Immunological Characterization of Proteins Detected by Phosphotyrosine Antibodies in Cells Transformed by Rous Sarcoma Virus

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Phosphotyrosine antibodies were used to identify tyrosine-phosphorylated proteins in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts. A large number of tyrosine phosphoproteins were detected. A similar set of proteins was observed in RSV-transformed murine cells. An 85,000-dalton protein, however, was present in transformed avian cells but missing in transformed murine cells. Neither the 85,000-dalton protein nor any of the other tyrosine phosphoproteins appeared to be viral structural proteins. Use of RSV mutants encoding partially deleted *src* gene products enabled us to identify a 60,000-dalton cellular tyrosine phosphoprotein that comigrated with wild-type pp60^{v-src}. With the exception of calpactin I, the major tyrosine phosphoproteins detected in immunoblots appeared to be different from several previously characterized substrates of pp60^{v-src} with similar molecular masses (ezrin, vinculin, and the fibronectin receptor).

Oncogenic transformation of cells infected with Rous sarcoma virus (RSV) is initiated and maintained by the *src* gene product, pp60^{v-src} (27), which is a protein kinase with specificity for tyrosine residues (30). A number of approaches have been used to identify substrates for pp60^{v-src} (reviewed in reference 37). Proteins that have been identified as presumptive substrates for pp60^{v-src} include the cytosolic protein p50 (4, 25, 31), which may be involved in transport of newly synthesized pp60^{v-src} to its membrane location; three enzymes of the glycolytic pathway: enolase, phosphoglycerate mutase, and lactate dehydrogenase (12); the cytoskeleton-associated proteins vinculin (53), calpactin I (p36) (46), and ezrin (26); and p42 (10), which is also a substrate for several growth factor receptor tyrosine kinases (2, 13, 24). In addition, talin (16, 44), calmodulin (20), and the fibronectin receptor (FNR) (28) are phosphorylated on tyrosine in a transformation-specific manner in cells infected with RSV. So far, there has been no clear demonstration of how phosphorylation on tyrosine within any of these proteins is related to the process of transformation.

More recently, antiphosphotyrosine antibodies (Ptyr antibodies) have been used to detect cellular proteins phosphorylated on tyrosine in response to viral transformation or treatment of cells with growth factors (51). These antibodies recognize the tyrosine-phosphorylated forms of the receptors for epidermal growth factor (18), platelet-derived growth factor (18), and insulin (42). The transforming proteins of the *src* (9, 17, 58), *abl* (17, 19, 51, 58), *fes* (17), and *fps* (17) oncogenes are also detected by Ptyr antibodies.

In this study, we used immunoblots with Ptyr antibodies to compare the tyrosine phosphoproteins found in RSV-transformed chicken embryo fibroblasts (RSV-CEF) with those found in RSV-transformed murine cells. Except for an 85,000-dalton protein, the tyrosine phosphoproteins detected in the two types of cells had very similar molecular masses. None of the major proteins identified in Ptyr immunoblots appeared to be viral structural proteins. A previ-

ously unrecognized 60,000-dalton cellular substrate was detected in avian cells infected with RSV mutants encoding partially deleted *src* genes. We determined that, with the exception of calpactin I, the major tyrosine phosphoproteins detected by these antibodies are different from several previously characterized substrates of pp60^{v-src} with similar molecular masses.

MATERIALS AND METHODS

Cells and viruses. Fertilized eggs from line MR 60 chickens (c/o, negative for chicken helper factor and group-specific antigen) were obtained from SPAFAS, Inc. (Norwich, Conn.). Cloned stocks of Schmidt-Ruppin RSV, subgroup A (SR-RSV-A) and SR-RSV *src* gene mutants NY314 and NY309 (15) were a kind gift of H. Hanafusa (The Rockefeller University, New York, N.Y.). Chicken embryo cells were prepared from 10-day-old embryos, infected with virus, and grown by standard methods (47, 57). SR-RSV-D-transformed NIH 3T3 cells (NIHSD) (14) and uninfected NIH 3T3 cells were obtained from G. Cooper (Sidney Farber Cancer Institute, Boston, Mass.). BALB/c 3T3 cells transformed by SR-RSV-D (43) (SR-BALB) were provided by R. Ferracini (University of Turin, Turin, Italy).

Antisera. The Ptyr antibodies used for most of these experiments were prepared as follows. Rabbits were immunized with aminobenzylphosphonic acid (Sigma Chemical Co., St. Louis, Mo.) which had been diazotized and coupled to keyhole limpet hemocyanin (21). To purify the antibody, we prepared a resin in which diazotized aminobenzylphosphonic acid was coupled to bovine serum albumin (BSA) and the conjugate was covalently linked to Sepharose with cyanogen bromide (39). Immune serum was adsorbed to the resin, which had been equilibrated in phosphate-buffered saline (10 mM NaH₂PO₄ buffer [pH 7.4], 150 mM NaCl) containing 1 mM Na₃VO₄. The column was washed extensively with the same buffer, and then bound Ptyr antibodies were eluted, first with 40 mM phenyl phosphate-50 mM NaCl in 3.3 mM NaH₂PO₄ (pH 7.4) and then with 0.5 M NaCl in 0.1 M sodium acetate (pH 4). The two eluates were combined. Ptyr antiserum coupled to BSA with carbodiimide (18) was a gift of M. Cobb (University of Texas Health

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Science Center at Dallas). P_{tyr} antibodies prepared by immunization with tyrosine-phosphorylated, bacterially derived v-*abl* protein (58) were a kind gift of J. Wang (University of California at San Diego, La Jolla).

Antiserum against bacterially produced p60^{v-src} was prepared as described by Gilmer and Erikson (23). Antisera against the viral glycoproteins gp85 and gp35 were obtained from H. Deigelman (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Antisera against disrupted avian myeloblastosis virions were gifts of V. Vogt (Cornell University, Ithaca, N.Y.) and R. Eisenman (Fred Hutchinson Cancer Center, Seattle, Wash.). V. Vogt also provided antiserum to the p19 *gag* gene product of avian myeloblastosis virus. Antibodies against the FNR were a gift of A. Horwitz (University of Pennsylvania School of Medicine, Philadelphia), antiserum against calpactin I was provided by R. Erikson (Harvard University, Cambridge, Mass.), and antiserum to vinculin was provided by R. Hynes (Massachusetts Institute of Technology, Boston). Anti-p81 (ezrin) serum was a gift of A. Bretscher (Cornell University). Antiserum to a peptide corresponding to the carboxyl-terminal 17 amino acids of pp60^{v-src} was a gift of A. Laudano (University of New Hampshire, Durham).

Subcellular fractionation. Cells were fractionated by a modification of the method of Resh and Erikson (48). Before the cells were harvested, Na₃VO₄ was added to the incubation medium for 2 h to a final concentration of 50 μM to preserve phosphate on tyrosine phosphoproteins. The cells were washed in STE buffer (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.4], 1 mM EDTA), scraped from the dish, and pelleted. The cells were suspended in hypotonic buffer (10 mM Tris hydrochloride [pH 7.4], 0.2 mM MgCl₂, 1 mM Na₃VO₄) at 2×10^7 cells per ml and allowed to swell for 10 min and then were broken by 25 strokes in a Dounce homogenizer. The homogenate was brought to 0.25 M sucrose and 1 mM EDTA. A sample of this was set aside for subsequent immunoblotting of total cellular protein. Nuclei and unbroken cells were pelleted by low-speed centrifugation. The postnuclear supernatant was centrifuged at $100,000 \times g$ for 1 h in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The membrane pellet (P-100) was suspended in sucrose resuspension buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris hydrochloride [pH 7.4], 1 mM Na₃VO₄) by homogenization with five strokes in the Dounce homogenizer. For immunoprecipitation, the P-100 fractions were brought to 0.5% CHAPS detergent (Sigma), 2 mM EDTA, and 100 mM NaCl.

Immunoprecipitation of unlabeled cell extracts. Cells were incubated with 50 μM Na₃VO₄ for 2 h before extraction. For immunoprecipitation with antisera other than anti-p36 and anti-FNR, whole-cell extracts were solubilized in sodium dodecyl sulfate (SDS) immunoprecipitation buffer (0.5% SDS, 50 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 100 μM Na₃VO₄). The samples were heated at 100°C for 2 min and then centrifuged at $10,000 \times g$ for 2 min. The clarified extract was made 1% in Triton X-100, chilled to 4°C, and preabsorbed with Sepharose-immunoglobulin G (IgG) (Sigma) for 2 h before immunoprecipitation. Whole-cell extracts to be immunoprecipitated with antiserum against p36 were solubilized in RIPA buffer (22) containing 100 μM Na₃VO₄; then centrifuged and added to Sepharose-IgG for preadsorption before immunoprecipitation. P-100 fractions prepared as described above were the starting materials for immunoprecipitation with anti-FNR IgG. The P-100 fractions were preadsorbed with Sepharose-IgG before immunoprecipitation.

The immunoprecipitation protocol for all samples was as follows. Antiserum (or purified IgG) was incubated with protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C. The protein A-bound IgG was then washed free of other serum proteins with Triton-NET (0.05% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride, pH 7.4) and added to the preabsorbed cell extract. After overnight incubation at 4°C, the samples were centrifuged. The supernatant was removed and saved for subsequent analysis by immunoblotting. The immunoprecipitates were washed four times with Triton-NET and solubilized in SDS sample buffer. The supernatants were lyophilized and then rehydrated with 2% SDS–5% β-mercaptoethanol–10% glycerol in a volume equal to that of the precipitates. Samples immunoprecipitated with anti-FNR IgG were divided in half. One half of each sample was solubilized in buffer containing β-mercaptoethanol for electrophoresis under reducing conditions. The other half was solubilized in buffer with no β-mercaptoethanol and with 10 mM *N*-ethylmaleimide for electrophoresis under nonreducing conditions, since fibronectin receptor must be electrophoresed under nonreducing conditions to be detected in immunoblots with anti-FNR IgG. In all cases, equal aliquots of the supernatants and precipitates were electrophoresed and analyzed by immunoblotting.

Preparation of whole-cell extracts for immunoblotting. Cells were washed twice with phosphate-buffered saline and then lysed in sample buffer (38) containing 2.5% SDS (0.3 ml / 3×10^6 to 5×10^6 cells). Unless otherwise specified, 100 μl of cell extract was loaded in each lane for SDS-polyacrylamide gel electrophoresis (38).

Immunoblotting. Samples of cell extracts or samples from immunoprecipitation were resolved by electrophoresis on 5 to 15% gradient SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose by the method of Towbin et al. (56). Nitrocellulose was equilibrated in 10 mM Tris hydrochloride (pH 7.5)–0.15 M NaCl (TBS) and incubated in TBS containing 5% BSA at 45°C for 1 h. All subsequent incubations were at room temperature. In experiments with P_{tyr} antibodies, the nitrocellulose filter was incubated for 3 h with affinity-purified antibody at a concentration of 16 μg/ml in TBS containing 5% BSA. The concentration of anti-FNR IgG used for immunoblotting was 5 μg/ml. Antisera against gp85, p19, and p36 were diluted 1:500 in TBS containing 5% BSA; all other antisera were used at a 1:200 dilution in TBS containing 5% BSA. After extensive washing in TBS containing 0.05% Triton X-100, the nitrocellulose was rinsed in TBS and incubated for 1 h with ¹²⁵I-labeled protein A (2×10^5 cpm/ml) (50 μCi/μg; ICN Pharmaceuticals Inc., Irvine, Calif.) in TBS containing 5% skim milk powder. Filters were then washed in detergent as above, dried, and exposed to Kodak X-Omat AR film with Du Pont intensifying screens at –70°C.

Immunoprecipitation of ³²P-labeled cell extracts. Cells were labeled with ³²PO₄ as described by Sefton et al. (52). After 4 h of incubation with ³²PO₄ (carrier free, 1 mCi/ml; ICN) 10^6 cells were solubilized in 0.5 ml of SDS immunoprecipitation buffer. The viscosity of the samples was reduced by shearing through a 27-gauge needle. The samples were then desalted on Sephadex G-50 (Sigma) equilibrated in SDS immunoprecipitation buffer containing 0.3% SDS, to remove nucleotides that competitively inhibit binding of P_{tyr} antibodies to phosphotyrosine (9). Desalted samples were concentrated back to their original volume by ultrafiltration (Centricon PM10 filter; Amicon Corp., Lexington, Mass.), and then Triton X-100 was added to a final concentration of 1%. P_{tyr}

FIG. 1. Immunoblot with P_{tyr} antibodies of RSV-transformed avian and murine fibroblasts. Uninfected (lanes 1 to 4) and RSV-transformed (lanes 5 to 10) cells were incubated for 2 h in medium with (even-numbered lanes) or without (odd-numbered lanes) 50 μ M Na_3VO_4 and then solubilized in SDS sample buffer (38). Equal amounts of protein (60 μ g) were loaded onto each lane of an SDS-polyacrylamide gel and then immunoblotted with P_{tyr} antibodies as described in Materials and Methods. Lanes 1 and 2, Uninfected CEF; lanes 3 and 4, uninfected NIH 3T3 cells; lanes 5 and 6, RSV-transformed SR-BALB 3T3 cells; lanes 7 and 8, SR-RSV-D-transformed NIH 3T3 cells; lanes 9 and 10, RSV-transformed CEF.

antibodies were added to a final concentration of 180 μ g/ml, together with Sepharose-IgG. After 7 h of incubation at 5°C, the Sepharose-IgG was removed by centrifugation and protein A-Sepharose was added. After 30 min of incubation, the protein A-Sepharose beads were washed four times in RIPA buffer (22) containing 100 μ M Na_3VO_4 and 0.05% SDS instead of the usual 0.1% SDS, followed by three washes with Triton-NET. The immune complexes were then solubilized in SDS sample buffer.

RESULTS

Detection of phosphotyrosine-containing proteins in cells transformed by RSV. We examined the phosphotyrosine content of proteins from transformed avian and murine cells, using P_{tyr} antibodies in immunoblots. In Fig. 1, the tyrosine phosphoproteins in RSV-transformed CEF and two RSV-transformed murine cell lines can be compared. NIHSRD is a cell line derived from transformed foci arising after transfection of NIH 3T3 cells with integrated proviral DNA obtained from chicken cells transformed by SR-RSV-D (14). SR-BALB is a transformed cell line derived from tumors induced by injection of SR-RSV-D-transformed chicken

cells into newborn BALB/c mice (43). Many phosphotyrosine-containing proteins were detected in both types of RSV-transformed cells. Major proteins observed in RSV-CEF have molecular weights of 36,000, 60,000, 63,000, 80,000, 85,000, 95,000, 115,000, 125,000, 135,000, 185,000, and 200,000. The transformed murine cells have a number of tyrosine phosphoproteins with a range of molecular weights similar to those found in RSV-CEF, although the intensity of many bands was quite reduced, particularly for the 60,000-dalton protein and for proteins with M_r s greater than 125,000. With longer exposure times, however, these proteins were also visible in both murine cell lines (data not shown). One notable difference was an 85,000-dalton protein which was present in RSV-CEF but missing in both mouse cell lines. This 85,000-dalton protein was not seen in mouse cells even after a long exposure. A protein of M_r approximately 75,000 present in RSV-CEF in the experiment shown in Fig. 1 was also absent in mouse cells. The relative intensity of this band, however, as well as that of a 90,000-dalton species varied in our experiments. There were no major differences in the pattern of tyrosine phosphorylation between the two murine cell lines, except that proteins of M_r 125,000 to 130,000 exhibited a reduced signal in the NIHSRD cells. In both RSV-murine cell lines there was, in addition to the M_r -63,000 species observed in RSV-CEF, a prominent, diffuse band of M_r 62,000 which was not observed in RSV-CEF.

Incubation of cells with vanadate has been shown to increase the amount of protein-tyrosine phosphorylation (3, 8, 35, 60). We observed an increase in the intensity of the bands detected by P_{tyr} antibodies when cells were treated with 50 μ M Na_3VO_4 (Fig. 1, even-numbered lanes).

The presence of detectable phosphotyrosine on these proteins was transformation specific since these proteins were not observed in uninfected CEF or NIH 3T3 cells. The antibodies appeared to be recognizing phosphotyrosine specifically, because incubation of the immunoblots with P_{tyr} antibodies in the presence of 5 mM phosphotyrosine blocked the signal. Incubation with 5 mM phosphothreonine or 5 mM phosphoserine had no effect (38a).

P_{tyr} antibodies have been reported to react with phosphohistidine within ATP-citrate lyase (19). Cross-reactivity of our P_{tyr} antibodies with phosphohistidine was tested by incubation of the nitrocellulose after protein transfer in 0.5 N HCl for 10 min at 50°C. These conditions hydrolyze more than 95% of phosphohistidine (29, 41). No reduction in signal was observed after exposure to low pH (data not shown).

We compared the reactivity of our antibodies prepared against azobenzenephosphonate, a phosphotyrosine analog, with that of P_{tyr} antibodies prepared by two other methods (18, 58). All three antibodies showed very similar reactivities with the tyrosine phosphoproteins in uninfected and RSV-infected CEF (data not shown).

Phosphotyrosine-containing proteins detected by P_{tyr} antibodies are not viral structural proteins. Because pp60^{v-src} is such an active kinase, it has been suggested that the phosphorylation of some proteins by pp60^{v-src} in RSV-transformed cells is merely fortuitous (59). Viral structural proteins could be accessible to the *src* enzyme in the cell and might be phosphorylated on tyrosine. Therefore, we determined whether any of the proteins detected by the antibody were viral structural proteins. In particular, we wished to determine whether the 85,000-dalton protein that was recognized only in RSV-CEF was of viral origin.

Cell extracts were immunoprecipitated with antiserum to the avian myeloblastosis virus *gag* protein, p19. This anti-

FIG. 2. Immunoprecipitation with p19 antiserum. RSV-CEF were immunoprecipitated with either p19 antiserum or nonimmune (NI) serum as described in Materials and Methods. The immunoprecipitates (Ip; lane 1, immune; lane 3, nonimmune) and the supernatants (S; lane 2, immune; lane 4, nonimmune) were then immunoblotted with either p19 antiserum (A) or P-tyr antibodies (B).

serum cross-reacts with p19 and the *gag* protein precursors, including Pr76, encoded by RSV. Parallel samples were immunoprecipitated with nonimmune serum. The immune complexes were bound to protein A-Sepharose, and the samples were centrifuged. The resulting immunoprecipitates and supernatants were resolved on SDS-polyacrylamide gels and then transferred to nitrocellulose for immunoblotting. Duplicate samples were prepared to allow immunoblotting with both P-tyr antibodies and p19 antibodies. In Fig. 2, the filter in panel A was probed with anti-p19 antiserum. Based on this immunoblot, the efficiency of immunoprecipitation of the *gag* proteins was estimated to be 70%. The duplicate nitrocellulose filter probed with P-tyr antibodies is shown in Fig. 2B. A small amount of an 85,000-dalton tyrosine phosphoprotein was in fact immunoprecipitated by the p19 antiserum (Fig. 2B, lane 1). Furthermore, one of the major proteins immunoprecipitated by the p19 antiserum, a species we tentatively identify as Pr76, migrated in SDS-polyacrylamide gels very close to the protein we designated as M_r 85,000. However, the major 85,000-dalton species detected by our P-tyr antibodies was probably not this product of the *gag* gene, because it remained in the supernatant (Fig. 2B, lane 2) under conditions in which the bulk of Pr76 was immunoprecipitated (Fig. 2A, lanes 1 and 2).

The same protocol was used with antiserum against gp85, the viral glycoprotein encoded by the *env* gene, as well as with antiserum against disrupted avian myeloblastosis viruses. Neither of these antisera immunoprecipitated any of the major tyrosine phosphoproteins detected with P-tyr anti-

bodies. We concluded from these experiments that the major proteins detected in immunoblots with P-tyr antibodies are not viral structural proteins but rather are of cellular origin.

The 60,000-dalton protein detected by P-tyr antibodies in immunoblots of RSV-transformed cells is a mixture of pp60^{v-src} and another cellular protein. We wished to determine whether the 60,000-dalton protein detected by P-tyr antibodies in immunoblots was pp60^{v-src}. We therefore used the same protocol described above, using antibodies made against a synthetic peptide corresponding to the carboxyl-terminal 17 amino acids of pp60^{v-src} for immunoprecipitation. We have confirmed that this antiserum (anti-v-src peptide serum) immunoprecipitates pp60^{v-src} (J. Lui and J. Burr, unpublished observations). The duplicate immunoblots of both immunoprecipitates and supernatants were probed with either P-tyr antibodies or p60^{src} antiserum. The p60^{src} antiserum used for immunoblotting is a polyclonal serum made against bacterially produced pp60^{v-src} and is not sensitive to small conformational changes in pp60^{v-src}, as are some tumor-bearing rabbit sera (48) and monoclonal antibodies (7). We used this serum rather than the anti-v-src peptide serum because the latter antiserum worked poorly in immunoblots. The results of the experiment can be seen in Fig. 3.

Approximately 80% of the pp60^{v-src} was immunoprecipitated (Fig. 3A, lanes 1 and 2), as determined by immunoblotting with p60^{src} antiserum. However, little pp60^{v-src} was detected by P-tyr antibodies in the immunoprecipitate (Fig. 3B, lane 1). Moreover, the amount of the 60,000-dalton species recognized by P-tyr antibodies in the supernatant after immunoprecipitation of 80% of the pp60^{v-src} was only slightly diminished (Fig. 3B, lane 2). There was a discrepancy, then, in the amount of pp60^{v-src} immunoprecipitated versus the relative amounts of 60,000-dalton protein in the supernatant and the precipitate as detected by P-tyr antibodies. One explanation for this discrepancy would be that a second, cellular protein of M_r 60,000 is recognized in immunoblots by P-tyr antibodies.

To explore this possibility further, we did immunoblots with P-tyr antibodies of proteins from cells infected with two RSV mutants, NY309 and NY314 (15). The *src* proteins of these viruses are fully active as tyrosine kinases, but have deletions in the amino terminus such that they encode proteins of M_r 51,000 and 50,000, respectively (Fig. 4A). (The faint 60,000-dalton band detected by anti-p60^{src} antiserum in uninfected and mutant RSV-infected cells [Fig. 4B, lanes 1, 3, and 4] may be pp60^{c-src}. It is not pp60^{v-src}, possibly derived from contaminating wild-type RSV infection, because it remained undiminished in the supernatant after immunoprecipitation of NY314-infected cells with anti-v-src peptide serum [data not shown].) The P-tyr immunoblot showed that cells infected with NY309 and NY314 have an amount of 60,000-dalton tyrosine phosphoprotein comparable to that found in cells infected with wild-type RSV (Fig. 4B). It therefore appeared that our antibodies recognize pp60^{v-src} poorly in immunoblots, but do recognize a 60,000-dalton cellular tyrosine phosphoprotein.

We also used P-tyr antibodies to immunoprecipitate ³²P-labeled tyrosine phosphoproteins from RSV-CEF. The pattern of proteins detected by immunoprecipitation was comparable to that observed in immunoblots, except that a ³²P-labeled band of M_r 60,000 was much more prominent in the immunoprecipitate (data not shown). This M_r -60,000 band consisted largely of pp60^{v-src}, as determined by peptide mapping (data not shown). We conclude that pp60^{v-src} can be immunoprecipitated from detergent-solubilized RSV-cell ex-

FIG. 3. Immunoprecipitation with anti-*v-src* peptide serum. RSV-CEF were immunoprecipitated with anti-*v-src* peptide serum or preimmune serum. The immunoprecipitates (Ip; lane 1, immune; lane 3, preimmune [PI]) and the supernatants (S; lane 2, immune; lane 4, preimmune) were then immunoblotted with either $p60^{src}$ antiserum (A) or Ptyr antibodies (B).

tracts by Ptyr antibodies, even though these antibodies recognize this protein poorly in immunoblots.

Calpactin I is one of the major tyrosine phosphoproteins detected in immunoblots with Ptyr antibodies, but ezrin, vinculin, and FNR are not. Several previously characterized substrates of $pp60^{v-src}$ have molecular masses similar to those of some of the major proteins we detected in immunoblots with Ptyr antibodies. We therefore used the same immunoprecipitation protocol to determine whether any of the proteins detected by our antibodies were the tyrosine-phosphorylated forms of calpactin I (p36), ezrin (p81), vinculin, or the FNR.

Using our standard protocol, we were able to immunoprecipitate approximately 70% of the calpactin I (p36) (Fig. 5A, lanes 1 and 2) from RSV-transformed cells. Similarly, most of the 36,000-dalton protein detected in immunoblots with Ptyr antibodies was immunoprecipitated by p36 antiserum under these circumstances (Fig. 5B, lanes 1 and 2). This strongly suggests that the 36,000-dalton tyrosine phosphoprotein is calpactin I.

Ezrin comigrated with the 80,000-dalton protein detected by Ptyr antibodies (Fig. 6). However, under conditions in which approximately 90% of the ezrin polypeptide was immunoprecipitated (Fig. 6A, lanes 1 and 2), there was no reduction in the signal of the 80,000-dalton tyrosine phosphoprotein detected in immunoblots with Ptyr antibodies (compare lanes 2 and 4 of Fig. 6B). Therefore, although the tyrosine-phosphorylated form of ezrin could be detected by

FIG. 4. Immunoblots of cells infected with RSV mutants containing partial deletions in the *src* gene. Uninfected CEF (lane 1) and CEF infected with wild-type RSV (lane 2), NY314 RSV (lane 3), or NY309 RSV (lane 4) were immunoblotted with either $p60^{src}$ antiserum (A) or Ptyr antibodies (B). Whole-cell extracts were used after being prepared as described in the subcellular fractionation section of Materials and Methods. Approximately 100 μ g of protein was loaded per lane of the gel.

Ptyr antibodies in the immunoprecipitate (Fig. 6B, lane 1), we conclude that the major 80,000-dalton protein detected in RSV-infected cells by Ptyr antibodies is not ezrin.

Vinculin is usually referred to as having a molecular weight of 130,000 (6). We found that vinculin comigrated with a tyrosine phosphoprotein that we designated as M_r 115,000 (Fig. 7). Under conditions in which approximately 80% of the vinculin was immunoprecipitated (Fig. 7, lanes 1 and 2), there was no significant immunoprecipitation of the M_r 115,000 protein detected by Ptyr antibodies (compare lanes 2 and 4, Fig. 7B). In fact, even the tyrosine-phosphorylated form of vinculin itself could not be detected by our antibodies in the immunoprecipitate.

The FNR in avian cells is a complex of three membrane glycoproteins of M_r s 160,000, 140,000, and 120,000. The 140,000- and 120,000-dalton proteins are phosphorylated on tyrosine in chicken cells infected with RSV (28). We found previously that approximately 80% of the FNR was associated with the membranous fraction derived from a 100,000 $\times$ *g* centrifugation (P-100) (38a). We therefore used affinity-purified polyclonal IgG made against the FNR (anti-FNR IgG) to immunoprecipitate P-100 fractions from RSV-CEF (Fig. 8). The anti-FNR IgG used in this experiment recognizes only the M_r 120,000 receptor subunit in immunoblots, but the entire three-subunit complex is immunoprecipitated by the antibodies when receptor has been solubilized with a nondenaturing detergent such as CHAPS (5, 36). P-100

FIG. 5. Immunoprecipitation with p36 (calpactin I) antiserum. RSV-CEF were immunoprecipitated with either p36 antiserum or nonimmune (NI) serum. The immunoprecipitates (Ip; lane 1, immune; lane 3, nonimmune) and supernatants (S; lane 2, immune; lane 4, nonimmune) were then immunoblotted with either p36 antiserum (A) or Ptyr antibodies (B).

FIG. 6. Immunoprecipitation with ezrin antiserum. RSV-CEF were immunoprecipitated with antiezin (α-Ez) serum or nonimmune (NI) serum. The immunoprecipitates (Ip; lane 1, immune; lane 3, nonimmune) and supernatants (S; lane 2, immune; lane 4, nonimmune) were then immunoblotted with either ezrin antiserum (A) or Ptyr antibodies (B).

FIG. 7. Immunoprecipitation with vinculin antiserum. RSV-CEF were immunoprecipitated with antivinculin (α-vinc.) serum or preimmune (PI) serum. The immunoprecipitates (Ip; lane 1, immune; lane 3, preimmune) and supernatants (S; lane 2, immune; lane 4, preimmune) were then immunoblotted with either antivinculin antiserum (A) or Ptyr antibodies (B).

fractions of RSV-CEF were therefore solubilized with 0.5% CHAPS before immunoprecipitation. The results of this immunoprecipitation are shown in Fig. 8. (The gel shown in Fig. 8A was run under nonreducing conditions because the anti-FNR IgG does not recognize reduced M_r -120,000 FNR subunits.) At least 60% of the FNR was immunoprecipitated from the P-100 fraction (Fig. 8A, lanes 1 and 2). However, there were no tyrosine phosphoproteins detected in the immunoprecipitate (Fig. 8B, lane 1). We conclude that none of the major bands of M_r 115,000 to 140,000 detected in immunoblots with Ptyr antibodies is the FNR.

From this series of experiments, we concluded that the tyrosine-phosphorylated form of calpactin I is a prominent protein in immunoblots with Ptyr antibodies. However, other putative substrates of pp60^{v-src}, ezrin, vinculin, and the FNR, are not among the major tyrosine phosphoproteins detected by Ptyr antibodies.

DISCUSSION

Identification and characterization of the physiological substrates of pp60^{v-src} is an essential step toward understanding the mechanism of transformation by RSV. Identification of cellular proteins phosphorylated on tyrosine in a transformation-dependent manner has been the criterion used to characterize proteins as putative substrates (37). We used Ptyr antibodies to examine the tyrosine phosphoproteins that are found in primary chicken embryo cells after transformation by RSV. We found a large number of proteins that appear to be phosphorylated in a transformation-specific manner. When the tyrosine phosphoproteins of transformed avian cells were compared with those of murine cells, in general there seemed to be a higher content of

FIG. 8. Immunoprecipitation with anti-FNR antibodies. High-speed particulate fractions (P-100) from RSV-CEF were immunoprecipitated with either antibodies to the FNR or preimmune serum (PI). The immunoprecipitates (Ip; lane 1, immune; lane 3, preimmune) and supernatants (S; lane 2, immune; lane 4, preimmune) were then immunoblotted with purified anti-FNR IgG (A) or Ptyr antibodies (B). The samples immunoblotted with anti-FNR IgG (A) were electrophoresed under nonreducing conditions.

phosphotyrosine in the avian cells. This might reflect higher levels of *src* protein expression in RSV-CEF than in RSV-transformed murine cell lines. An 85,000-dalton protein was present in RSV-CEF but missing in the two murine cell lines. We thought it possible that this protein was of viral origin, since it was found only in cells actively producing virus. However, removal of viral structural proteins by immunoprecipitation did not diminish the signal of the 85,000-dalton tyrosine phosphoprotein in immunoblots. We concluded from these experiments that the protein was of cellular origin.

In RSV-CEF, we observed proteins of molecular masses similar to those previously reported by others for RSV-transformed murine cells (9, 45, 54, 58, 60). The use of certain *src* deletion mutants, however, enabled us to identify a 60,000-dalton protein that has not been previously reported. This protein appears to be distinct from pp60^{v-src}. The 60,000-dalton protein is probably not tyrosine-phosphorylated pp60^{c-src}, since Iba et al. (32) have reported that there is no change in the state of tyrosine phosphorylation of pp60^{c-src} upon transformation and we did not detect this tyrosine phosphoprotein in immunoblots or by immunoprecipitation in uninfected cells. The 60,000-dalton protein may therefore be a novel substrate.

Except for pp60^{v-src}, the same proteins appeared to be detected by Ptyr antibodies when assayed by immunoprecipitation or by immunoblotting. The insensitivity of Ptyr antibodies to pp60^{v-src} in immunoblots was also noted by Wang (58). It appears that these antibodies also bind poorly

to a number of lower-molecular-weight proteins, since both Martinez et al. (40) and Beemon et al. (1) found that more than half of the total tyrosine phosphoproteins of RSV-infected cells had a molecular mass of less than 60,000 daltons. Although many proteins can be identified in this range of molecular mass if our immunoblots are exposed to film for a longer time, none of these bands are as intensely labeled as the proteins of higher molecular mass. Thus, the major proteins which we and others have identified using Ptyr antibodies are probably a subset of the total population of tyrosine-phosphorylated species in RSV-CEF.

The basis for this selectivity is unclear. It is possible that the antibody recognizes phosphotyrosine only when it is in a context with other protein determinants. We obtained very similar results with Ptyr antibodies prepared by three different methods. The protein environment of the hapten was different with each immunogen, so it is unlikely that this is a basis for selectivity. The antibodies have a high affinity for proteins tyrosine phosphorylated in the detergent-insoluble cytoskeletal matrix in an in situ kinase assay (9; J. Burr, unpublished observations). We have found that the tyrosyl phosphate on these proteins turns over very rapidly (J. Burr, unpublished observations). These sites of phosphorylation must therefore be readily accessible to kinases and phosphatases, and perhaps to the antibody as well. One might expect that after SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose, these proteins would be denatured; therefore, the accessibility of phosphotyrosine in any particular protein would be unrelated to its accessibility in the native form. There is some evidence, however, that the electrophoretic removal of SDS during transfer permits proteins to refold before their attachment to nitrocellulose (55). This may explain why we were able to immunoprecipitate denatured pp60^{v-src} out of a mixture of 0.5% SDS and 1% Triton X-100 with Ptyr antibodies, but were not able to detect the transferred protein in immunoblots with any significant efficiency.

Although we were able to detect the tyrosine-phosphorylated forms of ezrin and calpactin I with Ptyr antibodies, we could not detect the tyrosine-phosphorylated forms of vinculin or the FNR. An important consideration in this regard may be the stoichiometry of phosphorylation and the relative abundance of the protein. Until more of the tyrosine phosphoproteins recognized by the antibodies are purified and characterized, it is not possible to determine the significance of these parameters to immunoreactivity with the antibody. We are able to detect as little as 60 fmol of tyrosine-phosphorylated insulin receptor in immunoblots with these antibodies (as determined by ³²P content of an identical sample of receptor labeled with [γ -³²P]ATP) (M. Linder and M. Cobb, unpublished observations.)

Kamps et al. (34) studied the phosphorylation of eight well-characterized substrates of pp60^{v-src} in cells infected with transformation-defective mutants of pp60^{v-src} which encode a nonmyristylated yet fully active *src* tyrosine kinase. There was no qualitative difference in the tyrosine phosphorylation of these eight substrates in cells infected with the transformation-defective mutant versus cells infected with wild-type virus. DeClue and Martin (16) reported that there was no demonstrable correlation between elevation of phosphotyrosine in talin and the induction of morphological transformation. These studies and others (11, 33, 49, 50) suggest that other cellular substrates important in transformation remain to be identified. We showed that with the exception of p36, the major proteins of M_r 60,000, 63,000, 80,000, 85,000, 95,000, 115,000, 125,000, 135,000,

and 200,000 that we and others have identified with P_{tyr} antibodies are cellular tyrosine phosphoproteins which are different from any of the previously characterized substrates. These proteins therefore merit further study to determine whether they play a role in the process of transformation.

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