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# Differences in intracellular location of pp60$^{src}$ in rat and chicken cells transformed by Rous sarcoma virus

(protein kinase/membrane protein/viral oncogenesis)

JAMES G. KRUEGER, EUGENIA WANG, ELLEN A. GARBER, AND ALLAN R. GOLDBERG

The Rockefeller University, New York, New York 10021

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**ABSTRACT** **We have investigated the intracellular location of pp60$^{src}$ in Rous sarcoma virus-transformed rat cells (RR1022) by indirect immunofluorescence microscopy and cell fractionation. Immunofluorescence data suggest that pp60$^{src}$ is predominantly associated with the nuclear envelope and the juxtanuclear reticular membrane structures. The bulk of pp60$^{src}$ and of the associated phosphotransferase activity fractionated with nuclei and not with plasma membranes in disrupted cells. This localization contrasts strikingly with the association of pp60$^{src}$ with the plasma membrane of Rous sarcoma virus-transformed chicken fibroblasts. We propose that pp60$^{src}$ is a membrane protein that associates with cellular membranes through hydrophobic regions and that this membrane association is a general feature of the interaction of pp60$^{src}$ with avian and mammalian cells. Although there are major differences in the intracellular localizations of pp60$^{src}$, it may interact with cellular membranes through one or more $NH_2$-terminal hydrophobic regions.**

Knowledge of the intracellular location of pp60$^{src}$, the transforming protein specified by the *src* gene of Rous sarcoma virus (RSV), may provide an approach to the understanding of the mechanism by which pp60$^{src}$ transforms cells. Although studies using immunofluorescence microscopy have suggested that pp60$^{src}$ is present in the cytoplasm of RSV-transformed cells (1, 2), more recent studies using immunocytochemistry (3) and cellular fractionation (4) have indicated that pp60$^{src}$ is predominantly associated with plasma membranes of RSV-transformed avian and mammalian cells. To investigate further the nature of the association of pp60$^{src}$ with membranes we examined mammalian cells derived from RSV-induced tumors. In the choice of the cell type we were guided by two criteria: first, that the cells be large and well spread to permit good microscopic visualization of intracellular structures; and second, that the transformed mammalian cells contain a level of pp60$^{src}$ approximately equivalent to that found in RSV-transformed chicken cells. For these reasons, we studied RR1022 cells that were isolated from an *in vivo* sarcoma induced in an Amsterdam rat by infection with the Schmidt–Ruppin strain (subgroup D) of RSV (5).

## MATERIALS AND METHODS

**Cells.** RR1022 cells were isolated from a tumor induced in newborn rats by injection of the Schmidt–Ruppin strain (subgroup D) of RSV (5). These cells were obtained from the American Type Culture Collection. Rat 1 cells (normal rat fibroblasts) were a kind gift from Steve Anderson of this University. Culture and infection of chicken embryo fibroblasts were as described (6).

**Antiserum.** Tumor-bearing rabbit (TBR) serum was obtained as described (4, 7). It contains antibodies both to virion structural proteins and to pp60$^{src}$. To remove antibodies to structural proteins, TBR serum (30 ml) was allowed to react with an Affigel affinity support (10 ml) containing covalently linked viral proteins (40 mg). The unadsorbed serum fraction, containing antibodies to pp60$^{src}$, was purified further by adsorption of the IgG fraction to protein A-Sepharose (Pharmacia). The IgG fraction was then eluted with 8 M urea. Urea was removed by dialysis against 50 mM Tris·HCl (pH 8.1), and IgG was stored in that buffer. We refer to TBR serum prepared in this fashion as "pp60$^{src}$-specific IgG." Antisera to avian oncornavirus proteins p27 and gp85 were generous gifts from J. H. Chen. Antibodies to tubulin and 10-nm-filament protein have been described (8). Antiserum against total virion proteins of an avian myeloblastosis virus (AMV) was a generous gift of Erwin Fleissner.

**Indirect Immunofluorescence Microscopy.** Cells were fixed and processed for indirect immunofluorescence microscopy as described (9), except that 0.1% Triton X-100 in phosphate-buffered saline was used to render the fixed cells permeable to antisera. Fluorescein-conjugated goat anti-rabbit IgG (Antibodies, Davis, CA) was preadsorbed with the appropriate cell type before use and was diluted to 0.5 mg/ml in phosphate-buffered saline. Equal volumes of the secondary antibody were used in all experiments, and all micrographs were taken with a ×63 oil objective, epifluorescent illumination, and a 30-sec exposure.

**Other Methods.** Labeling of cells with [$^{35}$S]methionine and immunoprecipitation have been described (4). Assay of the pp60$^{src}$-associated phosphotransferase activity in immune complexes was performed as described (4). Analysis of pp60$^{src}$ peptides, obtained by partial digestion with *Staphylococcus aureus* V8 protease, was done on one-dimensional NaDodSO$_4$/polyacrylamide gels as described by Cleveland *et al.* (10), with minor modifications (11). NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography were by published procedures (12, 13).

## RESULTS

**Characterization of pp60$^{src}$-Specific IgG.** Immunoprecipitation of RR1022 cells with TBR serum showed that pp60$^{src}$ was the major virus-specified protein present in these cells (Fig. 1, lane A). TBR serum immunoprecipitated only pr76 in addition to pp60$^{src}$. When detergent-disrupted virus was included in the immunoprecipitation, only pp60$^{src}$ was immunoprecipitated (lane B). Low levels of pr76 and an unidentified $M_r$ 48,000 protein were immunoprecipiated from RR1022 cells by antibody against AMV (lane C). Little if any viral glycoprotein was immunoprecipitated by antibody to gp85 (lane D). The pp60$^{src}$-specific IgG used for immunofluorescent localization recognized only pp60$^{src}$ in RR1022 cells (lane E). Immu-

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Abbreviations: RSV, Rous sarcoma virus; TBR, tumor-bearing rabbit; AMV, avian myeloblastosis virus.

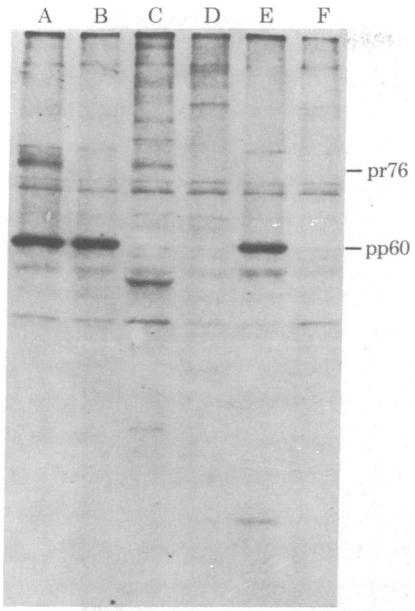


FIG. 1. Immunoprecipitation of [$^{35}$S]methionine-labeled proteins from RR1022 cells with various antisera. Lanes: A, TBR; B, TBR plus detergent-disrupted virus; C, anti-AMV; D, anti-gp85; E, pp60$^{src}$-specific IgG; F, normal rabbit serum.

noprecipitation of radiolabeled RSV-infected chicken cells showed that the purified IgG did not recognize pr76 or any structural virion proteins in these cells (data not shown).

**Localization of pp60 $^{src}$ by Indirect Immunofluorescence Microscopy.** We used pp60$^{src}$-specific IgG to localize the transforming protein in RR1022 cells (Fig. 2 *A* and *B*) and in RSV-transformed chicken cells (Fig. 2*C*). The most characteristic feature of RR1022 cells that had reacted with pp60$^{src}$-specific IgG was an intense fluorescent ring in the position of the nuclear envelope. As would be expected for a protein in association with the nuclear envelope, the fluorescence pattern remained a sharp circular ring that became smaller when we focused through the cytoplasm of RR1022 cells from a low to a high focal plane. We also observed fluorescent staining in the cytoplasm. Cytoplasmic fluorescence was seen as either patches or reticular structures, as illustrated in Fig. 2*B*. Because the majority of pp60$^{src}$ fractionates with intracellular membranes and is detergent extractable (see below), we infer from the immunofluorescence micrographs that the cytoplasmic fluorescence observed in RR1022 cells is due to the association of pp60$^{src}$ with intracellular membranes. The cytoplasmic fluorescence was more intense in the perinuclear region. It is likely that this increased fluorescence reflects the abundance of cytoplasmic structures in this region. In comparison, RSV-transformed chicken cells showed a diffuse cytoplasmic pattern of immunofluorescence (Fig. 2*C*). Little or no fluorescence was observed in the region of the nuclear membrane, but there was intense staining in the region of the plasma membrane, particularly in regions of cell-to-cell contact. In contrast, we did not observe staining at the plasma membrane or at regions of intercellular contact in RR1022 cells. Control experiments indicated that pp60$^{src}$-specific IgG produced dim and diffuse fluorescence in untransformed Rat 1 cells (Fig. 2*D*) and that normal rabbit serum did not stain any structure in RR1022 cells (data not shown). The pp60$^{src}$-specific IgG staining pattern did not resemble the immunofluorescence pattern obtained with antibodies against gp85 (plate E), p27 (plate F), or tubulin (Fig. 2*G*). Comparison of Fig. 2 *A* and *H* indicates that the nuclear envelope staining by pp60$^{src}$-specific IgG is distinct from the fluorescent staining pattern generated by antibodies to 10-nm filaments.

**Subcellular Localization of pp60 $^{src}$ by Cell Fractionation.** Immunofluorescence microscopic localization of pp60$^{src}$ on the nuclear envelope and juxtanuclear reticular membranes of RR1022 cells was confirmed by cell fractionation. RR1022 cells were lysed by mechanical homogenization and cells were separated into three fractions by differential centrifugation: N, a nuclear fraction containing nuclei with associated membranes; M, a membrane fraction containing mitochondria, a part of the smooth or rough endoplasmic reticulum, and plasma membranes; and S, a soluble protein fraction. The RR1022 N fraction contained 58% of the total pp60$^{src}$ associated phosphotransferase activity (Fig. 3) and most of the pp60$^{src}$ polypeptide (data not shown). In comparison, the bulk of the phosphotransferase activity in RSV-transformed chicken embryo fibroblasts was associated with the M fraction. Our fractionation methods can separate nuclei with associated membranes (nuclear envelope membrane and attached endoplasmic reticulum) from the bulk of plasma membranes. In the fractionation shown in Fig. 3, more than 60% of the plasma membranes, as judged by the distribution of 5′-nucleotidase activity, was found in the M fraction derived from either RR1022 or chicken cells. Thus, the chicken cell fractionation data are consistent with a specific plasma membrane localization of pp60$^{src}$, whereas the RR1022 results suggest that the bulk of pp60$^{src}$ is associated with other intracellular membranes. In this regard it is important to note that the RR1022 N fraction contains more than 60% of cellular endoplasmic reticulum membranes, as determined by the distribution of NADH-cytochrome $b_5$ reductase (data not shown). Therefore, the fractionation of pp60$^{src}$ in RR1022 cells correlates with the distribution of nuclear envelope and endoplasmic reticulum membranes. We attempted further fractionation of the RR1022 M fraction on sucrose gradients to determine whether pp60$^{src}$ was associated with the endoplasmic reticulum or with plasma membranes in this fraction. Two types of sucrose gradients failed to separate these membranes sufficiently, and we therefore cannot distinguish between association of pp60$^{src}$ with plasma membranes or with endoplasmic reticulum in the M fraction. However, an upper limit can be placed on the amount of pp60$^{src}$ that may associate with plasma membranes in RR1022 cells. Based on the distribution of 5′-nucleotidase, an enzyme specifically associated with plasma membranes, we estimate that no more than 24% of pp60$^{src}$ could associate with plasma membranes in RR1022 cells. Similar estimation of the amount of pp60$^{src}$ contained in purified plasma membranes from RSV-transformed chicken cells (4) suggests that >90% is plasma membrane-associated in these cells. These calculations do not consider the pp60$^{src}$ in the S fraction in either cell type because the polypeptide is degraded ($M_r$ 52,000) in this fraction and is believed to originate from a $M_r$ 60,000 membrane-associated molecule (see below).

**Peptide Maps of pp60 $^{src}$ from Avian and Mammalian Cells.** To determine whether the association of pp60$^{src}$ with different membrane fractions in RR1022 and RSV-transformed chicken cells might be due to a difference in the primary structure of pp60$^{src}$ in these cells, we compared these molecules by the technique of limited proteolysis in one-dimensional NaDodSO$_4$/polyacrylamide gels. Digestion of [$^{35}$S]methionine-labeled pp60$^{src}$ from RSV-transformed chicken cells with *S. aureus* V8 protease yielded peptides of 34, 26, 21, 18, 15, 14, 12, and 11 kilodaltons (Fig. 4, lane D). All of these peptides, with the exception of those of 14 and 12 kilodaltons, were labeled by $^{32}$PO$_4$ (result not shown). The *S. aureus* V8 protease peptide map of pp60$^{src}$ from RR1022 cells (Fig. 4, lane C) is similar to

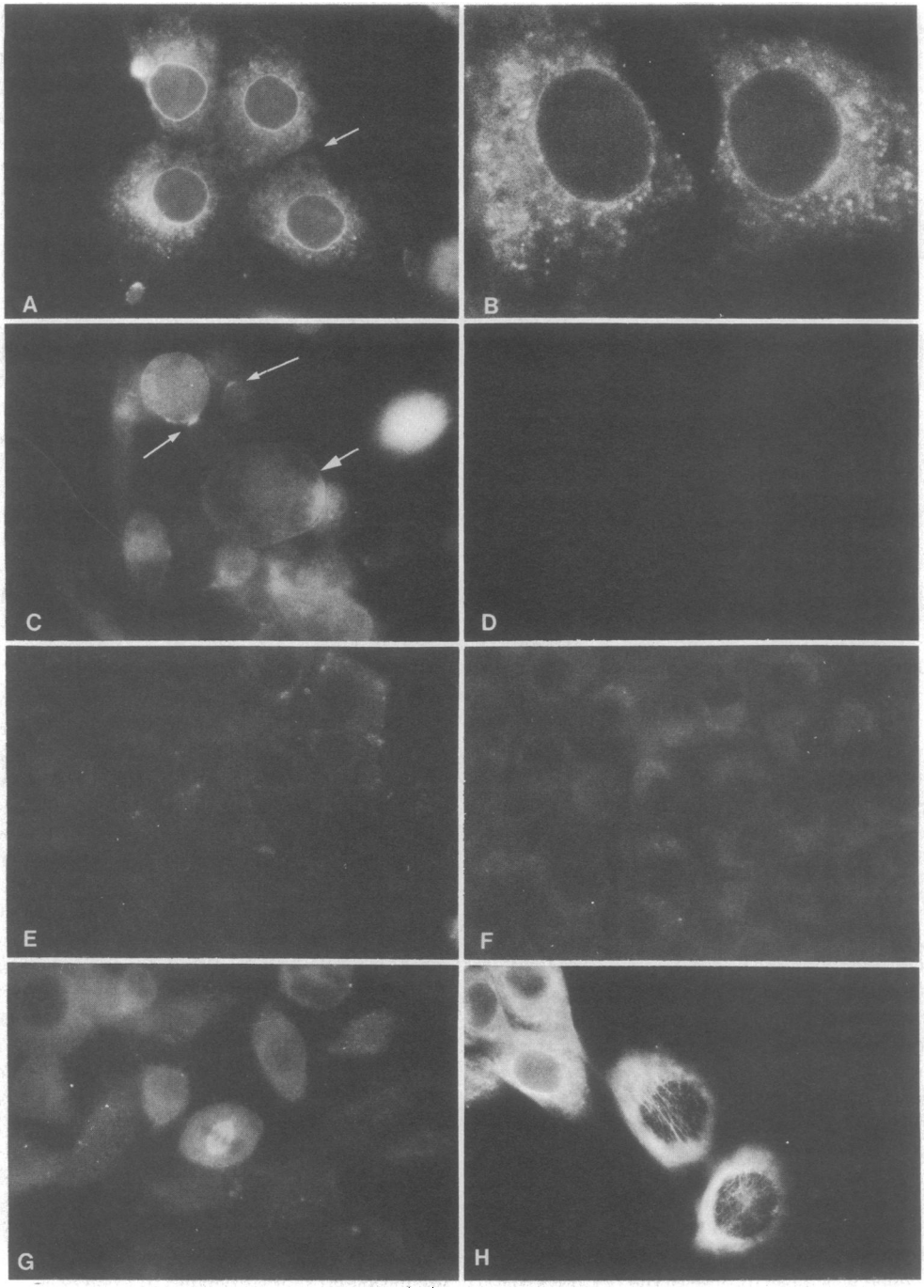


FIG. 2. Immunofluorescent localization of pp60$^{src}$ in RR1022 and RSV-transformed chicken cells. (*A*) RR1022 cells with pp60$^{src}$-specific IgG (×410); (*B*) RR1022 cells with pp60$^{src}$-specific IgG (×1000); (*C*) Schmidt–Ruppin RSV-transformed chicken cells with pp60$^{src}$-specific IgG (×300); (*D*) Rat 1 cells with pp60$^{src}$-specific IgG (×300); (*E*) RR1022 cells plus anti-gp85 serum (×300); (*F*) RR1022 cells plus anti-p27 serum (×300); (*G*) RR1022 cells plus anti-tubulin serum (×300); (*H*) RR1022 cells plus anti-10-nm filament protein serum (×410). In *A* the arrow indicates where two cells are touching; note the absence of fluorescence at this junction. In *C* the large arrow indicates the ring of increased fluorescence at the free cell edge, a characteristic pattern of plasma membrane staining; the small arrows indicate increased fluorescence at boundaries between two cells that are touching.

that of RSV (chicken cell) pp60$^{src}$: all the V8 protease peptides are present except for the 21- and 18-kilodalton species. The V8 protease peptide map of phosphate-labeled RR1022 pp60$^{src}$ was identical to that of the RSV pp60$^{src}$, except that the 21-kilodalton phosphorylated peptide was absent (result not shown). The *S. aureus* V8 protease peptide pattern of the soluble pp52$^{src}$ (4) from RSV-transformed chicken cells labeled with [$^{35}$S]methionine (Fig. 4, lane E) is a subset of that of pp60$^{src}$. The principal V8 protease digestion products of RSV pp60$^{src}$ are an $NH_2$-terminal 34-kilodalton peptide and a COOH-terminal 26-kilodalton peptide (14). The 26-kilodalton peptide is an end product of V8 protease digestion (14); the $NH_2$-terminal 34-kilodalton peptide could be further digested to yield the 21-, 18-, 15-, 14-, 12-, and 11-kilodalton peptides (complete data not shown). The 34-, 18-, 12-, and 11-kilodalton peptides are absent from the peptide map of pp52$^{src}$. We conclude that the 8-kilodalton fragment cleaved from pp60$^{src}$ to produce pp52$^{src}$ is the $NH_2$ terminus of the protein.

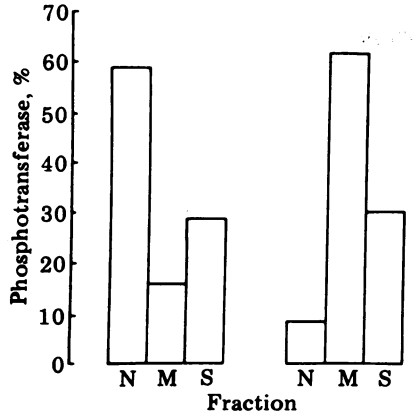


FIG. 3. Subcellular fractionation of RR1022 (*Left*) and RSV-transformed chicken embryo fibroblasts (*Right*) by differential centrifugation. RSV-transformed chicken cells were fractionated as described (4). RR1022 cells were lysed by homogenization in a Potter–Elvehjem homogenizer (20 up/down strokes at 600 rpm) in homogenization buffer containing 5 mM magnesium chloride and were fractionated by the methods used for chicken cells. N, nuclear fraction; M, membrane fraction; S, soluble protein fraction (M corresponds to a 200,000 × *g* pellet of a postnuclear supernatant, and S corresponds to the supernatant from this centrifugation). The M fraction of RSV-transformed chicken cells contains 61% of the plasma membranes and the M fraction of RR1022 cells contains 62% of the plasma membranes as judged by the distribution of 5′-nucleotidase activity (4).

## DISCUSSION

To gain insight into possible mechanisms by which pp60$^{src}$ transforms avian and mammalian cells, we have investigated the intracellular location of pp60$^{src}$ in chicken and rat cells transformed by the Schmidt–Ruppin strain of RSV. Results of immunofluorescence microscopy and cellular fractionation suggest that the bulk of pp60$^{src}$ is associated with different membranes in the two cells examined. In RR1022 cells, more than 75% of pp60$^{src}$ appears to be associated with the nuclear envelope and internal cytoplasmic membranes, whereas in RSV-transformed chicken cells, pp60$^{src}$ appears to associate predominantly with plasma membranes. Although the most intense immunofluorescence occurs at the nuclear envelope membrane in RR1022 cells stained with pp60$^{src}$-specific IgG, a considerable cytoplasmic fluorescence is also evident. Our cell fractionation data also indicate that pp60$^{src}$ is membrane-associated in RR1022 cells, and this view is further supported by the solubilization of pp60$^{src}$ from cells with detergents. These data suggest that the localization, by fluorescence, of pp60$^{src}$ in cytoplasmic areas in RR1022 cells reflects association of pp60$^{src}$ with membranous structures in the cytoplasm. It is possible that the immunofluorescent staining of juxtanuclear reticular structures by pp60$^{src}$-specific IgG is indicative of an association of some pp60$^{src}$ with the membranes of the endoplasmic reticulum. It is well known that the outer nuclear membrane occurs in morphological continuity with the rough endoplasmic reticulum (15). The fluorescent staining pattern of pp60$^{src}$ in RR1022 cells shows a striking similarity to that observed with antibodies against cytochrome $b_5$, a protein characteristically associated with endoplasmic reticulum and other intracellular membranes (16).

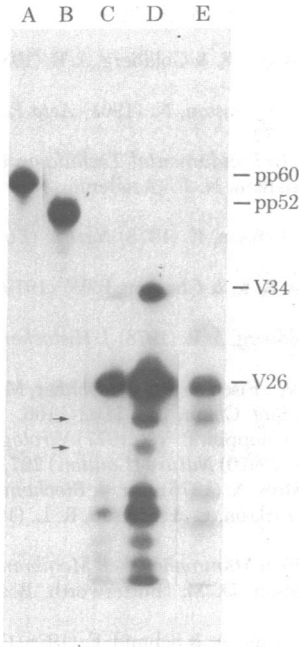


FIG. 4. Analysis of pp60$^{src}$ from RR1022 and RSV-transformed chicken cells by limited proteolysis with *S. aureus* V8 protease. Cells were labeled with 0.5 mCi of [$^{35}$S]methionine for 4 hr in methionine-free medium at 37°C. Lanes: A, undigested pp60$^{src}$; B, undigested pp52$^{src}$; C, RR1022 pp60$^{src}$ digested with 0.25 μg of V8 protease; D, RSV (chicken) pp60$^{src}$ digested with 0.25 μg of V8 protease; E, RSV (chicken) pp52$^{src}$ digested with 0.25 μg of V8 protease. All lanes were from a single experiment run on one $NaDodSO_4$/polyacrylamide gel and exposed to film. The figure was assembled from this film. Arrows indicate 21- and 18-kilodalton peptides absent from RR1022 pp60$^{src}$.

The intracellular distribution of pp60$^{src}$ has been investigated by immunofluorescence microscopy in a number of other cells. Rohrschneider (1) examined RSV-transformed chicken cells and RSV-transformed rat kidney cells and reported that immunofluorescence was mainly cytoplasmic with some intensification of staining at contact regions between two cells. Brugge *et al.* (2) observed only diffuse cytoplasmic immunofluorescence in RSV-transformed Syrian hamster cells. Using both immunofluorescence and immunocytochemical techniques, Willingham *et al.* (3) examined the intracellular location of pp60$^{src}$ in RSV-transformed rat kidney cells. They observed by immunofluorescence microscopy a generally diffuse cytoplasmic fluorescence with increased staining intensity at regions of intercellular contact. Immunocytochemical methods revealed a high concentration of pp60$^{src}$ at the plasma membrane, with the highest concentration at gap junctions. We have shown by cell fractionation that pp60$^{src}$ is associated primarily with plasma membranes in RSV-transformed chicken cells (4). The present localization of pp60$^{src}$ in RR1022 cells by immunofluorescence microscopy shows new features of the association of pp60$^{src}$ with specific intracellular membranes and the lack of immunofluorescence in regions that are visible in other cells. We wish to emphasize, however, that it is difficult to compare directly all the features of immunofluorescence observed in cell types with different shapes—e.g., the rounded shape of RSV-transformed chicken cells allows plasma membrane fluorescence to be readily detected whereas the flatter shape of RR1022 and other RSV-transformed mammalian cells could prevent visualization of fluorescence at the plasma membrane.

The nuclear membrane immunofluorescence pattern that we have demonstrated in RR1022 cells differs markedly from the fluorescence pattern of pp60$^{src}$ in RSV-transformed rat kidney cells reported by Rohrschneider (1) and Willingham *et al.* (3). The fluorescence pattern, representing the distribution of pp60$^{src}$ in RR1022 cells, is characterized by a sharp ring that encircles the whole nucleus. The immunofluorescence staining pattern of the nuclear envelope with pp60$^{src}$-specific IgG differs markedly from that observed with antisera directed against 10-nm filaments, microtubules, or microfilaments. It

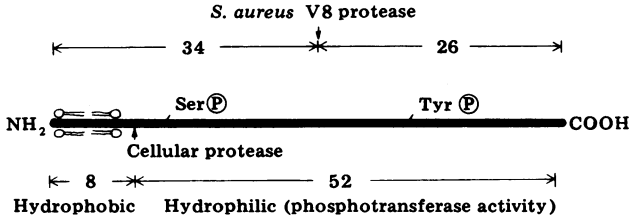


FIG. 5. Model for pp60$^{src}$ interaction with membranes, which incorporates several known features. The $NH_2$-terminal 8-kilodalton fragment contains one or more hydrophobic regions which allow pp60$^{src}$ to interact directly with membrane lipids. Given this orientation, pp60$^{src}$ could be synthesized on membrane-bound ribosomes (18, 19), which would couple synthesis, and a membrane association, or pp60$^{src}$ could be synthesized on free cytoplasmic polysomes (19, 20) and would then associate with membranes after synthesis through a hydrophobic region. It is not clear yet whether a small hydrophilic portion of pp60$^{src}$ is exposed on the ectoplasmic side of the membrane as suggested by immunofluorescence data on fixed, intact cells (17). During cell fractionation a protease present in the lysate, which could be of either viral or cellular origin, can cleave pp60$^{src}$ at the indicated point to release a water-soluble fragment (pp52$^{src}$) which retains the phosphotransferase activity. The other details of the model were suggested by Collett *et al.* (14) and, as previously emphasized, the position of the phosphorylated amino acid residues in the diagram only indicates that they are contained in indicated V8 fragments. The model is corrected to indicate that the phosphorylated amino acid residue in the 26-kilodalton V8 fragment is probably phosphotyrosine instead of phosphothreonine (21).

does not appear likely that the pp60$^{src}$ staining is due to staining of cytoskeleton structures by the pp60$^{src}$-specific IgG.

Fractionation of RSV-transformed chicken cells and of RR1022 cells has established that the bulk of pp60$^{src}$ and its associated phosphotransferase activity sediments with cellular membranes. In both of these cell types a small fraction of phosphotransferase activity remained in the high-speed supernatant or soluble protein fraction. Attempts to immunoprecipitate radiolabeled pp60$^{src}$ from RSV-transformed chicken cell high-speed supernatant fractions gave an immunoprecipitation product of 52 kilodaltons, which we termed pp52$^{src}$ (4, 17). We proposed that pp52$^{src}$ resulted from proteolytic degradation of pp60$^{src}$ during cell fractionation and that pp52$^{src}$ retained its associated phosphotransferase activity. Immunoprecipitation of high-speed supernatants from RR1022 cells also shows that pp60$^{src}$ is present as the degraded pp52$^{src}$ (unpublished data). Because pp52$^{src}$ fractionates as a water-soluble protein, we conclude that the 8 kilodalton fragment which has been lost is responsible for the ability of pp60$^{src}$ to associate with membranes.

The *S. aureus* V8 protease digestion of pp52$^{src}$ shown in Fig. 4 suggests that the missing 8-kilodalton fragment is derived from the 34-kilodalton V8 digestion product of pp60$^{src}$ and is therefore removed from the $NH_2$ terminus of pp60$^{src}$ (14). We have shown previously that membrane-associated pp60$^{src}$ resists high-salt extraction, that it remains membrane-associated in the presence of divalent cation chelators, and that it is efficiently extracted by nonionic detergents (4, 17). Furthermore, we have observed recently that extraction of membranes from RSV-infected chicken cells with 8 M urea released little if any pp60$^{src}$. These properties of pp60$^{src}$ are characteristics of integral membrane proteins which interact directly with lipid and have hydrophobic regions governing this association. These data thus suggest the model of pp60$^{src}$ that is presented in Fig. 5. We propose that pp60$^{src}$ is an integral membrane protein which associates directly with the membrane lipids. A protease present in cell lysates cleaves some of the pp60$^{src}$ molecules at the indicated junction, with the release of a soluble 52-kilodalton fragment of pp60$^{src}$ which retains phosphotransferase activity.

We do not know whether pp60$^{src}$ is a transmembrane protein. An 8-kilodalton polypeptide would contain approximately 75 amino acid residues and this number is sufficient to span a lipid bilayer more than once.

The conspicuous plasma membrane association of pp60$^{src}$ in RSV-transformed rat kidney (3) and RSV-transformed chicken cells (4) has given rise to the notion that the plasma membrane could be an important intracellular target for the action of pp60$^{src}$. We were therefore surprised to find the bulk of pp60$^{src}$ associated with internal cytoplasmic and nuclear membranes in RR1022 cells. These cells are tumorigenic *in vivo* and show anchorage-independent growth *in vitro*. Therefore, at least two possibilities need to be considered to explain the relationship between the observed localization of pp60$^{src}$ and its function in RR1022 cells. One possibility is that a small fraction of intracellular pp60$^{src}$ in association with the plasma membrane could be sufficient to maintain the transformed state. A second possibility is that pp60$^{src}$ may be able to express its function from different intracellular locations. If the latter possibility is correct, then an association of pp60$^{src}$ with cellular membranes through hydrophobic amino acids in the $NH_2$-terminal 8-kilodalton region may be the most general feature of the interaction of pp60$^{src}$ with RSV-transformed chicken and RR1022 cells. However, the absolute requirement of a membrane association of pp60$^{src}$ for the transformation of cells remains to be determined.

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