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Monoclonal Antibodies to Rous Sarcoma Virus pp60^{src} React with Enzymatically Active Cellular pp60^{src} of Avian and Mammalian Origin

SARAH J. PARSONS,* DEBORAH J. McCARLEY, CONSTANCE M. ELY, DAVID C. BENJAMIN, AND J. THOMAS PARSONS

Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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The derivation and characterization of 22 hybridoma clones producing monoclonal antibodies (Mabs) specific for the transforming protein of Rous sarcoma virus, pp60^{src}, are described. All Mabs reacted with pp60$^{v\text{-}src}$ encoded by Prague, Schmidt-Ruppin, and Bratislava 77 strains of Rous sarcoma virus. Of these Mabs, 10 efficiently immunoprecipitated pp60$^{c\text{-}src}$ from chicken embryo cells. Of these 10 Mabs, 2 (GD11 and EB8) readily detected pp60$^{c\text{-}src}$ from a variety of rodent and human cultured cells and from rat brain tissue in an in vitro immune complex kinase assay. Mapping experiments have tentatively localized the determinant(s) recognized by GD11 and EB8 to a region of the *src* protein bounded by amino acid residues 82 to 169, whereas the remaining Mabs appeared to recognize determinants residing within residues 1 to 82 or 169 to 173. Most of the Mabs complexed denatured pp60$^{v\text{-}src}$ in a Western immunoblot, and several were used to localize pp60$^{v\text{-}src}$ in Rous sarcoma virus-transformed chicken embryo cells by indirect immunofluorescence microscopy.

Functional expression of pp60$^{v\text{-}src}$, the transforming protein encoded by Rous sarcoma virus (RSV), is required for manifestation of the oncogenic events which are initiated upon RSV infection (1, 17). The initial identification of pp60$^{v\text{-}src}$ and the subsequent studies which have led to its partial characterization were carried out with sera from rabbits bearing RSV-induced tumors (TBR sera) (1, 5). These antisera provided critical reagents for the determination of many of the properties of pp60^{src}, including its intrinsic tyrosine-specific phosphotransferase activity (10, 23), its localization to the inner face of the plasma membrane (11, 20, 41), and its association with two cellular cytoplasmic proteins, pp90 and pp50 (3, 4, 27). These antisera have also been used to identify the normal cellular homolog of pp60$^{v\text{-}src}$, termed pp60$^{c\text{-}src}$ (9, 28, 34). In addition, other antisera reactive with pp60^{src} have been generated utilizing small peptides as immunogens. This approach has resulted in the production of rabbit antisera to a peptide containing tyrosine 419 (residues 415 to 424) (40), to the pentadecapeptide corresponding to residues 498 to 512 (15), to the carboxy-terminal 6 amino acids (residues 521 to 526) of pp60$^{v\text{-}src}$ (36), and to a variety of synthetic peptides corresponding to sequences distributed throughout the pp60$^{v\text{-}src}$ molecule (38). Most antipeptide sera immunoprecipitated in vivo-labeled pp60$^{v\text{-}src}$, but the cross-reactivity of these sera for pp60$^{c\text{-}src}$ is uncertain. TBR antisera which detect c-*src* are not reproducibly obtained.

Several recent findings have accentuated the need for antisera highly cross-reactive with c-*src* from a variety of species. First is the observation that anti-c-*src* TBR immunoglobulin G (IgG) becomes phosphorylated when it is incubated with membranes of cells stimulated with epidermal growth factor, but not when it is incubated with unstimulated membranes (1, 7, 21). This result suggests that c-*src* may be involved in the response of the cell to growth-stimulating factors, such as epidermal growth factor. Second, the observed association of the c-*src* protein with polyoma middle T antigen has provoked speculation that this interaction is necessary for transformation by polyomavirus (12). Monoclonal antibodies (Mabs) reactive with the c-*src* protein would provide important tools to probe the associative or synergistic role of c-*src* in regulating growth in normal cells and in mediating neoplastic transformation. In addition, Mabs to nonoverlapping epitopes within the *src* protein would provide important reagents for comparative studies of the structure and function of normal cell and v-*src* proteins.

In this communication, we report the isolation and characterization of 22 mouse hybridoma clones producing Mabs which react with pp60$^{v\text{-}src}$ as specified by Prague subgroup A, Schmidt-Ruppin subgroup D, and Bratislava 77 subgroup C strains of RSV (PrA-RSV, SRD-RSV, and B_{77} C-RSV, respectively). Ten of these Mabs cross-reacted strongly with pp60$^{c\text{-}src}$ from chicken embryo (CE) cells, and 2 of these 10 Mabs (GD11 and EB8) detected enzymatically active pp60$^{c\text{-}src}$ from a variety of rodent and human cells. Preliminary mapping experiments suggested that GD11 and EB8 recognize an antigenic determinant(s) bounded (in part) by amino acid residues 82 to 169, whereas the remaining Mabs appeared to recognize determinants mapping within residues 1 to 82 and 169 to 173 of pp60$^{v\text{-}src}$. Most of the Mabs bound pp60$^{v\text{-}src}$ in a Western immunoblot, although the efficiency of binding differed among the individual Mabs. Several Mabs were used to localize pp60$^{v\text{-}src}$ in RSV-transformed CE cells by indirect immunofluorescence microscopy.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Primary cultures of CE cells were prepared from 10-day-old gram-negative, chf-negative, Marek's disease-negative embryos (SPAFAS, Norwich, Conn.) and maintained in culture as described previously (30). Secondary cultures of CE cells were infected with the following strains of RSV: PrA-RSV, SRD-RSV, B_{77}C-RSV, or mutants of PrA-RSV, *ts*CH*dl*119, CH*dl*121, or CH*dl*300 (6; J. T. Parsons, D. Bryant, V. Wilkerson, G. Gilmartin,

* Corresponding author.

and S. J. Parsons *in* A. Levine, W. Topp, and G. Vande Woude, ed., *Cancer Cells, Vol. II, Oncogenes and Viral Genes*, in press). SR-1T, SRD-RSV-transformed field vole cells; 3Y1, normal rat fibroblasts; BALB/c, normal mouse fibroblasts; HeLa, a human carcinoma line; normal mink fibroblasts; J.L.M., a normal human fibroblast cell line adapted to culture by T. Kelly (University of Virginia); and NHF, normal human foreskin fibroblasts, kindly provided by John Herr (University of Virginia) were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% calf serum. *Escherichia coli* cells bearing the bacterial plasmid p*lac-src* (16), which directs the synthesis of PrA-RSV p60^{src}, or the plasmid pLSZ (29), which encodes the fusion protein *src*-β-galactosidase containing 204 amino acids of *src* fused to β-galactosidase, were propagated as previously described (16).

Purification of *src* protein and immunization of mice. BALB/c mice were immunized with biweekly subcutaneous injections of purified *src* antigen emulsified in Freund adjuvant. pp60^{src} from SR-1T cells was prepared by immunoaffinity chromatography of detergent extracts with TBR and tumor-bearing mouse immunoglobulins conjugated to Affi-Gel 10 as described by Erikson et al. (13). Bound pp60^{src} was eluted with 3 M KSCN, dialyzed extensively in 10 mM NH_4HCO_3 and lyophilized. About 25 μg of pp60^{src} was obtained from 10^9 SR-1T cells. The antigen was resuspended in phosphate-buffered saline (PBS) and emulsified in Freund adjuvant, and 5 to 25 μg of protein was administered to each mouse per injection. Based on Coomassie blue and silver nitrate staining of polyacrylamide gels, this material was judged to be ca. 85 to 90% pure. Mice immunized for 4 months with pp60^{src} antigen purified in this manner exhibited a weak anti-pp60^{src} response. Subsequently, these mice were challenged with p60^{src} purified from extracts of *E. coli* containing the plasmid p*lac-src*. Bacterial p60^{src} was prepared as follows. Spheroplasts, obtained by treatment of cells in 50 mM Tris-hydrochloride (pH 8.0)–2 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml–10 mM EDTA–25% sucrose for 15 min at 4°C, were incubated with DNase (Sigma) (0.25 mg/ml in 5 mM $MgCl_2$) and lysed in 10 mM Tris-hydrochloride (pH 7.2) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, and 1 mM EDTA. Extracts were clarified at 10,000 × *g*, and the insoluble fraction, which contained about 95% of the p60^{src}, was washed one time in 10 mM Tris-hydrochloride (pH 7.2) containing 1 M NaCl and 1 mM EDTA and three times with 1 M urea in RIPA buffer. The washed pellet was dissolved in sample buffer (22) without boiling and applied to a 9% preparative polyacrylamide gel. p60^{src} was localized by staining with acid-free, water-solubilized Coomassie blue dye and excised from the gel. p60 was eluted in PBS overnight and filtered before injection. Approximately 0.5 to 1.0 mg of p60^{src} was obtained from 1 liter of fully induced bacterial culture and was judged to be greater than 90% pure by polyacrylamide gel electrophoresis. Mice were boosted with biweekly injections of 50 to 100 μg of p60^{src} for an additional 2 months. Sera from such mice exhibited anti-pp60^{src} titers in a standard enzyme-linked immunosorbent assay (ELISA), 300- to 1,000-fold greater than those from mice immunized with live or irradiated RSV-transformed cells (30).

Fusion and screening of hybridomas for anti-pp60^{src} production. Splenocytes from immunized mice were fused in the presence of 37% polyethylene glycol 1000 (Koch-Light) with SP2/O BALB/c myeloma cells, and the resulting hybridomas were portioned into microtiter wells and cultured in conventional media as previously described (26, 31). Culture supernatants were tested for the presence of *src*-specific antibodies by two methods. Initial screening of the supernatants was accomplished with an ELISA with purified bacterial *src* as antigen (applied to microwells at concentrations of 2 μg/ml) and alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed) as the secondary antibody. Specific binding of antibody was detected by addition of the substrate *p*-nitrophenyl phosphate (Sigma) as described elsewhere (19). Culture supernatants of the clone, R2D2 (29), producing anti-*src* Mab and SP2/O myeloma cells were used as positive and negative controls, respectively. All culture supernatants exhibiting a positive ELISA reaction were further tested for their ability to immunoprecipitate pp60$^{v\text{-}src}$ from extracts of PrA-RSV-transformed CE cells labeled with $^{32}P_i$. Hybridoma cultures yielding antibodies which immunoprecipitated pp60$^{v\text{-}src}$ were immediately subcloned by limiting dilution (2 to 3 times) and injected into Pristane-primed mice for production of ascites fluid.

Purification of immunoglobulin. Immunoglobulins were purified from mouse ascites fluid as follows. Lipid was removed by dextran sulfate precipitation, and immunoglobulin was precipitated by addition of 50% ammonium sulfate (31). After extensive dialysis, the immunoglobulin was chromatographed on protein A-Sepharose by the method of Ey et al. (14). Immunoglobulin prepared in this manner was >95% pure as judged by Coomassie blue staining. Alternatively, immunoglobulin was partially purified and rendered protease-free by chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) by a modification of the method of Bruck et al. (2). Briefly, 2 ml of ascites fluid was dialyzed against 20 mM Tris-hydrochloride (pH 7.2) and applied to a column containing 7 ml of DEAE Affi-Gel Blue equilibrated in the same buffer. The column was washed with the starting buffer to remove unadsorbed material. Stepwise elution with 20 mM NaCl removed the majority of bound transferrin, after which the immunoglobulin was eluted was 45 mM NaCl. Albumin and proteases remained bound to the column at this salt concentration. Immunoglobulin purified in this manner was judged to be about 75 to 80% pure.

Isotyping. The isotypes of individual Mabs (obtained from either culture supernatants or diluted ascites fluid) were determined with a commercial isotyping kit (Boehringer Mannheim Corp., New York, N.Y.). Assignment of the isotypes was confirmed by double diffusion and chromatography on protein A-Sepharose.

Radiolabeling of cells and immunoprecipitation. Cells were labeled for 2 to 3 h in phosphate-free medium 199 (GIBCO) containing 5% dialyzed calf serum and 500 μCi of carrier-free $^{32}P_i$ (ICN Pharmaceuticals, Irvine, Calif.) per ml. Extracts were prepared as described previously (30). For screening experiments, samples of labeled extract were immunoprecipitated with 500 μl of culture supernatant and 3 μl of rabbit anti-mouse IgG as secondary antibody. For subsequent experiments, extracts were immunoprecipitated with 1 to 10 μl of ascites fluid or 2 to 25 μg of purified immunoglobulin. The amounts of each immunoglobulin used were determined to be in antibody excess for v-*src* in an extract derived from 2×10^5 RSV-infected CE cells and for c-*src* in an extract derived from 1×10^6 uninfected CE cells. Immune complexes were collected on formaldehyde-fixed *Staphylococcus aureus* Cowan I, washed, and subjected to electrophoresis on 9% polyacrylamide gels. Gels were dried and exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.), with Cronex intensifying screens.

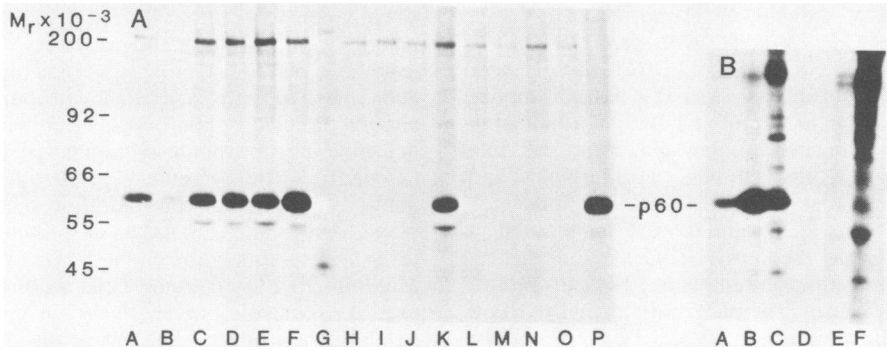

FIG. 1. Screening of hybridoma culture supernatants for RSV *src*-specific antibody. PrA-RSV-infected CE cells were labeled with $^{32}P_i$ for 2 h, and extracts were prepared as described in the text. Samples, corresponding to 2×10^5 cells, were immunoprecipitated with 500 μl of culture supernatant (A, lanes A through O; B, lanes A through F) or 3 μl of rabbit anti-p60^{src} (A, lane P), and the labeled proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography. Molecular weights of labeled proteins were determined relative to the migration of known molecular weight standards (200,000, myosin; 92,000, phosphorylase A; 66,000, BSA; 55,000, human immunoglobulin heavy chain; and 45,000, ovalbumin). The autoradiogram was exposed for 12 h.

Immune complex protein kinase assay. To measure pp60^{src} protein kinase activity, cells were lysed in RIPA buffer lacking sodium dodecyl sulfate, and extracts were immunoprecipitated with Mabs as described above. Immune complexes were collected, washed in PBS, suspended in 30 μl of kinase buffer (20 mM PIPES [pH 7.2], 10 mM $MnCl_2$, 10 μCi of [γ-^{32}P]ATP [5,000 Ci/mmol; ICN]), and incubated at 22°C for 30 min. The reactions were stopped by the addition of 15 μl of sample buffer concentrated three times. Labeled samples were heated at 100°C and subjected to electrophoresis on 9% polyacrylamide gels, and the phosphorylated proteins were visualized by autoradiography.

Phosphoamino acid analysis. pp60 was eluted from crushed gel slices in 50 mM ammonium bicarbonate–0.1% sodium dodecyl sulfate–5% β-mercaptoethanol overnight at room temperature, precipitated in cold acetone, and hydrolyzed at 110°C for 2 h in 6 N HCl–0.1 M phenol. Phosphoamino acids were resolved by electrophoresis in two dimensions on cellulose thin-layer plates as described by Hunter and Sefton (18). Labeled amino acids were visualized by autoradiography, and the position of phosphoamino acid standards was determined by ninhydrin staining.

Western immunoblot analysis. pp60$^{v\text{-}src}$ in whole-cell extracts was detected on nitrocellulose paper by the method outlined by Towbin et al. (39). Briefly, RSV-infected CE cells from a 100-mm tissue culture dish were harvested, washed, and solubilized in 400 μl of sample buffer with boiling for 4 min. The extract was clarified by centrifugation at 100,000 × *g* for 30 min and applied to a 9% polyacrylamide gel. The separated proteins were electrophoretically transferred to 0.2-μm nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.). The unbound sites on the paper were blocked by incubation in 3% bovine serum albumin (BSA) in PBS for 1 h at 43°C or overnight at 4°C. Excess BSA was removed by washing the paper in PBS at room temperature for 5 min. The paper was cut in strips, and each strip was incubated in a sealed bag with an individual Mab (about 0.1 μg/ml) overnight at 4°C. The strips were removed from the bags and washed for 5 min in PBS three times at room temperature with gentle agitation. The primary antibody was detected by incubation of the strips with horseradish peroxidase-conjugated goat anti-mouse IgG (Tago, Inc.) for 2 h at room temperature, followed by three additional washes in PBS for 5 min each and subsequent incubation with the substrates dianisidine and hydrogen peroxide for 10 to 20 min. The strips were then washed in distilled water for several minutes, allowed to air dry, and photographed.

Indirect immunofluorescence staining of cells. RSV-infected and uninfected CE cells were seeded on cover slips and allowed to grow overnight. Cells were fixed in 3% paraformaldehyde, permeabilized in 0.4% Triton X-100 (25), and incubated with 100 μl of anti-*src* Mab (at concentrations of 5 to 20 μg/ml) for 30 min at room temperature. The binding of the Mabs was localized by incubation of the cells with goat F(ab')$_2$ anti-mouse IgG (Jackson Immuno Research) (10 μg/ml) for 30 min, followed by incubation with fluorescein-conjugated rabbit F(ab')$_2$ anti-goat F(ab')$_2$ (Jackson Immuno Research) (10 μg/ml) for an additional 30 min. Between incubations with antibody, the cover slips were washed three times with PBS by addition of the buffer, followed by gentle swirling and aspiration. The stained cells were examined with a Leitz fluorescence microscope equipped with epi-illumination.

RESULTS

Isolation and identification of Mabs to v-*src* protein. Hybridoma cell lines producing anti-pp60^{src} antibodies were obtained by fusing splenocytes from immune BALB/c mice with SP2/O mouse myeloma cells as described above. Ten to 14 days after fusion, growth of hybrid cells was observed in 64% (246/384) of the microtiter wells seeded. Culture supernatants were assayed by ELISA to determine specific antibody production. Of the wells, 82% (201/246) contained antibody which bound to bacterial p60^{src} in the ELISA. Culture supernatants which exhibited a positive binding in the ELISA were further tested for their ability to immunoprecipitate pp60$^{v\text{-}src}$ from extracts of $^{32}P_i$-labeled PrA-RSV-infected CE cells. In total, 21% (42/201) of the culture supernatants tested in this manner immunoprecipitated a phosphoprotein of 60,000 M_r (Fig. 1A). The majority of these supernatants precipitated a single phosphoprotein of 60,000 M_r; however, four supernatants precipitated multiple phosphoproteins ranging in molecular weight from 25,000 to >200,000 in addition to the 60,000-M_r protein (Fig. 1B, lanes C and F).

To verify that the Mabs immunoprecipitated pp60$^{v\text{-}src}$, the Mabs were used to immunoprecipitate a structurally altered *src* protein encoded by the mutant of PrA-RSV, *ts*CH*dl*119. *ts*CH*dl*119 encodes a *src* protein, pp53^{src}, containing a deletion of amino acids 173 to 227 and is described in detail

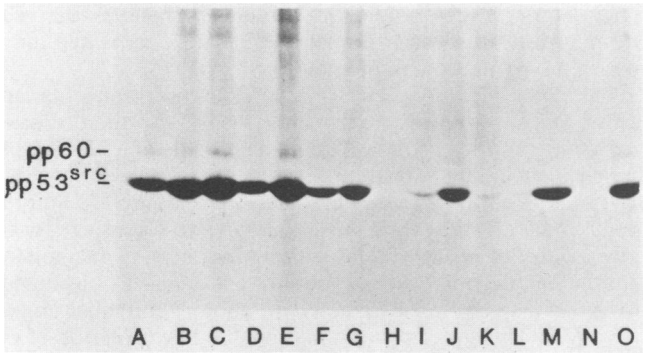

FIG. 2. Immunoprecipitation of pp53^{src} by culture supernatants containing *src*-specific antibodies. Samples of an extract of $^{32}P_i$-labeled *ts*CH*dl*119 RSV-infected CE cells were immunoprecipitated with 500 μl of *src*-specific culture supernatants (lanes A through M), 3 μl of normal rabbit serum (lane N), or 3 μl of rabbit anti-p60^{src} serum (lane O). Polyacrylamide gel electrophoresis and autoradiography were performed as described in the legend to Fig. 1. pp60 denotes the position in the gel of wild-type pp60^{src}.

elsewhere (6). Culture supernatants from 13 individual hybridomas, all of which precipitated a 60,000-M_r phosphoprotein from wild-type RSV-infected CE cells, readily immunoprecipitated pp53^{src} from extracts of $^{32}P_i$-labeled *ts*CH*dl*119-infected CE cells (Fig. 2). Culture supernatants which immunoprecipitated diminished amounts of pp53^{src} (e.g., Fig. 2, lanes H, I, K, and L) exhibited marked increases in antibody titer upon subcloning and continued propagation. In addition to the immunoprecipitation of pp53^{src}, the specificity of the Mabs for v-*src* protein was verified by *S. aureus* V-8 protease digestion and phosphoamino acid analysis of the immunoprecipitated 60,000-M_r phosphoprotein and by immunoprecipitation of a 60,000-M_r protein from extracts of [^{35}S]methionine-labeled CE cells infected with PrA-RSV, SRD-RSV, and B_{77}C-RSV (data not shown).

Immunoprecipitation of pp60$^{c\text{-}src}$ from uninfected CE cells. To test the cross-reactivity of the Mabs with pp60$^{c\text{-}src}$, purified immunoglobulin from a representative panel of 22 hybridomas was used to immunoprecipitate pp60$^{c\text{-}src}$ from $^{32}P_i$-labeled, uninfected CE cells. Concurrently, equal amounts of purified Mab were used to precipitate pp60$^{v\text{-}src}$ from extracts of $^{32}P_i$-labeled PrA-RSV-infected CE cells. Figure 3A (lanes D through I) shows the immunoprecipitation of pp60$^{v\text{-}src}$ by six representative Mabs, and Fig. 3B shows the immunoprecipitation of pp60$^{c\text{-}src}$ from uninfected CE cells by the same six Mabs. Three Mabs (Fig. 3B, lanes D through F) immunoprecipitated significant levels of pp60$^{c\text{-}src}$, whereas the remaining three Mabs (lanes G through I) precipitated pp60$^{c\text{-}src}$ with low efficiency, in spite of their apparently high reactivity with pp60$^{v\text{-}src}$. Such differences in reactivity could be due to affinity differences for the two proteins which might reflect the presence of an altered determinant on pp60$^{c\text{-}src}$. Figure 3C shows the immunoprecipitation of pp60$^{c\text{-}src}$ by nine additional Mabs. In total, 10 of 22 Mabs immunoprecipitated readily detectable levels of pp60$^{c\text{-}src}$.

To confirm that the 60,000-M_r phosphoprotein precipitated from CE cells was pp60$^{c\text{-}src}$, an *S. aureus* V-8 proteolytic digestion of the immunoprecipitated protein was carried out. Figure 4 shows the V-8 protease map of pp60$^{c\text{-}src}$ immunoprecipitated with Mabs EB8 and GD11 and rabbit anti-p60^{src}. Two major c-*src*-specific peptides of 34 and 26 kilodaltons as well as two smaller peptides (28) were observed. In addition, phosphoamino acid analysis of the immunoprecipitated 60,000-M_r protein revealed the presence of both *p*-tyrosine and *p*-serine (data not shown). These results, therefore, support the conclusion that the 60,000-M_r phosphoprotein precipitated by the Mabs from uninfected CE cells is pp60$^{c\text{-}src}$.

In vitro tyrosine protein kinase activity of Mab immune complexes. To determine whether pp60^{src} in immune complexes formed with Mabs exhibited protein kinase activity, kinase assays were carried out as described above, and the in vitro-labeled products were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography. Figure 5 shows that both pp60$^{v\text{-}src}$ (Fig. 5A, lanes C through J) and pp60$^{c\text{-}src}$ (Fig. 5B, lanes C through J) were phosphorylated in vitro, presumably by the intrinsic autophosphorylation activity of pp60^{src}. A further analysis of individual Mabs showed that all 22 Mabs immunoprecipitated pp60$^{v\text{-}src}$ as

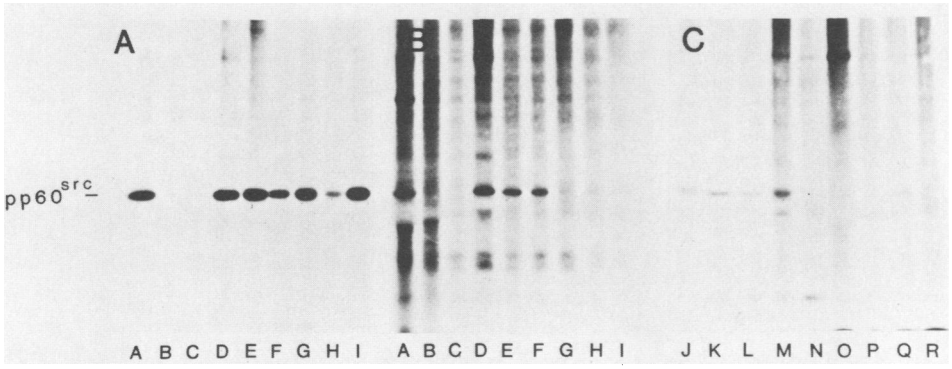

FIG. 3. Cross-reactivity of pp60$^{v\text{-}src}$-specific Mabs with pp60$^{c\text{-}src}$. Samples of extracts from $^{32}P_i$-labeled PrA-RSV-infected CE cells (A) or uninfected CE cells (B and C) were immunoprecipitated with ascites fluid or protein A-Sepharose-purified Mab, and the resulting immune complexes were analyzed by polyacrylamide gel electrophoresis and autoradiography as in Fig. 1. For the experiments shown in A, individual samples contained extract from about 2×10^5 cells, whereas in B and C, each sample contained extract from about 1×10^6 cells. In A and B, labeled extracts were immunoprecipitated with 10 μl of rabbit anti-p60^{src} (lane A), 10 μl of normal rabbit serum (lane B), 17 μg of mouse anti-BSA immunoglobulin (lane C), or the following anti-*src* Mabs: lane D, FG1; E, GD11; F, EB8; G, EC10; H, GD1; and I, EB7. In C, labeled extracts were immunoprecipitated with the following Mabs: lane J, GE7; K, FC6; L, GB6; M, FG8; N, FH2; O, FD7; P, GF3; Q, GA1; and R, EE10.

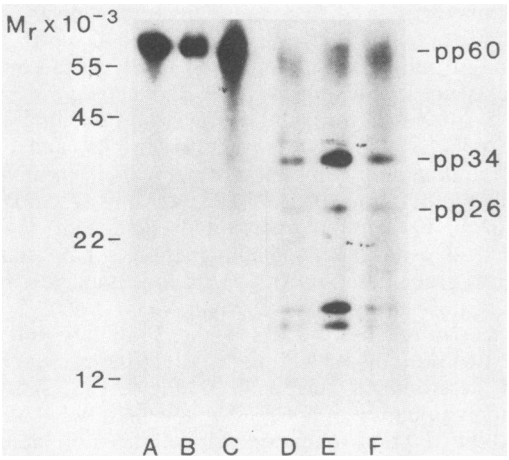

FIG. 4. Partial V-8 protease digestion patterns of pp60$^{c\text{-}src}$ immunoprecipitated by Mabs GD11 and EB8 and rabbit anti-p60^{src}. Extracts of $^{32}P_i$-labeled, uninfected CE cells were immunoprecipitated with Mabs GD11, EB8, or rabbit anti-p60^{src}, and the immune complexes were subjected to polyacrylamide gel electrophoresis and autoradiography. Radioactive proteins migrating with an apparent M_r of 60,000 were recovered from the gel and digested with *S. aureus* V-8 protease (lanes A through C, undigested control; lanes D through F, 50 ng of V-8 protease), and the products were analyzed by polyacrylamide gel electrophoresis by the method of Cleveland et al. (8). Lanes A and D, Mab EB8; lanes B and E, Mab GD11; lanes C and F, rabbit anti-p60^{src}. Molecular weights were determined as in the legend to Fig. 1 with the additional molecular weight standards of human immunoglobulin light chain, 22,000, and cytochrome *c*, 12,000.

detected in the immune complex kinase assay. The 10 Mabs which reproducibly immunoprecipitated in vivo $^{32}P_i$-labeled c-*src* also immunoprecipitated pp60$^{c\text{-}src}$ from normal CE cells as detected by in vitro phosphorylation (Table 1). In vitro phosphorylation of c-*src* was weak or undetectable in immune complexes formed with the 12 remaining Mabs, even when Mab concentrations were increased three- to fivefold. In no instance did we observe phosphorylation of mouse immunoglobulin heavy chain. The 60,000-M_r phosphorylated protein labeled in immune complexes derived from both transformed and normal CE cells contained only phosphotyrosine (data not shown).

Immunoprecipitation of pp60$^{c\text{-}src}$ from rodent and human cells. Mabs to pp60^{src} were also tested for their cross-reactivity with *src* proteins from normal cells of a number of species, including mouse, rat, mink, and human species. With the in vitro immune complex kinase assay to identify pp60$^{c\text{-}src}$, two Mabs, GD11 and EB8, were found to cross-react with the c-*src* protein from normal mouse, rat, mink, and human fibroblasts (Fig. 6A through D and F) but failed to detect c-*src* from HeLa cells, a tumor of epithelial origin (Fig. 6E). This latter result could be due to expression of c-*src* in these cells which contain an altered epitope not recognized by Mabs GD11 and EB8, or it could be due to expression of c-*src* at a level below the limits of detection by the assays. Roughly equal amounts of cell protein were used in all assays. In all cell lines tested, the amount of *src* protein detected by the Mab-immune complex kinase assay correlated well with the amount of pp60$^{c\text{-}src}$ immunoprecipitated from $^{32}P_i$-labeled cell extracts (data not shown).

The same panel of Mabs was also used to immunoprecipitate pp60^{src} from extracts of rat brain tissue (Fig. 7A). With the in vitro kinase assay, Mabs GD11 and EB8 were again found to be strongly cross-reactive, whereas rabbit anti-p60^{src} and Mabs GB6, FG1, FG8, HB7, and FB7 appeared to be weakly cross-reactive, and Mabs EE10, FC6, and FH2 were non-cross-reactive. The detection of a weak cross-reactivity with five Mabs and rabbit anti-p60^{src} which had previously been shown to be non-cross-reactive was probably due to the higher concentrations of c-*src* in rat brain extracts (H. Kim and J. T. Parsons, unpublished data) as compared with tissue culture cell extracts, thereby augmenting the ability of low-affinity Mabs to bind pp60$^{c\text{-}src}$. As before, the authenticity of the pp60$^{c\text{-}src}$ was established by its phosphotyrosine content (Fig. 7B) and its typical V-8 peptide profile (Fig. 7C), which shows the predominant tyrosine-specific phosphorylation of the 26-kilodalton carboxy-terminal peptide.

Mapping of epitopes recognized by Mabs. The epitopes recognized by individual Mabs were localized within the primary amino acid sequence of pp60^{src} by determining the

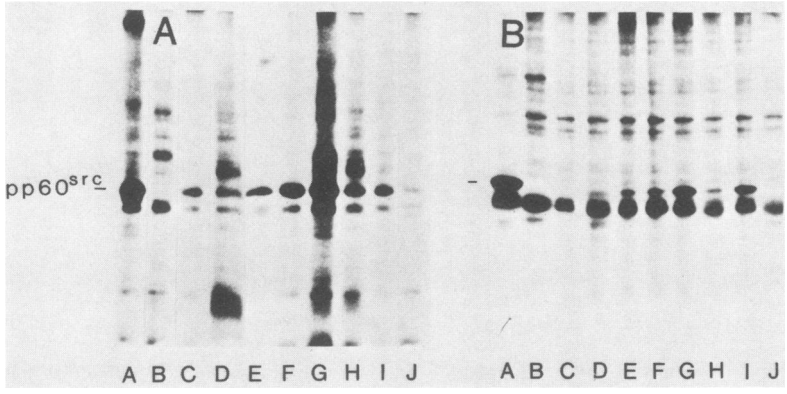

FIG. 5. In vitro kinase activity of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ in immune complexes formed with Mabs. Unlabeled extracts of uninfected or PrA-RSV-infected CE cells were immunoprecipitated with Mabs, and the immune complexes were collected, washed, and incubated in a kinase reaction mixture containing [γ-^{32}P]ATP as described in the text. The products of the reaction were subjected to electrophoresis on 9% polyacrylamide gels and autoradiographed. RSV-infected CE cells (A) or uninfected CE cells (B) were immunoprecipitated with anti-p60^{src} (lane A) or with the following Mabs: lane B, anti-BSA Mab; C, EB7; D, EE10; E, FC6; F, GD11; G, FG1; H, GB6; I, EB8; and J, GF3. Autoradiogram exposures: A, 12 h and B, 48 h.

TABLE 1. c-*src* cross-reactivity[a]

Anti-v-*src* clone	Immunoglobulin isotype	Cross-reactivity with cells from:						Western blot pattern for v-*src*
		CEs	Mice	Rats	Rat brain tissue	Minks	Humans	
EB7	IgG2a	±	−	−	−	−	−	++
EB8	IgG1	+	+	+	+	+	+	−
EC10	IgG2b	±	−	−	±	−	−	++
R2D2	IgG3	−	−	NT[b]	−	−	−	+ (MB)[c]
EE10	IgG1	±	−	−	−	−	−	±
FB7	IgG2a	+	−	−	±	−	−	+ (MB)
FC6	IgG2a	+	−	−	−	−	−	+
FD7	IgG2a	±	NT	NT	−	−	NT	NT
FF10	IgG2a	±	−	NT	−	−	NT	+
FG1	IgG2a	+	−	−	±	−	−	± (MB)
FG8	IgG2a	+	−	−	±	−	−	± (MB)
FH2	IgG1	±	−	NT	−	−	NT	+
GA1	IgG1	+	−	−	±	−	−	+
GB6	IgG1	+	±	±	±	±	±	+
GC6	IgG1	−	NT	NT	NT	NT	NT	+
GD1	IgG1	±	−	−	±	−	−	+
GD11	IgG1	+	+	+	+	+	+	−
GE7	IgG2b	+	+	−	+	−	±	NT
GF3	IgG1	−	NT	NT	NT	NT	NT	+
HB7	IgG2a	+	−	−	±	−	−	NT
HC5	IgG1	−	NT	NT	−	NT	NT	+
HC6	IgG1	±	−	NT	±	−	NT	+
HD4	IgG1	±	NT	NT	±	NT	NT	+

[a] Determined by in vitro immune complex kinase assay. Results are a consensus of a minimum of three experiments with two or more different sources of antibody, i.e., culture supernatant, ascites fluid, or purified immunoglobulin.
[b] NT, Not tested.
[c] MB, Multiple bands.

capability of each of the Mabs to immunoprecipitate *src* proteins encoded by *src* deletion mutants, as well as a β-galactosidase fusion protein (p130) containing the amino-terminal 204 amino acids of *src* fused to β-galactosidase (29) (Fig. 8). All 22 Mabs recognized epitopes contained within the amino-terminal 204 residues of the *src* molecule, as judged by their ability to immunoprecipitate bacterial p130 (Fig. 8A) and their failure to immunoprecipitate wild-type β-galactosidase (data not shown). Further mapping was accomplished by analysis of immunoprecipitations of two *src* deletion mutants, one containing a deletion spanning amino acid residues 173 to 227 (*ts*CH*dl*119) and a second containing a deletion of residues 82 to 169 (CH*dl*121) (Parsons et al., in press). As previously shown in Fig. 2, all Mabs precipitated pp53^{src} from *ts*CH*dl*119-infected CE cells. A similar analysis of CH*dl*121 showed that all but two Mabs (EB8 and GD11) were able to immunoprecipitate pp53^{src} from CH*dl*121-infected CE cells (Fig. 8B). The assignment of the epitope recognized by GD11 and EB8 to that region bounded by amino acid residues 82 to 169 cannot be definitively made since the deletion of CH*dl*121 may disrupt the confirmation of epitopes outside that region. However, residues critical to the correct formation of the epitope(s) recognized by EB8 and GD11 reside, at least in part, within amino acid residues 82 to 169. By inference, then, the remaining epitopes can be placed within amino acid residues 1 to 82 and 169 to 173. The placement of several of the epitopes in the amino third of the molecule has been verified by the binding of 10 Mabs to the amino-terminal p34 V-8 protease fragment of *src* in a Western immunoblot analysis (data not shown). The amino-terminal designation of the major epitopes was further documented by testing the ability of the Mabs to precipitate an altered *src* protein encoded by mutant CH*dl*300. This mutant encodes an enzymatically inactive *src* protein (pp58^{src}) containing a deletion of residues 504 to 506 and terminates with 24 amino acids unrelated to pp60^{src} (Parsons et al., in press). Figure 8C shows that all 22 Mabs immunoprecipitated pp58^{src}, indicating that carboxy-terminal amino acids were not involved in epitope recognition by any of the Mabs.

Western blot analysis with *src* Mabs. The binding of the Mabs to denatured, immobilized pp60$^{v\text{-}src}$ was tested in a Western immunoblot, as described above. Figure 9 shows several patterns of binding. Mabs EC10 and EB7 (Fig. 9, lanes C and D) bound strongly to a single protein of 60,000 molecular weight (i.e., pp60^{src}), whereas Mabs FG1, FG8, and FB7 (lanes F, G, and N) exhibited weak binding to v-*src* and to multiple cellular proteins. Interestingly, these three Mabs also immunoprecipitated multiple proteins from normal and transformed cells (Fig. 1B), supporting the argument that these cellular proteins share determinants with pp60^{src}. The majority of Mabs bound poorly to pp60^{src}, and the Mabs EB8 and GD11 appeared not to bind at all (Fig. 9, lanes B and K). This observation suggests that the epitopes recognized by GD11 and EB8 are denaturation sensitive.

Detection of pp60^{src} by indirect immunofluorescence staining with Mabs. The binding of the Mabs to pp60^{src} in PrA-RSV-infected CE cells was tested in an indirect immunofluorescence staining assay. The pp60$^{v\text{-}src}$ staining pattern of one Mab, EB7, is shown in Fig. 10. Incubation of formaldehyde-fixed PrA-RSV-infected cells with EB7 revealed a diffuse staining of the cytoplasm, with a more intense staining apparent in the perinuclear region, at the plasma membrane, and at cell-to-cell junctures. Such a pattern has previously been described by Rohrschneider (32, 33) with TBR antisera. Background staining of uninfected cells is shown in Fig. 10D. Similar staining of transformed cells was observed with Mabs EB8 and GD11.

DISCUSSION

We have previously reported that a bacterially expressed oncogene product, p60^{src}, encoded by the plasmid p*lac-src*, could be used to hyperimmunize mice for the production of Mabs to pp60^{src} (29). This method of immunization has proven to be more effective in inducing an immune response to the *src* protein than immunization with live RSV-transformed mouse tumor cells or with small amounts of *src* protein purified from tissue culture cells. Lipsich et al. have also employed a similar immunization protocol with bacterial *src* protein to obtain 13 Mabs to pp60^{src} (24). In an extension of our original results, we report here the isolation and characterization of 22 additional *src*-specific Mabs. All were found to react with *src* proteins as encoded by PrA-RSV, SRD-RSV, and B_{77}C-RSV. Ten of the Mabs efficiently immunoprecipitated the chicken cellular homolog of *src*, pp60$^{c\text{-}src}$. The specificity of the Mabs for pp60^{src} and the utility of the Mabs were demonstrated by *S. aureus* V-8 proteolytic digestion pattern and phosphoamino acid analysis of the phosphorylated p60 protein immunoprecipitated by the Mabs, the autophosphorylation of pp60^{src} in immune complexes, the immunoprecipitation of structurally altered *src* proteins containing defined amino acid deletions, the binding of certain Mabs to pp60^{src} in a Western immunoblot, and the binding of Mabs to pp60^{src} by immunocytochemical techniques.

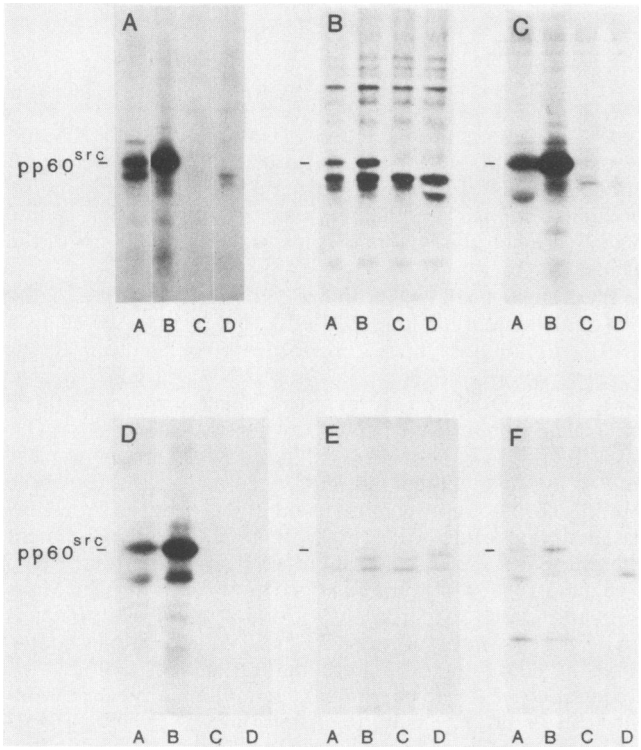

FIG. 6. Immunoprecipitation of pp60$^{c\text{-}src}$ from rodent and human cell lines with Mabs. Samples of extracts from cells were immunoprecipitated with individual Mabs, and the immune complexes were incubated in a kinase reaction mixture containing [γ-^{32}P]ATP and analyzed as described in the legend to Fig. 5. A, normal BALB/c mouse fibroblasts (samples contained extracts from about 2×10^6 cells); B, 3Y1, normal rat fibroblasts (3×10^6 cells); C, normal mink fibroblasts (2×10^6 cells); D, NHF, normal human foreskin fibroblasts (1×10^6 cells); E, HeLa (2×10^6 cells); and F, JLM, normal human fibroblast (2×10^6 cells). Lanes A, Mab EB8; B, Mab GD11; C, anti-BSA Mab; and D, Mab FG1.

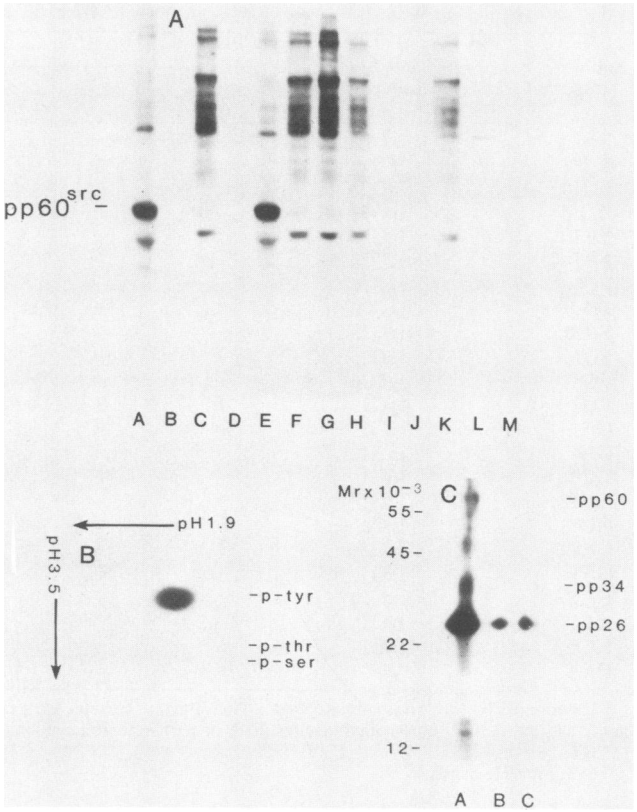

FIG. 7. Detection of pp60$^{c\text{-}src}$ in rat brain tissue. A, Immunoprecipitation by Mabs of pp60$^{c\text{-}src}$ from rat brain tissue. Extracts of cerebellum tissue of a 12-day-old rat were prepared and immunoprecipitated with Mabs as described in the text. Immune complexes were incubated with [γ-^{32}P]ATP, and the labeled proteins were analyzed by polyacrylamide gel electrophoresis. Mabs: lane A, GD11; B, anti-BSA Mab; C, HB7; D, EE10; E, EB8; F, FG1; G, FG8; H, GB6; K, FB7; L, FC6; and M, FH2. Lane I, 10 μl of rabbit anti-p60^{src} and lane J, 10 μl of normal rabbit serum. B, Two-dimensional phosphoamino acid analysis of in vitro-labeled rat brain pp60$^{c\text{-}src}$ immunoprecipitated by Mab GD11. C, Partial V-8 protease digestion pattern of pp60$^{c\text{-}src}$ phosphorylated in vitro. pp60$^{c\text{-}src}$ immunoprecipitated from rat brain extracts with Mab GD11 (lane A) or EB8 (lanes B and C) was labeled in an immune complex kinase assay. The labeled protein was recovered from a polyacrylamide gel, subjected to partial proteolysis by *S. aureus* V-8 protease (5 ng) by the method of Cleveland et al. (8), and analyzed as described in the legend to Fig. 4.

Two Mabs were of particular interest. GD11 and EB8 readily immunoprecipitated enzymatically active pp60$^{c\text{-}src}$ from extracts of uninfected cultured cells, including CE, mouse, rat, mink, and human fibroblasts, and from rat brain tissue. The epitope(s) specified by GD11 and EB8 appears to be highly conserved and was tentatively localized to a region of the *src* amino acid sequence bounded by amino acid residues 82 to 169. However, since the deletion of *src* used to map this epitope(s) could also result in the perturbation of epitopes outside the deleted region, a definitive placement of the epitope(s) cannot be made. These Mabs appeared to bind native pp60^{src} more efficiently than denatured pp60^{src}, as judged by their poor reactivity in a Western immunoblot. It is also not known if EB8 and GD11 recognize the same

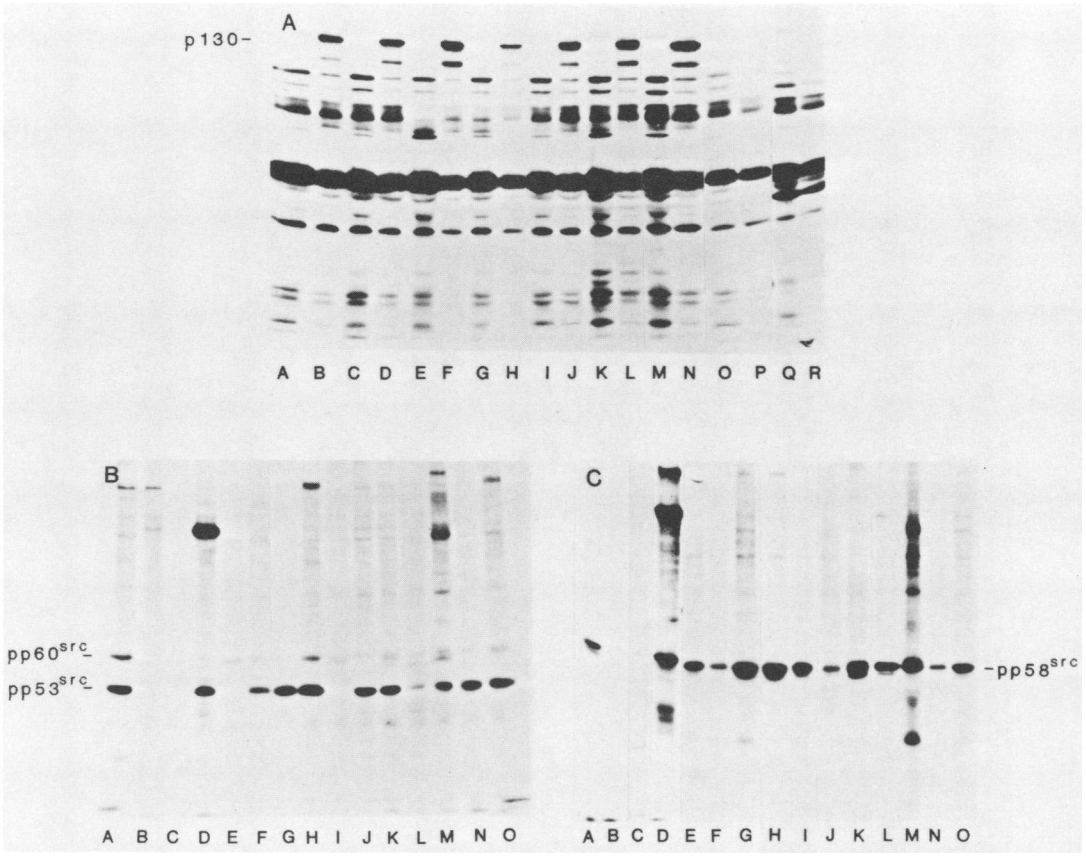

FIG. 8. Immunoprecipitation of *src*-β-galactosidase fusion protein and structurally altered *src* proteins encoded by RSV deletion mutants. A, Cultures of *E. coli* carrying plasmid pLSZ and expressing an *src*-β-galactosidase hybrid protein of M_r 130,000 were labeled for 2 h in sulfate-depleted growth medium with 100 μCi of [^{35}S]sulfate per ml. Extracts were prepared by the method of Gilmer et al. (16). As a negative control, cultures of *E. coli* containing a plasmid with the *src*-β-galactosidase coding sequence but lacking the lactose operator-promoter (pSZ) were grown and labeled in the same manner. Alternate lanes beginning with A denote immunoprecipitation of extracts of *E. coli*(pSZ) cells; alternate lanes beginning with B denote immunoprecipitation of extracts of pLSZ cells. Immunoprecipitations were carried out with 500 μl of culture supernatants of Mabs FG8 (lanes A and B), EB8 (C and D), GD11 (E and F), HC5 (G and H), GD1 (I and J), and EB7 (K and L) and 5 μl of rabbit anti-p60^{src} (M and N), 5 μl of normal rabbit serum (O and P), and anti-BSA (Q and R). B and C, Immunoprecipitation with Mabs of *src* protein encoded by RSV mutant CH*dl*121 or CH*dl*300. Samples of extracts prepared from CE cells infected with CH*dl*121 RSV (B) or CH*dl*300 RSV (C) were immunoprecipitated with individual Mabs, and the immune complexes were analyzed as in the legend to Fig. 1. Lanes A, 3 μl of rabbit anti-p60^{src}; B, 3 μl of normal rabbit serum; C, anti-BSA Mab; D, R2D2; E, GD11; F, FG1; G, EC10; H, EB7; I, EB8; J, EE10; K, FC6; L. FH2; M, FG8; N, GA1; and O, GD1.

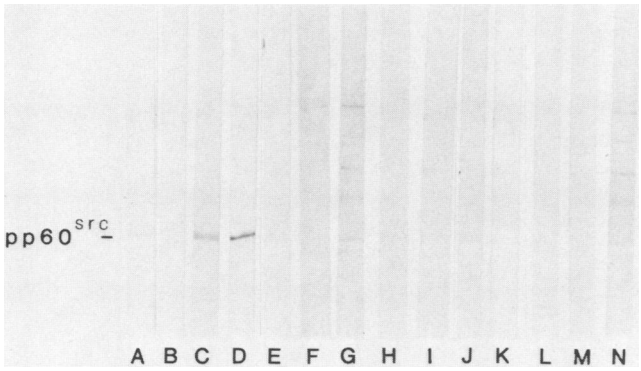

FIG. 9. Binding of Mabs to pp60$^{v\text{-}src}$ in a Western immunoblot. Extracts were prepared, subjected to polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose paper as described in the text. The paper was cut in strips, and each strip was incubated with an individual Mab (about 0.1 μg of antibody per ml) overnight at 4°C. The strips were stained for specific Mab binding as described in the text. Mabs: lane A, HC5; B, EB8; C, EC10; D, EB7; E, FC6; F, FG1; G, FG8; H, FH2; I, GA1; J, GD1; K, GD11; L. GC6; M, GF3; and N, FB7. The molecular weight of pp60^{src} was determined from the migration of standard proteins which had been transferred to nitrocellulose paper and stained with 0.1% amido black in 50% methanol and 10% acetic acid.

epitope, because competitive binding for p60^{src} has not been tested.

The epitopes defined by the remaining 20 Mabs appeared to map within the amino acid residues 1 to 82 or 169 to 173. Assignment of these epitopes (presumably more than one; see below) is based on the immunoprecipitation of structurally altered *src* proteins encoded by deletion mutants of RSV or *src*-β-galactosidase fusion proteins containing the first 204 amino acids of pp60^{src} (Fig. 8A) or the first 110 amino acids of *src* (unpublished results). Most of these Mabs bound

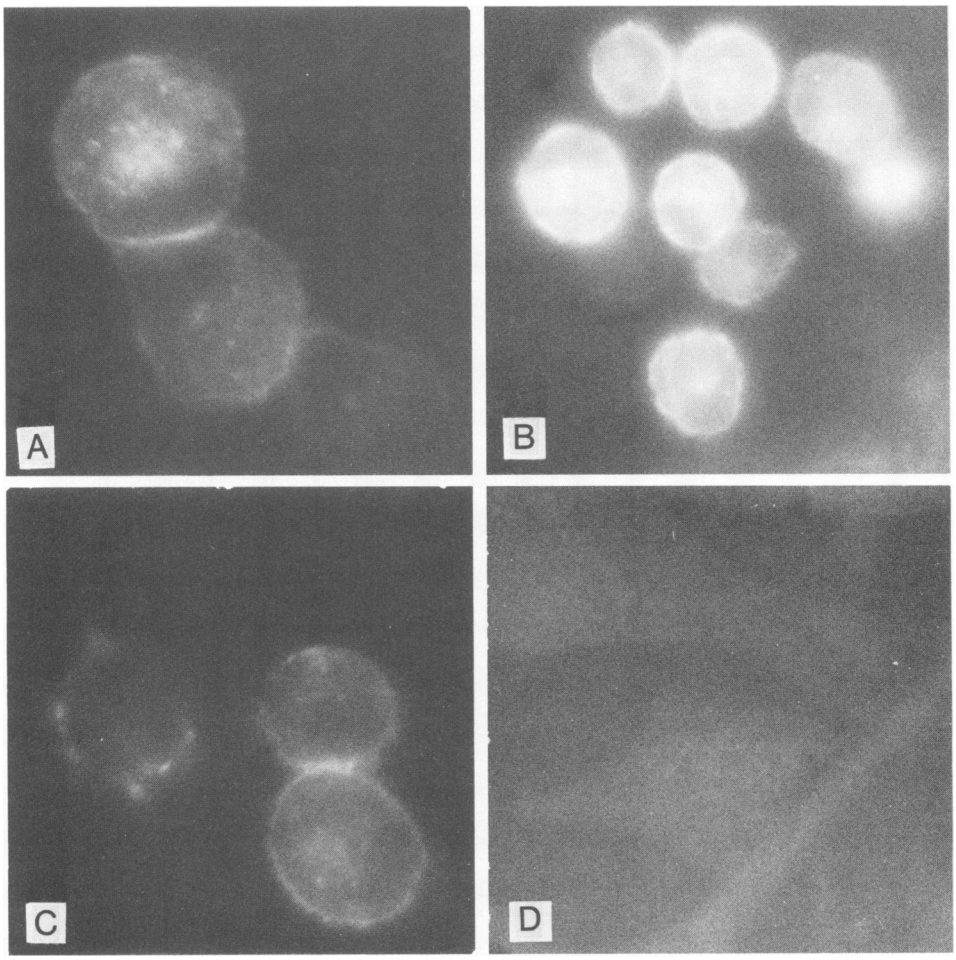

FIG. 10. Indirect immunofluorescence staining of cells with Mab EB7. Cells were grown on cover slips, fixed, and stained as described in the text. A through C, PrA-RSV-infected CE cells; and D, uninfected CE cells.

pp60$^{v\text{-}src}$ from unfractionated extracts of RSV-transformed cells in a Western immunoblot, although with various degrees of efficiency. Such variations in binding could readily represent differences in either titers or affinity for various epitopes. The total number of different epitopes recognized by this panel of Mabs is unknown at this time, since competitive binding studies for p60^{src} have not been completed. However, based on reactivity profiles of the Mabs with c-*src* from different species, with cellular phosphoproteins other than *src*, and with tyrosine kinase-transforming proteins of other retroviruses, a minimum of four to five epitopes can be identified. One such profile is represented by Mabs FG1, FG8, HB7, and FB7, which immunoprecipitated pp60^{src} as well as a mixture of phosphoproteins ranging in M_r from <25,000 to 200,000 from extracts of normal and transformed cells. These Mabs also bound to multiple proteins in Western immunoblots of RSV-transformed cell extracts (Fig. 9). The patterns of binding among these Mabs appeared virtually identical, suggesting that each of the Mabs recognized a determinant common to multiple cellular proteins. Whether such a common epitope(s) reflects the identification of proteins with similar function remains a matter for speculation. Additional determinants may be defined by those Mabs which immunoprecipitated CE pp60$^{c\text{-}src}$ poorly (e.g., GC6 and GF3; Table 1) and by those Mabs which readily immunoprecipitated CE pp60$^{c\text{-}src}$ but reacted poorly with pp60$^{c\text{-}src}$ from rodent or human cells (e.g., FC6 and GA1). A comparison of the deduced amino acid sequences of PrA-RSV pp60^{src} (35) and CE pp60$^{c\text{-}src}$ (37) reveals 7 amino acid differences within the first 100 amino acids and 10 differences within the first 200 residues. Thus it is not surprising that some Mabs directed toward epitopes within the first 100 amino acid residues of pp60$^{v\text{-}src}$ might exhibit a decreased affinity for an altered sequence in pp60$^{c\text{-}src}$. Similarly, the lack of strong reactivity with pp60$^{c\text{-}src}$ of other species likely reflects an alteration of the primary sequence of these epitopes.

Finally, three observations suggest that the previously described Mab R2D2 (29) appears to be directed against an amino-terminal determinant distinct from those described above. First, R2D2 immunoprecipitates the transforming proteins of Y73 sarcoma virus, p90$^{gag\text{-}yes}$, and Gardner Rasheed feline sarcoma virus, p70$^{gag\text{-}fgr}$ (S. J. Parsons, D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons, manuscript in preparation). Secondly, R2D2 does not efficiently immunoprecipitate pp60$^{c\text{-}src}$ from either avian or rodent cells. Third, pp60^{src} immunoprecipitated with R2D2 is not readily autophosphorylated in the immune complex kinase assay. In summary, the above data suggest that the Mabs described in this study recognize a minimum of four to five epitopes, each of which appears to be localized to the amino-terminal third of the *src* protein.

The utility of specific Mabs in studying structural and functional aspects of *src*-mediated transformation is exemplified by the demonstration of their reactivities in Western immunoblot assays and in immunofluorescence studies briefly described here. The use of specific Mabs to efficiently detect the cellular homolog of *src*, as shown by the in vitro immune complex kinase assays, may lead to a greater understanding of its function in nonneoplastic cells.

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