaction involving only pp60^{src}.

Further Characterization of the Complex Between pp60^{src} and Cellular Proteins. The 89-kDal protein induced by heat shock has been purified and used to raise antiserum in rabbits (16). We used this antiserum to characterize further the interaction between pp89^{cell} and pp60^{src}.

We first demonstrated that the precipitation of pp89^{cell} by tumor antisera was dependent upon the coincident precipitation of pp60^{src}. Repeated exposures of an extract of RSV-infected cells to fresh portions of tumor antiserum soon exhausted the precipitable pp60^{src} (Fig. 4*B*, lanes 1–3), at which point precipitation of pp89^{cell} also ceased to occur. Subsequent exposure of the extract to antiserum against the 89-kDal heat shock protein precipitated a large amount of an M_r 89,000 protein (Fig. 4*B*, lane 4) whose protease map was indistinguishable from that of pp89^{cell} (data not shown). From these data we conclude that: (*i*) pp60^{src} and pp89^{cell} are precipitated in conjunction with one another; (*ii*) the precipitation is not due to a reaction directly between the tumor antiserum and pp89^{cell}; and (*iii*) the amount of pp89^{cell} that precipitates with pp60^{src} represents only a small fraction of the total pp89^{cell} in the infected (or uninfected) cell.

We were surprised to learn that the antiserum directed against pp89^{cell} did not precipitate the apparent complex between pp89^{cell} and pp60^{src}. Two successive reactions with the antiserum precipitated large amounts of pp89^{cell} but failed to

precipitate any detectable pp60^{src} (Fig. 4*C*, lanes 1 and 2). After these reactions—which largely depleted the extract of precipitable pp89^{cell} (data not illustrated)—reaction with rabbit tumor antiserum still yielded characteristic amounts of pp60^{src}, pp89^{cell}, and pp50^{cell} (Fig. 4*C*, lane 3).

These findings raised the possibility that the pp89^{cell} in the complex with pp60^{src} was an antigenically different form that could not react with the antiserum directed against the protein induced by heat shock. We pursued this possibility by isolating the complex and then testing the ability of its dissociated components to react with various antisera. The complex was first bound to tumor antiserum immobilized on Sepharose, then released by washing with sodium thiocyanate. We recovered pp60^{src} and pp89^{cell} in typical amounts (Fig. 5*A*, lane 2); the cells in this experiment were labeled with [^{35}S]methionine, a fact that precluded evaluation of the behavior of pp50^{cell} (see above). Although the immunoadsorptions were not performed at high stringency (hence the large number of proteins in the lanes of Fig. 5*A*), the recovery of pp60^{src} and pp89^{cell} was specific. Neither protein was adsorbed from extracts of infected cells by an immunoadsorbent containing antibody against the viral structural protein p27 (Fig. 5*A*, lane 1), and the adsorbent containing rabbit tumor antiserum did not collect pp89^{cell} from an extract of uninfected chicken cells (Fig. 5*A*, lane 3).

After exposure to the chaotropic agent sodium thiocyanate, the isolated complex apparently dissociated, so that its individual components could now be precipitated only by their homologous antisera—pp89^{cell} by antiserum against the comparable heat shock protein (Fig. 5*B*, lane 3), and pp60^{src} by tumor antiserum (Fig. 5*B*, lane 2); the viral structural protein Pr76^{gag} was also recovered (Fig. 5*B*, lane 2) because, in this experiment, the adsorption of antisera with disrupted RSV was not exhaustive. Antiserum against pp50^{cell} is not available at present, but the protein was not precipitated in conjunction with pp60^{src} after the exposure to thiocyanate (Fig. 5*B*, lane 2). We conclude that the combination of pp60^{src}, pp50^{cell}, and pp89^{cell} represents a physical complex that can be isolated by immunoadsorption to antisera directed solely against pp60^{src}.

By contrast, it appears that the reactive antigenic determinants in the complexed form of pp89^{cell} are sheltered from antibodies but remain capable of reacting with homologous antiserum after release of pp89^{cell} from the complex. To date, we have made no effort to reassociate the components of the disrupted complex.

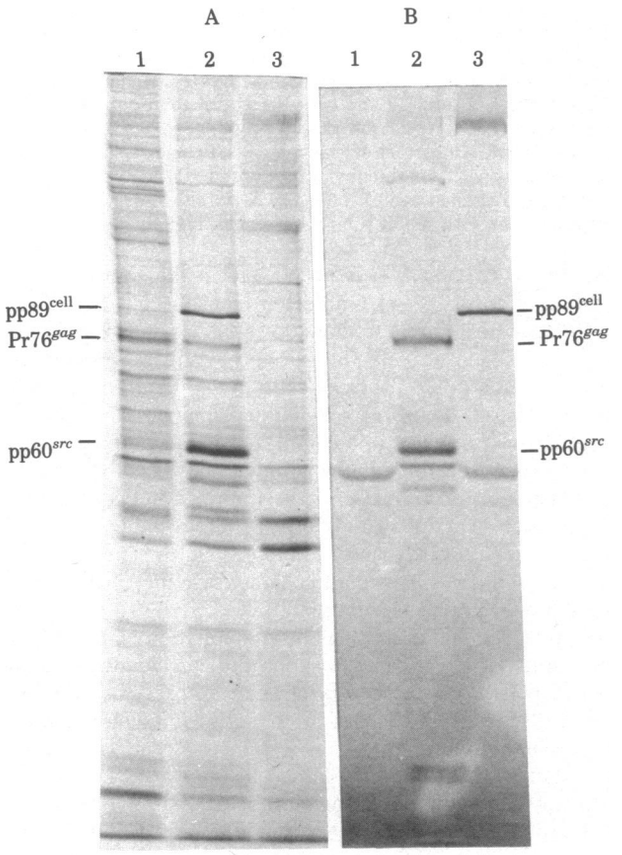

FIG. 5. Immunoprecipitation of pp60^{src} and pp89^{cell} after release from the complex. Extracts of chicken cells, either uninfected or transformed by SR-RSV and labeled with [^{35}S]methionine, were allowed to react with various antisera linked to Sepharose. Unlabeled disrupted RSV was present in the reaction mixtures as competitor. The adsorbent was washed, and bound proteins were eluted with 1 M sodium thiocyanate (8). The eluted proteins were then analyzed by immunoprecipitation with various antisera. (*A*) Proteins eluted from immunoadsorbents. Lane 1, infected cells, antibody against p27 of RSV; lane 2, infected cells, rabbit tumor antiserum; lane 3, uninfected cells, rabbit tumor antiserum. (*B*) Immunoprecipitation of proteins in the sample illustrated by lane 2 of *A*. Lane 1, normal rabbit serum; lane 2, rabbit tumor antiserum; lane 3, antiserum against pp89^{cell}.

DISCUSSION

The preceding data indicate that treatment of uninfected avian cells with sodium arsenite or unphysiological temperature amplifies the production of an 89-kDal protein that has also been implicated in neoplastic transformation by virtue of its ability to interact with a viral transforming protein. Evidence reported elsewhere indicates that pp89^{cell} and another cellular protein, pp50^{cell}, are bound to pp60^{src} in a relatively stable complex (6, *). Our present findings reinforce these conclusions: the complex could be isolated by immunoadsorption to antibodies that react with pp60^{src} but not with either pp50^{cell} or pp89^{cell}, and dissociation of the complex with a chaotropic agent yielded the individual components, which could thereafter be precipitated individually by homologous antisera.

The structure of pp89^{cell} recovered with pp60^{src} from avian and mammalian cells is highly conserved†; the same is true of the 89-kDal protein induced by arsenite and heat in widely diverged vertebrates (12). By contrast, the forms of pp50^{cell} recovered with pp60^{src} from avian and mammalian cells have very different compositions,† and there is no evidence that this protein participates in the response to noxious agents.

The induction of pp89^{cell} is part of an apparently coordinated response to cell distress recognized as "heat shock" in *Drosophila* (21) and since observed for a variety of species and inducing agents (12–14, 22). At present, we do not understand the metabolic purpose of this coordinated response, and we know nothing of the function of any of the proteins whose induction constitutes the response. On the other hand, the role of pp89^{cell} in the cellular response to pp60^{src} is also enigmatic. For example, the protein does not contain phosphotyrosine and is therefore not a likely substrate for the kinase activity of pp60^{src} (6). Our present findings may eventually assist in the solution of each of these puzzles. It seems reasonable to expect that pp89^{cell} may have overlapping roles in viral oncogenesis and the heat shock response, and that evidence on its function in either setting may illuminate its function in the other. In addition, the ability to experimentally amplify the production of pp89^{cell} should provide a welcome assist to tumor virologists in the purification of the protein and the characterization of its interaction with pp60^{src}. It would also seem wise to search for other overlaps between the heat shock response and virus-induced neoplastic transformation.

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