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

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ABSTRACT Antiserum to the Rous sarcoma virus (RSV)-transforming protein, $pp60^{v\text{-}src}$, was produced in rabbits immunized with p60 expressed in *Escherichia coli*. αp60 serum immunoprecipitated quantitatively more $pp60^{v\text{-}src}$ than did tumor-bearing rabbit (TBR) sera. When RSV-transformed cell lysates were preadsorbed with TBR serum, the remaining lysate contained additional $pp60^{v\text{-}src}$, which was recognized only by reimmunoprecipitation with αp60 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 $pp60^{v\text{-}src}$ was associated with the plasma membrane-enriched P100 fraction. However, αp60 serum revealed equal distribution of $pp60^{v\text{-}src}$ and its kinase activity between the P1 (nuclear) and P100 fractions. The same results were obtained for $pp60^{c\text{-}src}$ in uninfected CEFs. On discontinuous sucrose gradients nearly 50% of the P1-$pp60^{v\text{-}src}$ sedimented with nuclei, in fractions where no plasma membrane was detected. Indirect immunofluorescence microscopy of RSV-CEFs with αp60 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 $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ 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)[1] 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, $pp60^{v\text{-}src}$ (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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ and its associated kinase activity is associated with the plasma membrane (13, 29, 30). Based on immunocytochemical micrographs, $pp60^{v\text{-}src}$ has been localized to the cytoplasmic surface of the plasma membrane (47), with par-

[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; NRK, normal rat kidney cell line; 1T, Rous sarcoma virus-transformed field vole cell line; RSV, Rous sarcoma virus; RIPA, radioimmunoprecipitation assay; SR, Schmidt-Ruppin strain; 72-4, temperature-sensitive mutant of Rous sarcoma virus; TBR, tumor-bearing rabbit.

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ticularly 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ polypeptide in the infected cell is entirely due to the availability of antisera prepared from tumor-bearing rabbits (TBRs) 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 antiserum (22) (αp60) in rabbits immunized with $p60^{src}$ expressed in *E. coli* (23). We report here that this antiserum is highly specific for the amino-terminal portion of $pp60^{v\text{-}src}$. 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 $pp60^{v\text{-}src}$ 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 $pp60^{v\text{-}src}$ in the infected cell is associated with perinuclear membranous structures. The interaction of $pp60^{v\text{-}src}$ with intracellular membranes is apparent in both virus permissive (avian) and nonpermissive (mammalian) RSV-infected cells probed with αp60 serum. Furthermore, we now report that αp60 serum immunoprecipitates the normal cellular homologue of $pp60^{v\text{-}src}$ from uninfected CEFs, $pp60^{c\text{-}src}$, and that a substantial fraction of $pp60^{c\text{-}src}$ is also associated with intracellular membranes. These results imply that $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ should not be designated exclusively as plasma membrane proteins and suggest that we reconsider the possibility that $pp60^{v\text{-}src}$ 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 Schmidt–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).

Confluent cultures in 100-mm dishes were preincubated for 1 h in either phosphate-free or methionine-free medium. Cells were then radiolabeled with either 250–500 μCi/ml [^{32}P]orthophosphate (carrier-free, New England Nuclear, Boston, MA) or 100 μCi/ml [^{35}S]methionine (1,000 Ci/mmol, New England Nuclear) for 2–3 h in the appropriate above medium (37°C, 1T; 41°C, CEF). Identical results were obtained when cells were radiolabeled for 12 h.

Antisera: TBR antisera and antiserum from a rabbit immunized with bacterially produced p60 were used as described previously (3, 22). Monoclonal antibody to $pp60^{v\text{-}src}$ (No. 327) (35) and a cross-reacting TBR antiserum were kind gifts of Dr. J. Brugge, State University of New York, Stony Brook.

Subcellular Fractionation: Culture dishes were rinsed twice with STE buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.2) and pipetted (for 1T) or scraped off (for CEF) the dish with STE buffer. Cells were collected by low-speed centrifugation and resuspended in hypotonic buffer (10 mM Tris, 0.2 mM $MgCl_2$, pH 7.4) (0.8 ml per 100-mm dish). After a 10–15 min 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 respun at 1,000 *g* for 10 min, 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 Ty 65 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was removed and denoted S100; the resultant pellet was denoted P100.

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 Courtneidge et al. (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 recentrifuged at 100,000 *g* for 30 min. Each pellet was then solubilized and immunoprecipitated as described below.

Centrifugation through a sucrose cushion was also used to separate membranes on a simpler level. The P1 and P100 membrane fractions were suspended in 1.0 ml 20% sucrose/TE buffer, layered over 4.0 ml 50% sucrose/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 $MgCl_2$, and incubated with the appropriate extraction agent in a total volume of 500 μl 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 reisolated 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 αp60 serum (22) under conditions of antibody excess and analyzed by PAGE (31) on 10% polyacrylamide gels. The amount of radiolabel 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 $pp60^{v\text{-}src}$ 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% Na deoxycholate, 10 mM Tris, 1 mM EDTA, clarified, and immunoprecipitated with TBR and αp60 sera. The final wash was in 150 mM NaCl, 10 mM Tris, pH 7.4. Staph pellets were resuspended in 10 mM Tris, 5 mM $MgCl_2$, 20 μCi [γ-^{32}P]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 1:100 dilution of either nonimmune rabbit serum or αp60 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 × 75 microscope slides, and sealed with nail polish. Cells were observed through a × 100 oil-immersion objective on a Leitz microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epi-fluorescent illumination, and

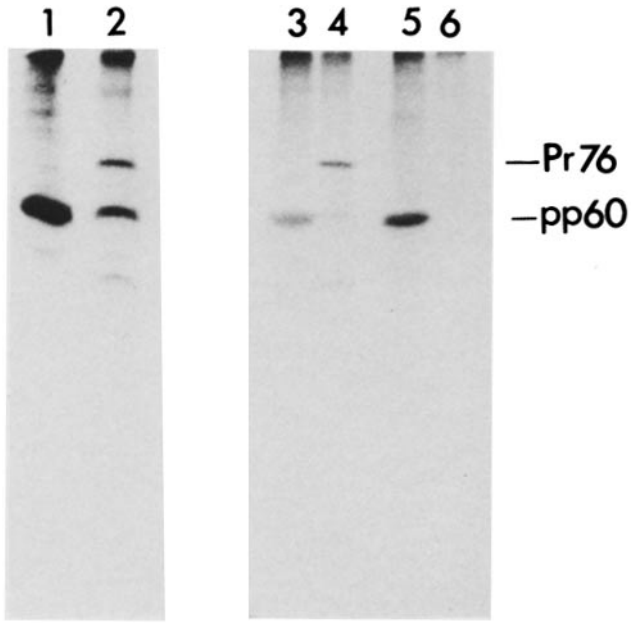

FIGURE 1 Reimmunoprecipitation of TBR- or αp60-adsorbed cell lysates. [^{32}P]phosphate-labeled 1T cell lysates in RIPA buffer were reacted with either αp60 or TBR serum under conditions of antibody excess. The immune complexes were collected by adsorption to *S. aureus*, and the supernatants from the first two washes were saved. These supernatant fractions were divided into two aliquots and reimmunoprecipitated with either αp60 or TBR serum. All of the immune complexes were washed with RIPA buffer and analyzed by SDS PAGE and autoradiography. Lane *1*, immunoprecipitation with αp60 serum alone; lane *2*, immunoprecipitation with TBR serum alone; lane *3*, reimmunoprecipitation of αp60-adsorbed lysate (from lane *1*) with αp60; lane *4*, reimmunoprecipitation of αp60-adsorbed lysate (from lane *1*) with TBR serum; lane *5*, re-immunoprecipitation of TBR-adsorbed lysate (from lane *2*) with αp60 serum; lane 6, re-immunoprecipitation of TBR-adsorbed lysate (from lane *2*) with TBR serum. The dried gel was exposed to Kodak XAR film for 6 h at −70°C with Cronex intensifying screen. The total amount of pp60$^{v\text{-}src}$ immunoprecipitated by αp60 serum was 3.8 times higher than that immunoprecipitated by TBR serum. The amount of pp60 recovered in lane *5* was 16 times greater than that in lane 6. The same results were obtained with two different TBR sera.

were photographed with Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) using an automatic exposure meter.

Other Assays: Protein was assayed by the method of Bradford (2). 5′-nucleotidase activity was monitored as described by Avruch and Wallach (1). To verify that this enzyme is a specific marker for plasma membrane, 5′-nucleotidase activity was measured in whole cell suspensions (in iso-osmotic sucrose) before and after Dounce homogenization. Because 5′-nucleotidase is an "ectoenzyme" with its active site exposed on the outside of the cell (44), all of the 5′-nucleotidase activity should be expressed when whole cells are assayed. In accordance with this, no additional 5′-nucleotidase activity was detected when cell homogenates were assayed and compared with unbroken cell activity.

RESULTS

Characterization of pp60$^{v\text{-}src}$ Population Immunoprecipitated by αp60 Antibody

Expression of p60^{src} in bacterial recombinants containing a lac-src plasmid (23) has facilitated the production of high-titer antibody to pp60$^{v\text{-}src}$ from a variety of RSV strains (22). This antibody, denoted αp60, differs from antisera raised in TBRs in its ability to recognize denatured antigen and the apparent lack of transfer of phosphate from [γ-^{32}P]ATP to the heavy chain of αp60 IgG. When immune complexes of pp60$^{v\text{-}src}$ immunoprecipitated with αp60 serum were incubated with [γ-^{32}P]ATP, a phosphopeptide of 60,000 mol wt was observed. This 60,000-mol-wt band was identified as autophosphorylated pp60$^{v\text{-}src}$, in that it was absent in phosphotransfer reactions using nonimmune complexes (data not shown). This reaction will be denoted as "autokinase" activity to distinguish it from the IgG kinase activity observed with TBR sera. These results demonstrate that αp60 serum can immunoprecipitate enzymatically active pp60$^{v\text{-}src}$.

During these studies it became apparent that αp60 serum immunoprecipitated quantitatively (1.5–4 times) more pp60$^{v\text{-}src}$ than did TBR serum, even under conditions of antibody excess. In light of these results, it was of interest to determine whether αp60 recognized a population of pp60$^{v\text{-}src}$ molecules previously undetected by TBR sera. Accordingly, cell lysates were immunoprecipitated with either αp60 or TBR serum, the immune complexes were collected by adsorption to *Staphylococcus aureus*, and the remaining supernatants were reimmunoprecipitated with αp60 or TBR. As can be seen in Fig. 1, in lysates previously reacted with αp60, a second αp60 immunoprecipitation brought down an additional amount of pp60$^{v\text{-}src}$ (~10–15% of the total) (lane *3*), whereas very little pp60$^{v\text{-}src}$ 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 pp60$^{v\text{-}src}$ molecules which were recognized by reimmunoprecipitation with αp60 (lane *5*) but not by TBR (lane *6*). The same results were obtained with [^{35}S]methionine and [^{32}P]orthophosphate-labeled cells, with IgG and autokinase activities, and in mammalian and avian RSV-infected cells. Thus there exists a population of pp60$^{v\text{-}src}$ molecules that are immunoreactive toward αp60 sera but are not recognized by TBR sera.

Subcellular Distribution of pp60$^{v\text{-}src}$

Based on the ability of αp60 antisera to immunoprecipitate additional pp60$^{v\text{-}src}$ differentially, it was of interest to determine whether this population was plasma membrane-associated, as had been demonstrated for the pp60$^{v\text{-}src}$ 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 membraneous 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 pp60$^{v\text{-}src}$ was found associated with the P100 fraction, in agreement with the results of other investigators (13, 29, 30). However, when αp60 antibody was used, a strikingly different pattern emerged. Approximately equal distribution of pp60$^{v\text{-}src}$ was detected in the P1 and P100 fractions (Fig. 2*A*). The amount of pp60$^{v\text{-}src}$ 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 pp60$^{v\text{-}src}$ distribution with a monoclonal antibody

TABLE I

Distribution of pp60$^{v\text{-}src}$ in Subcellular Fractions

	P1	P100	S100
	% of total activity		
Protein	21	26	55
5′-Nucleotidase	17	80	3
[^{32}P]pp60$^{v\text{-}src$:TBR*	10	61	28
[^{32}P]pp60$^{v\text{-}src$:αp60	42	45	13
[^{32}P]pp60$^{v\text{-}src$:mAb327‡	39	45	15
IgG kinase:TBR*	22	69	8
Autokinase:αp60	52	44	4
[^{32}P]pp60$^{v\text{-}src$:TBR§	24	67	9
[^{32}P]pp60$^{v\text{-}src$:αp60§	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, pp60$^{v\text{-}src}$, and pp60$^{v\text{-}src}$ 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 pp60$^{v\text{-}src}$ 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).

§ SR-A-CEF.

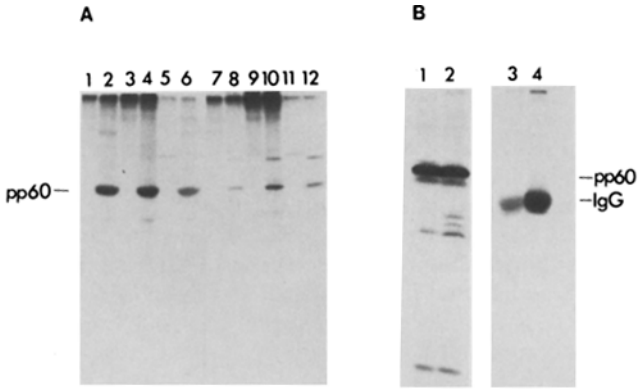

FIGURE 2 Subcellular distribution of pp60$^{v\text{-}src}$ immunoprecipitated with αp60 and TBR antisera. 1T cells were lysed by Dounce homogenization in hypotonic buffer and fractionated by differential centrifugation as described under Materials and Methods. (*A*) Analysis of pp60$^{v\text{-}src}$ in subcellular fractions from [^{32}P]phosphate-labeled cells. Each fraction was adjusted to RIPA buffer, immunoprecipitated with nonimmune rabbit serum (lanes *1*, *3*, *5*, *7*, *9*, and *11*), αp60 serum (lanes *2*, *4*, and *6*), or TBR serum (lanes *8*, *10*, and *12*), and analyzed by SDS-PAGE and autoradiography. Lanes *1*, *2*, *7*, and *8*, P1 fraction. Lanes *3*, *4*, *9*, and *10*, P100 fraction. Lanes *5*, *6*, *11*, and *12*, S100 fraction. (*B*) Autokinase and IgG kinase activities of membrane fractions. The P1 and P100 fractions were reacted with αp60 or TBR sera and the immune complexes were incubated with [γ-^{32}P]ATP. The reaction products were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane *1*, P1 fraction; αp60 antiserum; lane *2*, P100 fraction, αp60 antiserum; lane *3*, P1 fraction TBR serum; lane *4*, P100 fraction, TBR serum. The gels were exposed to Kodak XAR film at −70°C with Cronex intensifying screen for 2 h (lanes *1* and *2*) or 15 min (lanes *3* and *4*).

(35). Moreover, the presence of additional αp60-reactive pp60$^{v\text{-}src}$ in the P1 fraction was detected whether the cells were labeled with [^{32}P]phosphate or [^{35}S]methionine, and with both mammalian and avian RSV-infected cells. Identical results were obtained when IgG and autokinase activities were measured (Fig. 2*B*). No gross biochemical differences between the pp60$^{v\text{-}src}$ molecules in the P1 and P100 fractions were observed, based on one-dimensional V8 proteolytic maps. The molecular weights of the V8 fragments and the extent of amino- and carboxy-terminal phosphorylation were not appreciably different. No immunoprecipitable pp60$^{v\text{-}src}$ was detected in subcellular fractions of normal, uninfected vole cells. Thus, immunoprecipitation of pp60$^{v\text{-}src}$ with a highly specific antibody reveals the presence of additional active pp60 in the P1 (nuclear) fraction of RSV-infected cells, which had previously been undetected by TBR antisera.

TABLE II

Sucrose Gradient Distribution of pp60$^{v\text{-}src}$

Gradient location	0/20	20/35	35/40	40/50	Pellet
	% of total activity*				
P1: [^{32}P]pp60$^{v\text{-}src}$	0	34	11	11	44
P1: 5′-nucleotidase	0	18	81	<1	0
P100: [^{32}P]pp60$^{v\text{-}src}$	0	56	30	13	<1
P100: 5′-nucleotidase	<1	66	30	4	<1

	Autokinase activity (% of total)‡,§	
	20/50	Pellet
	%	
P1: pp60 kinase	12	32
P1: 5′-nucleotidase	9	<1
P100: pp60 kinase	47	9
P100: 5′-nucleotidase	90	<1

P1 and P100 fractions from 1T cells were fractionated over discontinuous sucrose gradients, and the amount of αp60-immunoprecipitable pp60$^{v\text{-}src}$ (*top*) and pp60$^{v\text{-}src}$ autokinase activity (*bottom*) was quantitated as described under Materials and Methods.

* Total calculated for αp60 immunoprecipitable [^{32}P]pp60$^{v\text{-}src}$ 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 αp60 serum.

§ Total = P1 + P100.

To further characterize the nature of pp60$^{v\text{-}src}$ 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 pp60$^{v\text{-}src}$ in the P100 fraction immunoprecipitated by αp60 fractionated in a nearly identical fashion to the plasma membrane marker enzyme activity (Table II). In contrast, the distribution of pp60$^{v\text{-}src}$ in the P1 fraction was significantly different from that of 5′-nucleotidase. Although some of the pp60$^{v\text{-}src}$ in this fraction could be accounted for by plasma membrane contamination, nearly 50% of the pp60$^{v\text{-}src}$ 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 pp60$^{v\text{-}src}$ in the RSV-infected cell is apparently associated with dense cellular structures other than the plasma membrane.

Extraction of pp60$^{v\text{-}src}$ from P1 and P100 Membrane Fractions

The efficiency of various agents in disrupting the interaction between pp60$^{v\text{-}src}$ and the membrane was investigated. Neither

TABLE III

Extraction of pp60$^{v\text{-}src}$ from P1 and P100 Membrane Fractions

Extraction conditions	P1		P100	
	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		3	97
DNase, 1 mg/ml	87	13	ND	

Membrane suspensions from ^{32}P-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.

treatment with EDTA nor high salt released pp60$^{v\text{-}src}$ from the P1 and P100 fractions (Table III), implying that ionic interactions were not solely responsible for pp60$^{v\text{-}src}$ association with either fraction. Extraction of pp60 from the P1 fraction was achieved by treatment with nonionic detergent (NP-40), whereas ionic detergent (deoxycholate) was required for effective solubilization of pp60$^{v\text{-}src}$ from the P100 fraction (Table III). The pp60$^{v\text{-}src}$ is apparently associated with the nuclear, P1 fraction of the cell through hydrophobic interactions, and is not simply nonspecifically adsorbed to it.

Preparation of Purified Nuclei

Because the P1 fraction of the cell is enriched for nuclei, it was logical to question whether any pp60$^{v\text{-}src}$ could be detected when nuclei were purified by more rigorous procedures. Nuclei were prepared from 1T cell homogenates by treatment with nonionic detergent and centrifugation through 2 M sucrose (30). Approximately 10% of the total pp60$^{v\text{-}src}$ recovered was present in the sucrose gradient-purified nuclei (data not shown). However, the absolute recovery of pp60$^{v\text{-}src}$ from all of the fractions was only 30% of the total p60 present in the starting cell homogenate. Further experimentation revealed that the conditions used to prepare nuclei (detergent and divalent cations) were particularly conducive to proteolytic degradation of p60. Moreover, the use of detergent, in removing contaminating membranes that stick to nuclei, also strips away the nuclear envelope and other nuclear-associated membrane components. Therefore, a less disruptive method was utilized to examine the intracellular distribution of pp60$^{v\text{-}src}$.

Immunocytochemical Localization of pp60$^{v\text{-}src}$

The distribution of αp60-reactive pp60$^{v\text{-}src}$ was probed by indirect immunofluorescence. The antibody staining patterns of uninfected (normal), SR-A-infected, and temperature-sensitive mutant (72-4)-infected CEFs as well as SR-D-transformed 1T cells were examined. The results of the immunofluorescent analyses are illustrated in Fig. 3. Staining of uninfected CEFs with nonimmune and αp60 sera (*a* and *b*) and of SR-A-transformed CEFs with nonimmune serum (*c*) was faint and diffuse. In contrast, αp60 serum yielded a distinct pattern of perinuclear fluorescence (*d* and *e*), which was especially prominent in cells where the nucleus was clearly visible. No staining of the nucleus itself was apparent. Plasma membrane fluorescence was most evident in regions of cell–cell contact. At the nonpermissive temperature (41°C), the 72-4-infected cells displayed a uniform staining pattern throughout the cytoplasm (*f*), in agreement with the results of Garber et al. (19). Within 24 h after shifting to the permissive temperature (35°C), 72-4 cells exhibited the distinct perinuclear fluorescence staining observed with wild-type SR-A-infected cells (*g*). The fluorescence pattern was abolished when the αp60 serum was preadsorbed with excess p60 (*h*). SR-D 1T cell staining with αp60 serum was especially prominent at the cell periphery and was particularly intense in regions of cell–cell contact (*i* and *j*). Although some staining of the interior perinuclear region of the cell was evident, it was difficult to focus on due to the extreme rounding of the cells. No specific staining of unpermeabilized cells was observed (not shown). Thus, the immunocytochemical evidence confirms the biochemical data indicating the presence of pp60$^{v\text{-}src}$ in membrane associated perinuclear structures.

Subcellular Distribution of pp60$^{c\text{-}src}$

Very little information is currently available concerning the function of the normal cellular homologue of pp60$^{v\text{-}src}$, denoted pp60$^{c\text{-}src}$. It was therefore interesting to determine whether αp60 serum could be utilized to study pp60$^{c\text{-}src}$. Immunoprecipitation of [^{32}P]phosphate-labeled, uninfected CEFs with αp60 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 pp60$^{v\text{-}src}$, 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-^{32}P]ATP to the immune complex, autophosphorylation of pp60$^{c\text{-}src}$ occurred on tyrosine in the carboxy-terminal half of the molecule (data not shown). Based on this evidence, we conclude that αp60 serum immunoprecipitates enzymatically active pp60$^{c\text{-}src}$.

Normal, uninfected CEFs were fractioned 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 Courtneidge et al. (13). In contrast, the majority of the autokinase activity (using αp60 serum) fractionated with the P1 fraction in a distinctly different fashion from the plasma membrane marker enzyme. Therefore pp60$^{c\text{-}src}$, 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 pp60$^{v\text{-}src}$-specific antiserum with pp60$^{v\text{-}src}$ from avian and mammalian RSV-infected cells. In cell lysates preadsorbed with excess TBR serum, we have detected an additional population of pp60$^{v\text{-}src}$ molecules that react only with αp60 serum (Fig. 1). This phenomenon was also observed with IgG and autokinase activities and in SR-CEFs and 1T cells. The one-dimensional peptide maps of pp60$^{v\text{-}src}$ immunoprecipitated by TBR and αp60 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 pp60$^{v\text{-}src}$ within the infected cell. We were interested in investigating the intracellular location of this

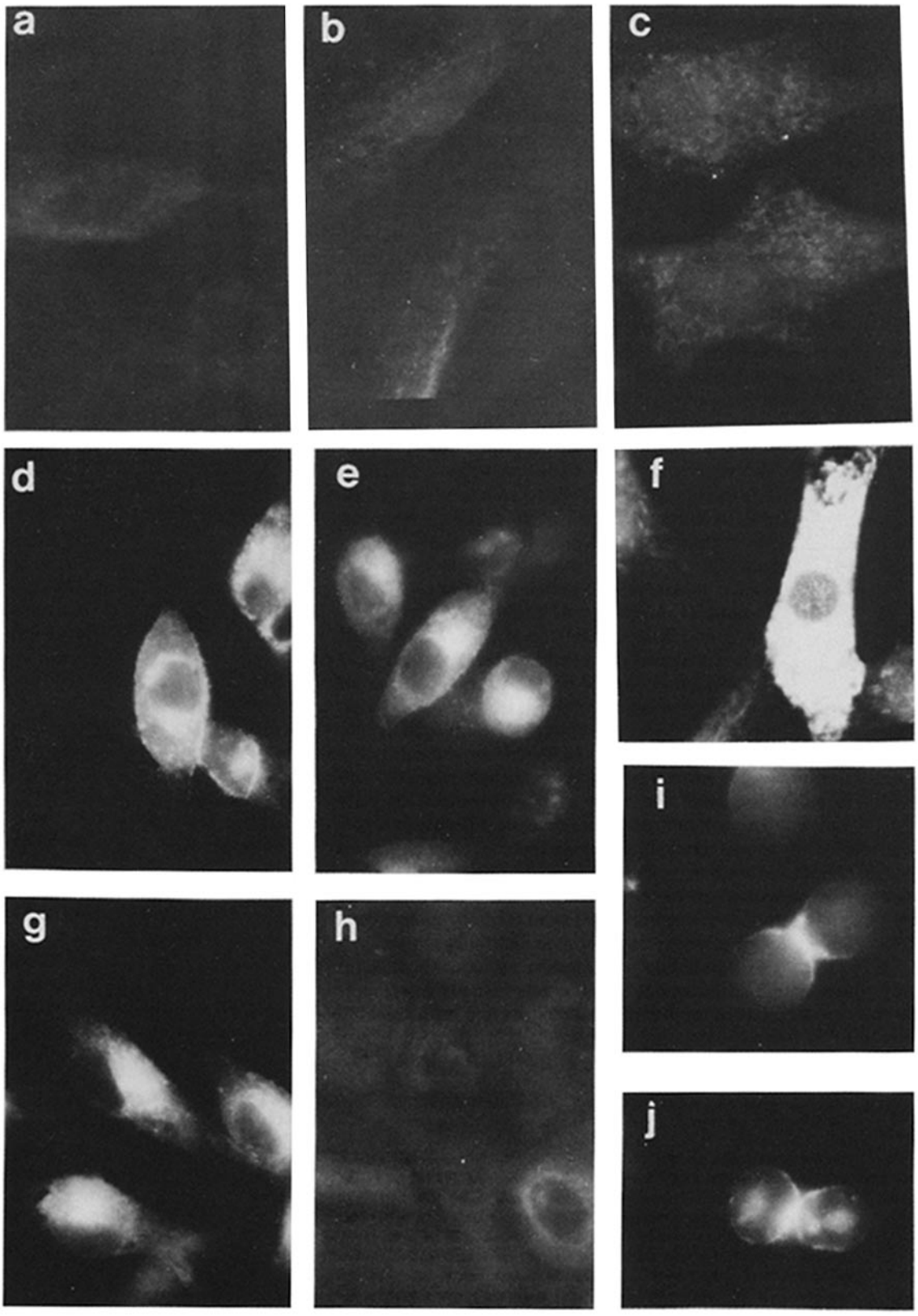

FIGURE 3 Indirect immunofluorescent localization of $pp60^{v\text{-}src}$ in uninfected and SR-RSV infected cells. All cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, reacted with primary antibody followed by reaction with fluorescein-conjugated goat anti-rabbit IgG second antibody. (*a*) Uninfected CEFs, nonimmune serum; (*b*) uninfected CEFs, αp60 serum; (*c*) SR-A-infected CEFs, nonimmune serum; (*d* and *e*) SR-A-infected CEFs, αp60 serum; (*f*) 72-4-infected CEFs grown at 41°C, αp60 serum; (*g*) 72-4 CEFs, temperature shifted to 35°C for 23 h, αp60 serum; (*h*) SR-A-infected CEFs, αp60 serum preadsorbed with 2 μg of p60 (purified from recombinant *E. coli*); (*i* and *j*) SR-D-transformed 1T cells, αp60 serum. All cells were viewed through a × 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.

TABLE IV

Distribution of pp60$^{c\text{-}src}$ in Subcellular Fractions

	P1	P100	S100
	% *of total activity*		
5′-Nucleotidase	34	63	3
IgG kinase:XR-TBR	28	54	18
Autokinase:αp60	63	24	13

Normal uninfected CEFs were fractionated as described under Table I and analyzed for the presence of pp60$^{c\text{-}src}$ kinase activity. Recoveries were from 85 to 90%. The same results were obtained with three different αp60 sera from three different rabbits. *XR-TBR*, cross-reacting TBR serum.

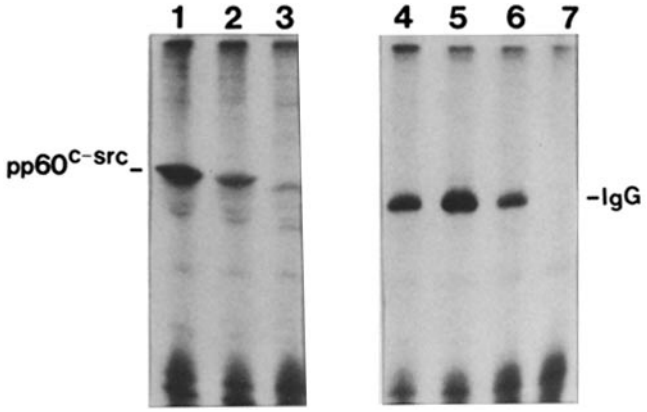

FIGURE 4 Subcellular distribution of pp60$^{c\text{-}src}$ kinase activity. Normal uninfected CEFs were lysed by Dounce homogenization using a procedure identical to that used for transformed cells. Each fraction was immunoprecipitated, and the immune complexes were assayed for IgG and autokinase activities and analyzed by SDS gel electrophoresis and autoradiography. Lane *1*, P1 fraction, αp60 serum; lane *2*, P100 fraction, αp60 serum; lane *3*, S100 fraction, αp60 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.

novel population of pp60$^{v\text{-}src}$. In particular, we wanted to determine if most of the αp60-immunoprecipitable pp60$^{v\text{-}src}$ was associated with the plasma membrane, as other investigators using TBR sera had claimed.

The results of subcellular fractionation analyses clearly demonstrated that more pp60$^{v\text{-}src}$ fractionated with the P1 (nuclear) fraction of RSV-transformed CEFs and 1Ts 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$^{v\text{-}src}$ 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$^{v\text{-}src}$ 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$^{v\text{-}src}$ 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$^{v\text{-}src}$ with both the plasma membrane and perinuclear membrane structures in both virus-permissive (CEF) and nonpermissive (1T) RSV-transformed cells.

We have also utilized αp60 serum to identify the normal cellular protein pp60$^{c\text{-}src}$ in uninfected chicken embryo fibroblasts. This protein was present in quantities 30–50-fold lower than pp60$^{v\text{-}src}$ in virally transformed cells. Under the conditions we used to examine SR-A–CEFs (Table I), the contribution of pp60$^{c\text{-}src}$ would have been negligible. The inability to observe any specific αp60 immunofluorescent staining pattern in normal CEFs (Fig. 3, *a* and *b*) is probably due to the extremely low amounts of pp60$^{c\text{-}src}$ that preclude detection by this technique. We have been unable to obtain cross-reaction of αp60 with pp60$^{c\text{-}src}$ of mammalian cells (Resh, M. D., unpublished observation).

The fact that a significant proportion of the active pp60$^{c\text{-}src}$ is present in the P1 fraction has interesting implications. One might argue that in a virally transformed cell, the superabundant expression of pp60$^{v\text{-}src}$ results in saturation of available plasma membrane binding sites, and that the excess pp60$^{v\text{-}src}$ simply partitions into the other available intracellular membranes. However, the presence of pp60$^{c\text{-}src}$, 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$^{v\text{-}src}$ 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 vole cells transformed by the wild-type RSV, subcellular fractionation studies support the notion that the majority of the immunoprecipitable pp60$^{v\text{-}src}$ is associated with the plasma membrane (29, 30). Although a plasma membrane localization of pp60$^{v\text{-}src}$ 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$^{v\text{-}src}$ and pp60$^{c\text{-}src}$. 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$^{v\text{-}src}$ interaction with the nuclear envelope and perinuclear membrane structures (17, 28). It is clear that, based on existing evidence, pp60$^{v\text{-}src}$ cannot be definitively localized to a single intracellular structure.

With which intracellular organelles does pp60$^{v\text{-}src}$ interact? Although the P1 fraction prepared from cell homogenates is highly enriched for nuclei, a direct association of pp60$^{v\text{-}src}$ with the nucleus cannot be inferred from our data. Preparations of purified nuclei did retain ~10% of the total cellular pp60$^{v\text{-}src}$, 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$^{v\text{-}src}$ (Table III), implying that interaction with the DNA was not mediating the pp60$^{v\text{-}src}$ association. Nonionic detergent did effectively solubilize pp60$^{v\text{-}src}$ from P1 membranes, and this is consistent with an interaction between pp60 and either nuclear envelope or perinuclear membrane regions. Finally, an obvious nuclear staining pattern was not evident

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$^{v\text{-}src}$ association with nuclear envelope and perinuclear membranous structures (possibly endoplasmic reticulum) in RR1022 rat cells and in CEFs infected with virus rescued from RR1022 cells. However, in these cells, the perinuclear localization was attributed to an alteration in the amino-terminal primary sequence of the pp60$^{v\text{-}src}$. We detected no such alteration in the pp60$^{v\text{-}src}$ expressed in our cell system. Thus we conclude that the interaction of pp60$^{v\text{-}src}$ 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$^{v\text{-}src}$. Subcellular fractionation methods are limited in that many organelles cofractionate 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$^{v\text{-}src}$ 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$^{v\text{-}src}$ represent newly synthesized protein? It has been shown that pp60$^{v\text{-}src}$ is synthesized on free polysomes 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$^{v\text{-}src}$ does not exhibit tyrosine kinase activity and is not phosphorylated on the carboxy-terminal domain. In contrast, the pp60$^{v\text{-}src}$ detected here in the P1 fraction possesses kinase activity and is phosphorylated in the C-terminal region, and is membrane bound. Moreover, no specific association of pp50 or pp90 was detected in the P1 or P100 membrane fractions using αp60 serum. Thus it is unlikely that all of the P1 material represents newly synthesized protein associated with the pp50:pp90 complex. It is probable, however, that the pp60$^{v\text{-}src}$ detected in the S100 fraction (Table I) is associated with this macromolecular complex, because it does not exhibit significant kinase activity (Table I) and is not phosphorylated on the carboxy-terminal 26,000-mol-wt domain (data not shown). The covalent attachment of fatty acid to the amino-terminus of pp60$^{v\text{-}src}$ has been implicated as an obligatory step for the interaction with the plasma membrane (18, 42). Preliminary experiments have revealed myristic acid binding to pp60$^{v\text{-}src}$ in both the P1 and P100 fractions (Resh, M. D., unpublished observation). This confirms the notion that fatty acid attachment occurs at an intracellular site, possibly in the endoplasmic reticulum (46). In conclusion, the association of active pp60$^{v\text{-}src}$ with intracellular membranes of RSV-transformed cells appears to be a genuine phenomenon. In view of the pleiotropic actions of pp60$^{v\text{-}src}$ on cellular processes, it should not be surprising that pp60$^{v\text{-}src}$ is found in multiple cellular locations. In that the membrane association of pp60$^{v\text{-}src}$ plays a vital role in the expression of tumorigenicity of RSV, it is hoped that these studies will aid in elucidating the molecular mechanism of transformation by oncogenic viruses.

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REFERENCES

1. Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta.* 233:334–347.
2. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
3. Brugge, J. S., and R. L. Erikson. 1977. Identification of a transformation specific antigen induced by an avian sarcoma virus. *Nature (Lond.).* 269:346–348.
4. Brugge, J. S., P. J. Steinbaugh, and R. L. Erikson. 1978. Characterization of the avian sarcoma virus protein pp60^{src}. *Virology.* 91:130–140.
5. Brugge, J. S., W. Yonemoto, and D. Darrow. 1983. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. *Mol. Cell. Biol.* 3:9–19.
6. Burke, B., and G. Warren. 1984. Microinjection of mRNA coding for an anti-Golgi antibody inhibits intracellular transport of a viral membrane protein. *Cell.* 36:847–856.
7. Cheng, Y-S. E., and L. B. Chen. 1981. Detection of phosphotyrosine-containing 34,000-dalton protein in the framework of cells transformed with Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 78:2388–2392.
8. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
9. Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma virus src gene product. *Proc. Natl. Acad. Sci. USA.* 75:2021–2024.
10. Collett, M. S., E. Erikson, and R. L. Erikson. 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. *J. Virol.* 29:770–781.
11. Collett, M. S., A. F. Purchio, and R. L. Erikson. 1980. Avian sarcoma virus transforming protein, pp60^{src}, shows protein kinase activity specific for tyrosine. *Nature (Lond).* 285:167–169.
12. Courtneidge, S. A., and J. M. Bishop. 1982. Transit of pp60^{src} to the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 79:7117–7121.
13. Courtneidge, S. A., A. D. Levinson, and J. M. Bishop. 1980. The protein encoded by the transforming gene of avian sarcoma virus and a homologous protein in normal cells are associated with the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 77:3783–3787.
14. Erikson, E., M. S. Collett, and R. L. Erikson. 1978. In vitro synthesis of a functional avian sarcoma virus transforming gene product. *Nature (Lond.).* 274:919–921.
15. Erikson, E., and R. L. Erikson. 1980. Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus-transforming gene product. *Cell.* 21:829–836.
16. Gallis, B., P. Bornstein, and D. L. Brautigan. 1981. Tyrosyl protein kinase and phosphatase activities in membrane vesicles from normal and Rous sarcoma virus-transformed rat cells. *Proc. Natl. Acad. Sci. USA.* 78:6689–6693.
17. Garber, E. A., J. G. Krueger, and A. R. Goldberg. 1982. Novel location of pp60^{src} in Rous sarcoma virus-transformed rat and goat cells and in chicken cells transformed by viruses rescued from these mammalian cells. *Virology.* 118:419–429.
18. Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg. 1983. Only membrane-associated Rous sarcoma virus src proteins have amino-terminally bound lipid. *Nature (Lond.).* 302:161–163.
19. Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg. 1983. Temperature-sensitive membrane association of pp60^{src} in tsNY68-infected cells correlates with increased tyrosine phosphorylation of membrane-associated proteins. *Virology.* 126:73–86.
20. Gentry, L. E., L. R. Rohrschneider, J. E. Casnellie, and E. G. Krebs. 1983. Antibodies to a defined region of pp60^{src} neutralize the tyrosine-specific kinase activity. *J. Biol. Chem.* 258:11219–11228.
21. Gilmer, T. M., and R. L. Erikson. 1981. Rous sarcoma virus transforming protein, p60^{src}, expressed in *E. coli*, functions as a protein kinase. *Nature (Lond.).* 294:771–773.
22. Gilmer, T. M., and R. L. Erikson. 1983. Development of anti-pp60^{src} serum with antigen produced in *Escherichia coli*. *J. Virol.* 45:462–465.
23. Gilmer, T. M., J. T. Parsons, and R. L. Erikson. 1982. Construction of plasmids for expression of Rous sarcoma virus transforming protein, p60^{src}, in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 79:2152–2156.
24. Hanafusa, H. 1977. Cell transformation by RNA tumor viruses. *In* Comprehensive Virology. Vol. 10. Plenum Press, New York. 401–483.
25. Hunter, T., and B. M. Sefton. 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA.* 77:1311–1315.
26. Krueger, J. G., E. A. Garber, and A. R. Goldberg. 1983. Subcellular localization of pp60^{src} in Rous sarcoma virus-transformed cells. *Curr. Top. Microbiol. Immunol.* 107:52–124.

27. Krueger, J. G., E. A. Garber, A. R. Goldberg, and H. Hanafusa. 1982. Changes in amino-terminal sequences of pp60^{src} lead to decreased membrane association and decreased in vivo tumorigenicity. *Cell.* 28:889–896.
28. Krueger, J. G., E. Wang, E. A. Garber, and A. R. Goldberg. 1980. Differences in intracellular location of pp60^{src} in rat and chicken cells transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:4142–4146.
29. Krueger, J. G., E. Wang, and A. R. Goldberg. 1980. Evidence that the src gene product of Rous sarcoma virus is membrane associated. *Virology.* 101:25–40.
30. Krzyek, R. A., R. L. Mitchell, A. F. Lau, and A. J. Faras. 1980. Association of pp60^{src} and src protein kinase activity with the plasma membrane of nonpermissive and permissive avian sarcoma virus-infected cells. *J. Virol.* 36:805–815.
31. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
32. Lee, J. S., H. E. Varmus, and J. M. Bishop. 1979. Virus-specific messenger RNAs in permissive cells infected by avian sarcoma virus. *J. Biol. Chem.* 254:8015–8022.
33. Levinson, A. D., H. E. Oppermann, and J. M. Bishop. 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. *J. Biol. Chem.* 255:11973–11980.
34. Levinson, A. D., H. Opperman, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell.* 15:561–572.
35. Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruss. *J. Virol.* 48:352–360.
36. Nigg, E. A., B. M. Sefton, T. Hunter, G. Walter, and S. J. Singer. 1982. Immunofluorescence localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic src peptide. *Proc. Natl. Acad. Sci. USA.* 79:5322–5326.
37. Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson. 1978. Identification of a polypeptide encoded by the avian sarcoma virus src gene. *Proc. Natl. Acad. Sci. USA.* 75:1567–1571.
38. Purchio, A. F., E. Erikson, and R. L. Erikson. 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. *Proc. Natl. Acad. Sci. USA.* 74:4661–4665.
39. Rohrschneider, L. R. 1979. Immunofluorescence on avian sarcoma virus-transformed cells: localization of the src gene product. *Cell.* 16:11–24.
40. Rohrschneider, L. R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514–3518.
41. Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell.* 24:165–174.
42. Sefton, B. M., I. S. Trowbridge, J. A. Cooper, and E. M. Scolnick. 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. *Cell.* 31:465–474.
43. Takeya, T., R. A. Feldman, and H. Hanafusa. 1982. DNA sequence of the viral and cellular src gene of chickens. *J. Virol.* 44:1–11.
44. Trams, E. G., and C. J. Lauter. 1974. On the sidedness of plasma membrane enzymes. *Biochim. Biophys. Acta.* 345:180–197.
45. Virtanen, I., P. Ekblom, and P. Laurila. 1980. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell. Biol.* 85:429–434.
46. Wen, D., and M. J. Schlessinger. 1984. Fatty acid-acylated proteins in secretory mutants of *Saccharomyces cerevisiae. Mol. Cell. Biol.* 4:688–694.
47. Willingham, M. C., G. Jay, and I. Pastan. 1979. Localization of the avian sarcoma virus src gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. *Cell.* 18:125–134.