Highly Specific Antibody to Rous Sarcoma Virus src Gene Product Recognizes a Novel Population of pp60^v-src and pp60^c-src Molecules

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ABSTRACT Antiserum to the Rous sarcoma virus (RSV)-transforming protein, pp60^v-src, was produced in rabbits immunized with p60 expressed in Escherichia coli. ap60 serum immunoprecipitated quantitatively more pp60^v-src than did tumor-bearing rabbit (TBR) sera. When RSV-transformed cell lysates were preadsorbed with TBR serum, the remaining lysate contained additional pp60^v-src, which was recognized only by reimmunoprecipitation with ap60 serum and not by TBR serum. In subcellular fractions of RSV-infected chicken embryo fibroblasts (RSV-CEFs) and field vole cells probed with TBR serum, the majority of the pp60^v-src was associated with the plasma membrane-enriched P100 fraction. However, ap60 serum revealed equal distribution of pp60^v-src and its kinase activity between the P1 (nuclear) and P100 fractions. The same results were obtained for pp60^c-src in uninfected CEFs. On discontinuous sucrose gradients nearly 50% of the P1-pp60^v-src sedimented with nuclei, in fractions where no plasma membrane was detected. Indirect immunofluorescence microscopy of RSV-CEFs with ap60 serum revealed a distinct pattern of perinuclear fluorescence, in addition to staining at the cell periphery. Thus the use of a highly specific antibody reveals that enzymatically active pp60^v-src and pp60^c-src molecules are present in other intracellular structures, probably juxtareticular nuclear membranes, in addition to the plasma membrane in normal, uninfected, and wild-type RSV-infected cells.

Transformation of cells by Rous sarcoma virus (RSV) is mediated by the expression of a single viral gene termed src (24). The polypeptide encoded by src is a 60,000-mol-wt phosphoprotein, pp60^v-src (3, 10), which has been identified by immunoprecipitation of RSV-transformed cell extracts with antiserum from tumor-bearing animals (3, 37), by in vitro translation of subgenomic viral RNA (14, 37, 38), and by expression from plasmids containing the cloned src gene in Escherichia coli (21). The amino-terminal domain of pp60^v-src contains a serine residue, which is phosphorylated by a cyclic AMP (cAMP)-dependent protein kinase (10). Phosphorylation at a second site occurs on a tyrosine residue (11, 25) in the carboxy-terminal region of the protein, through a cAMP-independent reaction (10). Because pp60^v-src itself possesses protein kinase activity (9, 15, 34), with the unusual ability to specifically phosphorylate tyrosine residues (11, 21, 25, 33), it has been suggested that tyrosine kinase activity may play an important role in neoplastic transformation.

The pleiotropic effects resulting from expression of pp60^v-src are most prominent on cell morphology and growth properties. One approach to understanding the mechanism of cell transformation is to determine the intracellular localization of pp60^v-src in RSV-infected cells. To this end, both cytologic and biochemical techniques have been employed (reviewed in reference 26). Subcellular fractionation studies of chicken embryo fibroblasts (CEFs), field vole cells (1T), and rat kidney cells (NRK) transformed by wild-type RSV support the notion that the majority of the immunoprecipitable pp60^v-src and its associated kinase activity is associated with the plasma membrane (13, 29, 30). Based on immunocytochemical micrographs, pp60^v-src has been localized to the cytoplasmic surface of the plasma membrane (47), with par-
particularly high concentrations evident in adhesion plaques (36, 40) and regions of cell–cell contact (28). Recent experiments indicate a good correlation between membrane association of pp60^c-src and expression of cellular transformation parameters (5, 12, 19, 27). In addition, several phosphotyrosine-containing proteins, which may serve as direct or indirect substrates for pp60^c-src tyrosine kinase activity, have been identified in membrane vesicles (16, 19) and cytoskeletons (7, 41) isolated from RSV-infected cells. Thus these studies have led many investigators to concentrate on the plasma membrane as the primary site of action for the oncogenic potential of p60.

The ability to study the biochemistry of the pp60^c-src polypeptide in the infected cell is entirely due to the availability of antisera prepared from tumor-bearing rabbits (TRRs) and antisera raised against synthetic peptides of various domains of p60. However, it is important to consider that the detection of an antigen is determined by the strength and the specificity of the antibody probe for that antigen. Recently, this laboratory reported the production of a high-titer p60-specific antisera (22) (ep60) in rabbits immunized with p60^c-src expressed in E. coli (23). We report here that this antisera is highly specific for the amino-terminal portion of pp60^c-src. Because the membrane binding domain of p60 has been localized to an 8,000-mol-wt amino-terminal region (27), we were interested in reexamining the cellular membrane distribution of pp60^c-src by using this new antibody. In this report, we present evidence that, in addition to the plasma membrane, a significant proportion (25–40%) of the total, active pp60^c-src in the infected cell is associated with perinuclear membranous structures. The interaction of pp60^c-src with intracellular membranes is apparent in both virus permissive (avian) and nonpermissive (mammalian) RSV-infected cells probed with ep60 serum. Furthermore, we now report that ep60 serum immunoprecipitates the normal cellular homologue of pp60^c-src from uninfected CEFs, pp60^c-src, and that a substantial fraction of pp60^c-src is also associated with intracellular membranes. These results imply that pp60^c-src and pp60^c-src should not be designated exclusively as plasma membrane proteins and suggest that we reconsider the possibility that pp60^c-src may function from other intracellular locations in normal and transformed cells.

MATERIALS AND METHODS

Cells and Viruses: European field vole (Microtus agrestis) cells, which were originally transformed with the Schmid–Ruppin strain of RSV (SR-RSV), subgroup D (SR-D), by P. Vogt (clone 1T), and normal vole cells were provided by A. Faras (University of Minnesota) and maintained in culture at 37°C. CEFs transformed with SR-RSV, subgroup A (SR-A), were used for experiments several passages after infection. CEFs infected with a temperature-sensitive mutant of RSV, 72-4, were a kind gift of Dr. H. Hanafusa (The Rockefeller University). These cells were provided by A. Faras (University of Minnesota) and maintained in culture at 0.2 mM MgCl2, pH 7.4) (0.8 ml per 100-mm dish). After a 10–15 rain incubation on ice, the cells were disrupted by 25–30 up and down strokes in a Dounce homogenizer with tight-fitting pestle. Under these conditions, >95% of the cells were broken and nuclei remained intact, as judged by light microscopy. The homogenate was adjusted to a final concentration of 0.25 M sucrose and 1 mM EDTA. At this stage, an aliquot representing 10% the total volume was removed and saved for quantitation of total recovery. The cell homogenate was centrifuged at 1,000 g for 10 min at 4°C and the supernatant was removed immediately and saved. The pellet was resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4 by five strokes in a Dounce homogenizer. This suspension was resuspended at 1,000 g, after which the supernatant was removed and combined with the supernatant from the first 1,000-g spin. The pellet, denoted P1, containing mostly nuclei, was resuspended in the appropriate buffer as required. The pooled 1,000 - g supernatants were centrifuged at 100,000 g for 1 h at 3°C in a SW55Ti rotor. Material that collected at each interface, as well as the gradient pellet, were diluted with 10 vol of TE buffer and centrifuged at 100,000 g for 1 h. Each pellet was then solubilized and immunoprecipitated as described below.

Membrane Fractionation on Discontinuous Sucrose Gradients: The P1 and P100 membrane fractions prepared as described above were resuspended in 1.0 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) by Dounce homogenization. The membrane suspension was layered over successive layers of 1.0 ml each: 50, 40, 35, and 20% sucrose (wt/wt) in TE buffer as described by Courtemp (13). The sucrose columns were spun at 100,000 g for 2.5 h at 3°C in an SW55Ti rotor. Material that collected at each interface, as well as the gradient pellet, were diluted with 10 vol of TE buffer and centrifuged at 100,000 g for 2.5 h at 3°C (SW55Ti rotor). Material accumulating at the sucrose interface and in the pellet was washed with TE buffer as described above.

Membrane Extractions: The P1 fraction was resuspended in 0.25 M sucrose, 10 mM Tris, pH 7.4, 0.2 mM MgCl2, and incubated with the appropriate extraction agent in a total volume of 500 al for 30 min at 0°C. After a 5-min centrifugation at 4°C in a Fisher microfuge (Fisher Scientific Co., Pittsburgh, PA) (10,000 g), the pellet and supernatant fractions were separated, adjusted to radioimmunoprecipitation (RIPA) buffer, clarified, and immunoprecipitated. The P100 fraction was treated in the same manner except that extractions were performed in 10 mM Tris, pH 7.4, 1 mM EDTA, and membranes were resolated by centrifugation at 100,000 g for 30 min.

Immunoprecipitation: Subcellular fractions were adjusted to RIPA buffer (3) and clarified at 40,000 g for 30 min at 3°C. Clarified lysates were immunoprecipitated with TBR serum (3) or ep60 serum (22) under conditions of antibody excess and analyzed by PAGE (31) on 10% polyacrylamide gels. The amount of radioactivity incorporated into pp60 was quantitated by excising the pp60 band from a dried gel and counting it in 4 ml of Aquasol (New England Nuclear, Boston, MA). For one-dimensional peptide mapping, the bands corresponding to pp60^c-src were excised from wet gels, treated with 0.5, or 50 ng of Protease V8 (Miles Laboratories Inc., Elkhart, IN), and reelectrophoresed through 10% polyacrylamide gels as described by Cleveland et al. (8).

Kinase Assays: Subcellular fractions were adjusted to 1% NP-40, 0.5% Nonidet-NP-40, 10 mM Tris, 1 mM EDTA, clarified, and immunoprecipitated with TBR and ep60 sera. The final wash was in 150 mM NaCl, 10 mM Tris, pH 7.4. Staph phabs were resuspended in 10 mM Tris, 5 mM MgCl2, 20 μCi [γ-32P]ATP in a final volume of 30–50 μl and incubated for 10–15 min at room temperature, at which time steady-state incorporation of phosphate was attained. The reaction was quenched by the addition of 1.0 ml of ice-cold STE buffer and, after centrifugation, the staph pellet was solubilized by boiling in sample buffer (31) and electrophoresed through 10% polyacrylamide gels.

Indirect Immunofluorescent Microscopy: Normal and RSV-infected CEF cells were grown on 25-mm glass coverslips. All subsequent operations were performed at room temperature. The coverslips were rinsed with PBS, and cells were fixed with 3.7% formaldehyde in PBS for 15 min. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and then washed with PBS. Reaction with the primary antibody was in PBS containing 10% fetal calf serum and a 1:10 dilution of either nonimmune rabbit serum or ep60 serum, using 100 μl per coverslip for 1–2 h in a humid chamber. The coverslips were washed five times for 5 min each with PBS; and then incubated in PBS containing 10% fetal calf serum and a 1:70 dilution of fluorescein-conjugated goat anti-rabbit IgG (affinity-purified, 30 μg/ml final concentration; Miles-Yeda, Miles Laboratories, Inc.). 100 μl per coverslip for 1 h. The coverslips were washed five times for 5 min each with PBS, mounted in 90% glycerol:0.1% p-phenylene diamine in PBS, pH 9, on 25 x 75 microscope slides, and sealed with nail polish. Cells were observed through a x 100 oil-immersion objective on a Leitz microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epi-fluorescent illumination, and
when cell homogenates were assayed and compared with unbroken cell activity.

of the 5'-nucleotidase activity should be expressed when whole cells are assayed.

To verify that this enzyme is a specific marker for plasma membrane, 5'-
sucrose) before and after Dounce homogenization. Because 5'-nucleotidase is
the cell lysates were immunoprecipitated with either ap60 or TBR serum, the immune complexes were collected by adsorption to Staphylococcus aureus, and the remaining supernatants were immunoprecipitated with ap60 or TBR. As can be seen in Fig. 1, in lysates previously reacted with ap60, a second ap60 immunoprecipitation brought down an additional amount of pp60v-src (~10-15% of the total) (lane 3), whereas very little pp60v-src was recovered (<2%) when TBR was the second antibody (lane 4). In the latter experiment, however, the viral structural protein, Pr76, was efficiently recovered by the second TBR immunoprecipitation (lane 4).

Interestingly, supernatants from TBR-adsorbed lysates contained additional pp60v-src molecules which were recognized by reimmunoprecipitation with ap60 (lane 5) but not by TBR (lane 6). The same results were obtained with [35S]methionine and [32P]orthophosphate-labeled cells, with IgG and autokinase activities, and in mammalian and avian RSV-infected cells. Thus there exists a population of pp60v-src molecules that are immunoreactive toward ap60 sera but are not recognized by TBR sera.

Subcellular Distribution of pp60v-src

Based on the ability of ap60 antisera to immunoprecipitate additional pp60v-src differentially, it was of interest to determine whether this population was plasma membrane-associated, as had been demonstrated for the pp60v-src recognized by use of TBR sera (13, 29, 30). RSV-infected cells were swollen in hypotonic buffer, Dounce-homogenized, and separated into three fractions by differential centrifugation: a P1 fraction, containing mostly nuclei; a P100 fraction, enriched for plasma membrane and containing other membranous organelles; and an S100 fraction, consisting of cytoplasmic and soluble components. 75-85% of the plasma membrane, as judged by the marker enzyme 5'-nucleotidase activity, fractionated with the P100 fraction (Table I). When probed with TBR serum, the majority of the pp60v-src was found associated with the P100 fraction, in agreement with the results of other investigators (13, 29, 30). However, when ap60 antibody was used, a strikingly different pattern emerged. Approximately equal distribution of pp60v-src was detected in the P1 and P100 fractions (Fig. 2A). The amount of pp60v-src detected in the P1 fraction was two- to fourfold higher than could be accounted for by plasma membrane contamination (Table I). This observation was confirmed by probing pp60v-src distribution with a monoclonal antibody...
gels were exposed to Kodak XAR film at -70°C with Cronex 3, P1 fraction TBR serum; lane 4, P100 fraction, TBR serum. The reaction products were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane 1, P1 fraction. Lanes 3, 4, 9, and 12, P100 fraction. Lanes 5, 6, and 11, S100 fraction. (B) Autokinase and IgG kinase activities of membrane fractions. The P1 and P100 fractions were reacted with op60 or TBR sera and the immune complexes were incubated with ap60 or TBR antisera. 1T cells were lysed by Dounce homogenization in hypotonic buffer and fractionated into a 1,000-g pellet (P1) and a 100,000-g pellet (P100) and supernatant (S100). Each fraction was analyzed for protein, plasma membrane marker enzyme 5'-nucleotidase, pp60src, and pp60src kinase activity as described under Materials and Methods. All experiments were with SR-D-1T cells, unless noted. Recoveries were from 65-95%, based on the total pp60src detected in the crude homogenate. Each data point represents the average of two to five separate determinations; standard deviations were ±1-5%.

* The same results were obtained using two different TBR sera.

Monoclonal antibody No. 327 (35).

Membrane suspensions in 20% sucrose from unlabeled 1T cells were fractionated through a 50% sucrose cushion (see Materials and Methods) and assayed for autokinase activity with op60 serum.

SR-A-CEF.

** Total = P1 + P100.

To further characterize the nature of pp60src interaction with cellular components, the P1 and P100 fractions were analyzed by centrifugation through discontinuous sucrose gradients. The distribution of various subcellular organelles on these gradients has been well characterized by several investigators (13, 29, 30). In agreement with other studies, the pp60src in the P100 fraction immunoprecipitated by ap60 fractionated in a nearly identical fashion to the plasma membrane marker enzyme activity (Table II). In contrast, the distribution of pp60src in the P1 fraction was significantly different from that of 5'-nucleotidase. Although some of the pp60src in this fraction could be accounted for by plasma membrane contamination, nearly 50% of the pp60src was present in the gradient pellet, where no plasma membrane was observed. In addition, this population of pp60 exhibited autokinase activity (Table II), and thus cannot simply represent inactive, aggregated material. These results demonstrate that at least 25-40% of the total pp60src in the RSV-infected cell is apparently associated with dense cellular structures other than the plasma membrane.

** Extraction of pp60src from P1 and P100 Membrane Fractions

The efficiency of various agents in disrupting the interaction between pp60src and the membrane was investigated. Neither

### Table I

| Protein          | P1   | P100 | S100 |
|------------------|------|------|------|
| % of total activity |     |      |      |
| 5'-Nucleotidase  | 21   | 26   | 55   |
| [3P]pp60src, TBR* | 17   | 80   | 3    |
| [3P]pp60src, ap60 | 42   | 45   | 13   |
| [3P]pp60src, mAb327* | 39   | 45   | 15   |
| IgG kinase, TBR* | 22   | 69   | 9    |
| Autokinase, ap60 | 52   | 44   | 4    |
| [3P]pp60src, TBR* | 24   | 67   | 9    |
| [3P]pp60src, ap60 | 46   | 43   | 11   |

Transformed cells were lysed by Dounce homogenization and fractionated by differential centrifugation into a 1,000-g pellet (P1) and a 100,000-g pellet (P100) and supernatant (S100). Each fraction was analyzed for protein, plasma membrane marker enzyme 5'-nucleotidase, pp60src, and pp60src kinase activity as described under Materials and Methods. All experiments were with SR-D-1T cells, unless noted. Recoveries were from 65-95%, based on the total pp60src detected in the crude homogenate. Each data point represents the average of two to five separate determinations; standard deviations were ±1-5%.

* The same results were obtained using two different TBR sera.

Monoclonal antibody No. 327 (35).

* Total calculated for ap60 immunoprecipitable [32P]pp60src in P1 and P100 fractions separately.

Membrane suspensions in 20% sucrose from unlabeled 1T cells were fractionated through a 50% sucrose cushion (see Materials and Methods) and assayed for autokinase activity with op60 serum.

A

B

![Figure 2](image)
TABLE III

| Extraction conditions | Pellet | Supernatant | Pellet | Supernatant |
|-----------------------|--------|-------------|--------|-------------|
| Control               | 78     | 22          | 96     | 4           |
| 10 mM EDTA            | 94     | 6           | 95     | 5           |
| NaCl                  | 72     | 28          | 88     | 12          |
| Nonidet P-40, 1%      | 23     | 77          | 81     | 19          |
| Deoxycholate, 1%      | ND     | ND          | 3      | 97          |
| DNase, 1 mg/ml        | 87     | 13          | ND     | ND          |

Membrane suspensions from 32P-labeled 1T cells were incubated with the indicated reagent for 30 min at 0°C, separated into pellet and supernatant fractions (see Materials and Methods), and immunoprecipitated. ND, not determined.

* P1 fraction: 0.2 M NaCl (higher salt concentrations disrupted the integrity of the nuclei); P100 fraction: 0.6 M NaCl.

Subcellular Distribution of pp60v-src

Very little information is currently available concerning the function of the normal cellular homologue of pp60v-src, designated pp60v-sr. It was therefore interesting to determine whether ap60 serum could be utilized to study pp60v-src. Immunoprecipitation of [32P]phosphate-labeled, uninfected CEFs with ap60 serum revealed a phosphoprotein of ~61,000 mol wt. The one-dimensional V8 proteolytic maps of this phosphoprotein were nearly identical to those generated by proteolysis of pp60v-src, with the exception that the carboxy-terminal fragment was slightly larger than the corresponding v-src fragment, as expected based on the predicted amino acid sequence (43). Upon addition of [γ-32P]ATP to the immune complex, autophosphorylation of pp60v-src occurred on tyrosine in the carboxy-terminal half of the molecule (data not shown). Based on this evidence, we conclude that ap60 serum immunoprecipitates enzymatically active pp60v-src.

Normal, uninfected CEFs were fractionated using a procedure identical to that utilized for SR-transformed cells. The IgG kinase activity (monitored with a cross-reacting TBR serum [XR-TBR]) was associated predominantly with the P100 fraction (Table IV, Fig. 4), in agreement with the results of Courtenedge et al. (13). In contrast, the majority of the autokinase activity (using ap60 serum) fractionated with the P1 fraction in a distinctly different fashion from the plasma membrane marker enzyme. Therefore pp60v-src, like its viral counterpart, also interacts with intracellular membranous structures other than the plasma membrane.

DISCUSSION

In this article, we have characterized the interaction of pp60v-src-specific antisera with pp60v-src from avian and mammalian RSV-infected cells. In cell lysates preadsorbed with excess TBR serum, we have detected an additional population of pp60v-src molecules that react only with ap60 serum (Fig. 1). This phenomenon was also observed with IgG and autokinase activities and in SR-CEFs and IT cells. The one-dimensional peptide maps of pp60v-src immunoprecipitated by TBR and ap60 were identical, as were the extents of phosphorylation of the N- and C-terminal fragments (data not shown). Thus there appears to be more than one immunoreactive pool of pp60v-src within the infected cell. We were interested in investigating the intracellular location of this
FIGURE 3  Indirect immunofluorescent localization of pp60 \textsuperscript{src} in uninfected and SR-RSV infected cells. All cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, reacted with primary antibody followed by reaction with fluorescein-conjugated goat anti-rabbit IgG second antibody. (a) Uninfected CEFs, nonimmune serum; (b) uninfected CEFs, ap60 serum; (c) SR-A-infected CEFs, nonimmune serum; (d and e) SR-A-infected CEFs, ap60 serum; (f) 72-4-infected CEFs grown at 41°C, ap60 serum; (g) 72-4 CEFs, temperature shifted to 35°C for 23 h, ap60 serum; (h) SR-A-infected CEFs, ap60 serum preadsorbed with 2 \(\mu\)g of p60 (purified from recombinant E. coli); (i and j) SR-D-transformed 1T cells, ap60 serum. All cells were viewed through a \(\times 100\) oil-immersion objective and photographed on Kodak Tri-X film. Exposure times for panels a-c and h were two to three times longer than the exposures for the remainder of the panels.
analyzed for the presence of pp60 src kinase activity. Recoveries were from 85 to 90%. The same results were obtained with three different pp60 sera from three different rabbits.

Normal uninfected CEFs were fractionated as described under Table I and analyzed for the presence of pp60 src kinase activity. Recoveries were from 85 to 90%. The same results were obtained with three different pp60 sera from three different rabbits. XR-TBR, cross-reacting TBR serum.

|                          | P1   | P100  | S100  |
|--------------------------|------|-------|-------|
|                          | % of total activity |
| 5′-Nucleotidase          | 34   | 63    | 3     |
| IgG kinase:XR-TBR        | 28   | 54    | 18    |
| Autokinase:pp60          | 63   | 24    | 13    |

Normal uninfected CEFs were lysed by Dounce homogenization using a procedure identical to that used for transformed cells. Each fraction was immunoprecipitated, and the immune complexes were assayed for IgG and autokinase activities and analyzed by SDS gel electrophoresis and autoradiography. Lane 1, P1 fraction, pp60 serum; lane 2, P100 fraction, pp60 serum; lane 3, S100 fraction, pp60 serum; lane 4, P1 fraction, XR-TBR serum; lane 5, P100 fraction, XR-TBR serum; lane 6, S100 fraction, XR-TBR serum; lane 7, cell homogenate equivalent to 10% of the total, nonimmune serum.

The results of subcellular fractionation analyses clearly demonstrate that more pp60 src fractionated with the P1 (nuclear) fraction of RSV-transformed CEFs and ITs than could be accounted for by plasma membrane contamination (Tables I and II). An exclusive plasma membrane localization for the marker enzyme 5′-nucleotidase activity was verified by showing that all of the 5′-nucleotidase activity in the cell was expressed on the cell surface (see Materials and Methods). The possibility that the P1 association of pp60 src was an artifact of cell lysis was unlikely in that a predominantly plasma membrane fractionation pattern was obtained when the same preparations were immunoprecipitated with TBR serum. The P1 pp60 src is probably not simply a large aggregate of inactive protein because it retained autokinase activity (Table I and II), and it could be released from the P1 fraction by mild detergent treatment (Table III). Finally, the presence of pp60 src in intracellular structures was confirmed by indirect immunofluorescent microscopy, and was shown to be transformation specific (Fig. 3). Thus we have demonstrated the association of pp60 src with both the plasma membrane and perinuclear membrane structures in both virus-permissive (CEF) and nonpermissive (IT) RSV-transformed cells.

We have also utilized pp60 src to identify the normal cellular protein pp60 src in uninfected chicken embryo fibroblasts. This protein was present in quantities 30–50-fold lower than pp60 src in virally transformed cells. Under the conditions we used to examine SR-A-CEFs (Table I), the contribution of pp60 src would have been negligible. The inability to observe any specific pp60 src immunofluorescent staining pattern in normal CEFs (Fig. 3, a and b) is probably due to the extremely low amounts of pp60 src that preclude detection by this technique. We have been unable to obtain cross-reaction of pp60 src with pp60 src of mammalian cells (Resh, M. D., unpublished observation).

The fact that a significant proportion of the active pp60 src is present in the P1 fraction has interesting implications. One might argue that in a virally transformed cell, the superabundant expression of pp60 src results in saturation of available plasma membrane binding sites, and that the excess pp60 src simply partitions into the other available intracellular membranes. However, the presence of pp60 src, a protein produced in 50-fold lower amounts, in cytoplasmic membranes implies that its localization in this compartment is indeed a genuine phenomenon.

There have been a number of other investigations of the intracellular distribution of pp60 src that use TBR sera and antisera to synthetic peptides. To date, the results obtained have been multifarious, and vary depending on the cell type and the investigator. In chicken embryo fibroblasts and field voles transformed by the wild-type RSV, subcellular fractionation studies support the notion that the majority of the immunoprecipitable pp60 src is associated with the plasma membrane (29, 30). Although a plasma membrane localization of pp60 src is evident in some indirect immunofluorescence micrographs (26), other studies revealed cytoplasmic staining patterns (4, 39) and concentration in adhesion plaques (40). In the mammalian rat kidney cell line transformed by RSV (SR-NRK), Courtneidge et al. (12) have presented biochemical evidence indicative of a predominantly plasma membrane location of pp60 src and pp60 src. However, cytological data also indicates cytoplasmic (47) and perinuclear association (39) with high concentrations in adhesion plaques (20, 36, 40). Finally, in RSV-infected rat cells (RR1022) and goat cells (Pc1), the immunofluorescence and cell fractionation studies are consistent with pp60 src interaction with the nuclear envelope and perinuclear membrane structures (17, 28). It is clear that, based on existing evidence, pp60 src cannot be definitively localized to a single intracellular structure.

With which intracellular organelles does pp60 src interact? Although the P1 fraction prepared from cell homogenates is highly enriched for nuclei, a direct association of pp60 src with the nucleus cannot be inferred from our data. Preparations of purified nuclei did retain ~10% of the total cellular pp60 src, but it is extremely difficult to ascertain whether this is due to retention of nuclear associated membranes. Treatment of nuclei with DNase I did not release the bound pp60 src (Table III), implying that interaction with the DNA was not mediating the pp60 src association. Nonionic detergent did effectively solubilize pp60 src from P1 membranes, and this is consistent with an interaction between pp60 src and either nuclear envelope or perinuclear membrane regions. Finally, an obvious nuclear staining pattern was not evident

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**Table IV**

|                          | P1   | P100  | S100  |
|--------------------------|------|-------|-------|
|                          | % of total activity |
| 5′-Nucleotidase          | 34   | 63    | 3     |
| IgG kinase:XR-TBR        | 28   | 54    | 18    |
| Autokinase:pp60          | 63   | 24    | 13    |

**Figure 4** Subcellular distribution of pp60 src kinase activity. Normal uninfected CEFs were lysed by Dounce homogenization using a procedure identical to that used for transformed cells. Each fraction was immunoprecipitated, and the immune complexes were assayed for IgG and autokinase activities and analyzed by SDS gel electrophoresis and autoradiography. Lane 1, P1 fraction, pp60 src serum; lane 2, P100 fraction, pp60 src serum; lane 3, S100 fraction, pp60 src serum; lane 4, P1 fraction, XR-TBR serum; lane 5, P100 fraction, XR-TBR serum; lane 6, S100 fraction, XR-TBR serum; lane 7, cell homogenate equivalent to 10% of the total, nonimmune serum.
in indirect immunofluorescence; rather, a distinct perinuclear fluorescence was observed (Fig. 3). This pattern is similar to observations made by Krueger and co-workers (17, 28), who reported pp60^src association with nuclear envelope and perinuclear membranous structures (possibly endoplasmic reticulum) in R1022 rat cells and in CEFs infected with virus rescued from R1022 cells. However, in these cells, the perinuclear localization was attributed to an alteration in the amino-terminal primary sequence of the pp60^src. We detected no such alteration in the pp60^src expressed in our cell system. Thus we conclude that the interaction of pp60^src with intracellular membranes, as well as plasma membrane, is a transformation-specific characteristic of wild-type SR-RSV infected cells.

It is obviously of interest to definitively identify the intracellular structures interacting with pp60^src. Subcellular fractionation methods are limited in that many organelles co-fractionate in density-gradient separation methods. The likely possibilities for the structures we have detected in the P1 fraction are the nuclear envelope, rough endoplasmic reticulum, and Golgi apparatus. It is curious to note the similarity between our immunofluorescence patterns and those recently obtained by staining with antibody to a Golgi protein (6). The fluorescent staining pattern with rhodamine-conjugated wheat germ agglutinin, a lectin reported to stain specifically the Golgi apparatus (45) closely resembles the pp60^src staining pattern in double labeled cells (Resh, M. D., unpublished observation). However, the specificity of this lectin for the Golgi complex in CEFs has not yet been established, and ultimately rigorous identification of these structures must rely on electron microscopic examination.

Does the P1 pp60^src represent newly synthesized protein? It has been shown that pp60^src is synthesized on free polymers in the cytoplasm (32), and is then transported to the membrane presumably in a soluble cytoplasmic complex with two cellular proteins, pp50 and pp90 (5, 12). These investigators have demonstrated that, while bound in the complex, pp60^src does not exhibit tyrosine kinase activity and is not phosphorylated on the carboxy-terminal domain. In contrast, the pp60^src detected here in the P1 fraction possesses kinase activity and is phosphorylated in the carboxy-terminal domain (Table I). It is curious to note the similarity between our immunofluorescence patterns and those recently obtained by staining with antibody to a Golgi protein (6). The fluorescent staining pattern with rhodamine-conjugated wheat germ agglutinin, a lectin reported to stain specifically the Golgi apparatus (45) closely resembles the pp60^src staining pattern in double labeled cells (Resh, M. D., unpublished observation). However, the specificity of this lectin for the Golgi complex in CEFs has not yet been established, and ultimately rigorous identification of these structures must rely on electron microscopic examination.

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