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# Association of pp60$^{src}$ and *src* Protein Kinase Activity with the Plasma Membrane of Nonpermissive and Permissive Avian Sarcoma Virus-Infected Cells

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The intracellular localization of pp60$^{src}$ and *src* protein kinase activity in avian sarcoma virus (ASV)-infected chicken embryo fibroblasts and transformed and morphologically reverted field vole cells was examined by subcellular fractionation procedures. Fractionation by differential centrifugation of Dounce-homogenized cellular extracts prepared from vole cells showed that 83 to 91% of pp60$^{src}$ sedimented with particulate subcellular components from both transformed and revertant vole cells. A slightly lesser amount (60 to 70%) of pp60$^{src}$ was found associated with the particulate fraction from ASV-infected chicken embryo fibroblasts. The distribution of *src* protein kinase activity in the cytosol and particulate cell fractions was identical to that of pp60$^{src}$, indicating no detectable differences in the activity of cytosol- and particulate-associated pp60$^{src}$. When subcellular components of the cell were fractionated by discontinuous sucrose gradient centrifugation, similar amounts of both pp60$^{src}$ and *src* protein kinase activity cosedimented with the plasma membrane fractions from both transformed and revertant vole cells, as well as from ASV-infected chicken embryo fibroblasts. *src* protein kinase activity associated with plasma membrane fractions prepared from vole cells and ASV-infected chicken embryo fibroblasts was resistant to extraction with high salt concentrations, but partial elution was achieved with nonionic detergent. Thus, in both transformed and morphologically reverted vole cells, pp60$^{src}$ is intimately associated with the plasma membrane. Since transforming virus can be rescued from revertant vole cells by fusion to chicken embryo fibroblasts, revertant vole cell pp60$^{src}$ is capable of inducing morphological transformation. Thus, although the data presented herein suggest that transformation requires the association of pp60$^{src}$ with the plasma membrane, the binding of pp60$^{src}$ to the plasma membrane per se is insufficient to induce morphological transformation and requires the additional interaction with a specific target membrane protein which appears to be defective in revertant vole cells.

Transformation of cells by avian sarcoma viruses (ASV) is mediated by a 60,000-dalton virus-coded polypeptide, pp60$^{src}$, which functions as a phosphotransferase (3, 6, 22, 25). The pp60$^{src}$ phosphotransferase activity has been implicated directly in morphological transformation of fibroblasts by the demonstration that temperature-sensitive mutations in the *src* gene resulted in heat-labile phosphotransferase activity (6, 25). Recent studies (8, 14) indicate that there is a high degree of specificity in the phosphorylation reaction since pp60$^{src}$ phosphorylates the amino acid tyrosine, a minor phosphoamino acid in eucaryotic cells. These findings have led investigators to propose that this specific reaction will be useful in identifying the cellular target protein(s) of pp60$^{src}$ by providing a tyrosine substrate for phosphorylation by the pp60$^{src}$ protein kinase. Using this approach, Hunter and Sefton (14) have recently identified a 50,000-dalton phosphoprotein containing phosphotyrosine which co-immunoprecipitates with pp60$^{src}$.

Another approach to identifying cellular targets for the pp60$^{src}$ activity has been to determine the intracellular localization of pp60$^{src}$ in transformed cells. Several immunofluorescence studies performed with ASV-transformed chicken embryo fibroblasts (CEF) and mammalian cells revealed a diffuse cytoplasmic fluorescence (4, 24, 28). In addition, in most of these studies, increased staining intensity was also observed at regions of intercellular contact (24, 28), suggesting that pp60$^{src}$ was associated with plasma membrane proteins. Support for the association of pp60$^{src}$ with the plasma membrane was provided by Willingham et al. (28), who employed electron microscopic immunocytochemical techniques which offer both greater sensitivity and resolution than immunofluores-

cence methods. These investigators observed a high concentration of pp60$^{src}$ at the inner surface of the plasma membrane of ASV-transformed mammalian cells. The difficulty with these kinds of studies, however, is that they do not discriminate between enzymatically active and inactive pp60$^{src}$.

Employing both aforementioned approaches, we initiated studies to determine the target site(s) of pp60$^{src}$ in a particular mammalian cell system, the European field vole, that we have been studying for the past several years. The interesting aspect of this particular cell system is that morphological revertants of ASV-transformed vole cells contain high levels of pp60$^{src}$ and exhibit levels of protein kinase activity similar to those of transformed cells (7, 20, 21). Since ASV rescued from these cells can transform CEF in vitro (17), indicating that the *src* gene of the rescued virus is biologically active, the lesion responsible for the normal morphological phenotype in these cells must represent a host cell component with which pp60$^{src}$ must interact to facilitate the morphologically transformed phenotype. Thus, we have an additional method available to help us identify the target site(s) of pp60$^{src}$ required for morphological transformation in mammalian fibroblasts. In an attempt to determine the nature of the specific lesion responsible for the morphological reversion in vole cells, we initiated studies to localize pp60$^{src}$ in these cells to determine whether differences in membrane association of pp60$^{src}$ could be detected between morphologically normal (revertant) and transformed cells containing pp60$^{src}$ protein kinase. Since at the time we began these studies no in-depth subcellular fractionation evidence was available to confirm the above immunological data, we directed our initial efforts to the detection and the quantitation of pp60$^{src}$ associated with the cell membrane in transformed and revertant vole cells by cell fractionation procedures. Moreover, since similar data were lacking for the ASV-transformed CEF system, we also analyzed these permissive cells for the location of pp60$^{src}$ by these procedures. In this communication, we demonstrate that pp60$^{src}$ and its protein kinase activity are indeed intimately associated with the plasma membrane in ASV-transformed CEF and nonpermissive ASV-transformed vole cells by employing cellular fractionation procedures that clearly distinguish between cytoplasmic and membrane-bound cellular enzymatic activities. Furthermore, employing fractionation procedures that further separate the plasma membrane from other cellular organelles, we have demonstrated that pp60$^{src}$ fractionates predominantly with the plasma membrane in both permissive and nonpermissive cells.

Interestingly, similar amounts of pp60$^{src}$ could be demonstrated in the plasma membrane fraction from morphologically reverted vole cells, indicating that the specific lesion that is responsible for reversion in this cell system was not a component necessary to attach pp60$^{src}$ to the cell membrane. While this work was being prepared for publication, we learned of two other cellular fractionation studies with ASV-transformed CEF indicating that the bulk of the pp60$^{src}$ is associated with the cell membrane (16; J. M. Bishop, personal communication). Thus, our studies not only serve to further support this contention, but more importantly extend these studies to an interesting nonpermissive mammalian cell system in which pp60$^{src}$ is present in the membranes of morphologically normal cells.

## MATERIALS AND METHODS

**Cells and virus.** The infection of an established cell line from the European field vole, *Microtus agrestis*, with the Schmidt-Ruppin (SR) (subgroup D) strain of ASV and the isolation of transformed (1), morphologically revertant (4R and 866R), and spontaneously retransformed (866RT) vole cell lines have been previously described (10, 21). The biological properties of these vole cell types have been extensively described elsewhere (7, 17–21). Field vole cells were grown in Eagle modified minimum essential medium supplemented with 10% calf serum, 10% tryptose phosphate broth, 0.12% sodium bicarbonate, 2 mM glutamine, 20 U of penicillin per ml, and 20 μg of streptomycin per ml. SR-ASV-infected CEF were grown in culture as previously described (2, 12). The various cell lines, grown on 100-mm culture dishes, were labeled with 10 to 20 μCi of [$^{35}$S]methionine (New England Nuclear Corp.) per ml for 2 h at 37°C as previously described (21).

**Cell disruption.** ASV-infected vole cells and CEF were disrupted by the following procedures performed at 4°C.

In method 1, cells were harvested from tissue culture plates by scraping, washed three times with TS buffer (20 mM Tris-hydrochloride [pH 7.4]–150 mM NaCl), resuspended in TS buffer containing 1% Trasylol (100 kallikrein inactivator units per ml), and then disrupted by sonication with an MSE Sonifier at a setting of 1.5 for two 10-s exposures. This treatment resulted in complete cell breakage and the fragmentation of all nuclei. The disrupted cell suspension was adjusted to RIPA buffer (10 mM Tris-hydrochloride [pH 7.4]–150 mM NaCl–1% Triton X-100–1% sodium deoxycholate–0.1% sodium dodecyl sulfate–1% Trasylol), incubated on ice for 15 min, and then centrifuged for 30 min at 20,000 × *g*. The supernatant was removed, and the pellet was resuspended in TS buffer containing 1% Trasylol, disrupted by sonication, adjusted to RIPA buffer, and then centrifuged as above. Both supernatant fractions were then subjected to immunoprecipitation as described below.

In method 2, cells were harvested as described above, suspended in TMT buffer (10 mM Tris-hydrochloride [pH 7.4]–0.2 mM $MgCl_2$–1% Trasylol), and disrupted by sonication for two 10-s exposures. The disrupted cell suspension was centrifuged at 200,000 × *g* for 30 min, and the pellet ($P_{200}$) was resuspended in TMT buffer; both the pellet suspension and supernatant ($S_{200}$) were sonicated a second time. Portions of both fractions were removed for enzyme assays, and the remainders were adjusted to RIPA buffer and centrifuged at 200,000 × *g* for 30 min; the resultant supernatants were immunoprecipitated.

In method 3, cells were treated as described in method 2 except that, after resuspension in TMT buffer, the cell suspension was incubated on ice for 10 min and then disrupted by 20 to 40 strokes of a tight-fitting Dounce homogenizer. Under these conditions, greater than 95% of the cells were disrupted with nuclei remaining morphologically intact as judged by phase-contrast microscopy. The disrupted cell suspension was centrifuged at 200,000 × *g* for 30 min, and the pellet and supernatant fractions were treated as described in method 2.

**Cellular fractionation by discontinuous sucrose gradient centrifugation.** [$^{35}$S]methionine-labeled cells were harvested by scraping and washed twice in TS buffer, and the cell pellet was suspended in 1.0 ml of TS buffer containing 3 × $10^6$ cpm of $^{125}$I-labeled wheat germ agglutinin (WGA). After a 10-min incubation at 4°C, the cell suspension was diluted 40-fold with TS buffer, collected by centrifugation, washed twice to remove unbound WGA, and suspended in TMT buffer. Cells were then disrupted by Dounce homogenization as described in method 3. Immediately after cell breakage, the suspension was adjusted to 0.25 M sucrose and 1 mM EDTA, and nuclei were removed by centrifugation at 750 × *g* for 10 min. The postnuclear supernatant was removed, and the nuclear pellet was suspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.25 M sucrose–1% Trasylol and resedimented. The two supernatant fractions were combined and centrifuged at 50,000 × *g* for 30 min. The pellet fraction containing subcellular organelles was washed twice in the above buffer, suspended by Dounce homogenization, and layered onto a discontinuous sucrose gradient composed of successive layers of 60% (0.5 ml), 55% (2.5 ml), 40% (2.5 ml), 35% (3.5 ml), 30% (2.5 ml), 25% (2.5 ml), and 20% (2.5 ml) sucrose (all weight/weight) containing 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.5% Trasylol. The gradient was centrifuged at 70,000 × *g* for 2.5 h in an SW27.1 rotor and then fractionated into 0.5-ml fractions which were assayed for subcellular organelle markers. Gradient fractions were subsequently pooled, adjusted to RIPA buffer, and immunoprecipitated.

**Isolation of nuclei.** [$^{35}$S]methionine-labeled cells were harvested by scraping and washed three times in TS buffer, and the cell pellet was suspended in buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM $MgCl_2$, and 1% Trasylol. After incubation on ice for 10 min, the cell suspension was disrupted by Dounce homogenization, adjusted to 0.25 M sucrose and 0.5% Nonidet P-40, and placed on ice for 20 min. The suspension was centrifuged at 750 × *g* for 15 min, and nuclei were suspended in buffer containing 10 mM Tris-hydrochloride (pH 7.4), 3 mM $CaCl_2$, 0.5% Nonidet P-40, 0.25 M sucrose, and 1% Trasylol, incubated on ice for 10 min, and resedimented. The postnuclear supernatants obtained from the above centrifugation steps were pooled, sonicated for two 10-s exposures, adjusted to RIPA buffer, and subjected to immunoprecipitation. The nuclear pellet was washed a third time in the above buffer except that the Nonidet P-40 concentration was 0.2% and, after 5 min on ice, nuclei were collected by low-speed centrifugation. At this stage of purification, nuclei appeared to be morphologically intact and free of cytoplasmic material as judged by phase-contrast microscopy. Nuclei were further purified by sedimentation through 2 M sucrose (15), and the nuclear pellet was suspended in TMT buffer, sonicated for two 10-s exposures, adjusted to RIPA buffer, and centrifuged at 200,000 × *g* for 30 min. The resultant supernatant fraction was then immunoprecipitated.

**Immunoprecipitation, assay of pp60$^{src}$ protein kinase activity, and preparation of antiserum.** Antiserum was obtained from New Zealand rabbits in which tumors had been induced by injection of purified SR-ASV as described previously (21). Several preparations of tumor-bearing rabbit serum (TBR serum) were employed in these studies. Immunoprecipitations were performed on [$^{35}$S]methionine-labeled cell extracts, and subcellular fractions were adjusted to RIPA buffer. The samples were divided into two equal portions and then incubated with either normal rabbit serum or a vast excess of TBR serum. After 30 min of incubation at 4°C, 300 to 400 μl of a 10% suspension of the protein A-containing bacterium *Staphylococcus aureus*, strain Cowan I, was added to absorb the immune complexes. After an additional incubation for 10 min, the immune complexes were collected by centrifugation and washed four times with RIPA buffer and once each with TS buffer and kinase buffer (20 mM Tris-hydrochloride [pH 7.4]–5 mM $MgCl_2$). In some experiments, the first two washes were performed with buffer containing 10 mM Tris-hydrochloride (pH 7.4), 1 M NaCl, 0.1% Nonidet P-40, and 1% Trasylol instead of RIPA. This wash schedule markedly decreased the amount of background due to the nonspecific binding of proteins to the immune complexes. The immune complexes were suspended in 250 μl of kinase buffer, and 50 μl was removed to assay for pp60$^{src}$ protein kinase activity as described below. The remainder of the suspension was centrifuged, and the immune complex pellet was suspended in electrophoresis sample buffer; this suspension was heated in a boiling water bath for 3 min and then analyzed on a sodium dodecyl sulfate-polyacrylamide slab gel (5 to 15% gradient) as described previously (21). The gels were then fluorographed, dried, and exposed on Kodak X-Omat R film. For quantitation, the pp60$^{src}$ band was excised, the gel slice was dissolved in 30% $H_2O_2$, and, after the addition of Bray solution, the activity was counted in a scintillation counter.

For the assay of *src* protein kinase activity, the reaction was started by the addition of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) to 0.1 to 0.05 μM. After incubation at

room temperature for 10 min, the reaction was terminated by adjusting the mixture to 10 mM EDTA and 2 mM ATP. TS buffer (10 volumes) was then added, and the immune complex was collected by centrifugation and analyzed by polyacrylamide gel electrophoresis as described above. The amount of $^{32}P$ transferred to the heavy chain of immunoglobulin G (IgG) was determined by excising this band out of the dried gel and assaying the radioactivity by Cerenkov radiation.

**Marker enzyme assays.** Acid phosphatase was used as a lysosome marker and assayed by using *p*-nitrophenylphosphate as the substrate (23). Endoplasmic reticulum membranes were assayed for by reduced nicotinamide adenine dinucleotide (NADH) diaphorase as described by Avruch and Wallack (1). Plasma membranes were assayed for by 5'-nucleotidase as described by Avruch and Wallack (1) with [$^3H$]AMP as the substrate, except that the assay solution contained 50 mM glycine (pH 9.1), 10 mM $MgCl_2$, and 0.1 mM AMP. Mitochondria were identified by assaying for cytochrome *c*-succinate reductase (13). Lactate dehydrogenase was used as a cytosol enzyme marker and assayed as described by Stolzenbach (27). Enzyme activity is expressed as the total amount of units of enzyme present in either a subcellular fraction or a sucrose gradient fraction.

**Iodination of WGA.** WGA (Sigma Chemical Co.) was iodinated as described by Cuatrecasas (9), except that the unreacted $^{125}I$ was removed by passing the mixture through a Sephadex G-50 column. The specific activity of $^{125}I$-labeled WGA varied between 2 and 4 mCi/mg. $^{125}I$-labeled WGA was employed as an alternate plasma membrane marker (5) in some studies because 5'-nucleotidase activity was too low in revertant vole cells and ASV-transformed CEF to be of use as a marker enzyme. To identify plasma membranes in sucrose gradients, the distribution of $^{125}I$-labeled WGA was determined by counting a sample of each gradient fraction in a gamma counter.

## RESULTS

**Distribution of pp60$^{src}$ and *src* protein kinase activity in cytosol and particulate fractions prepared from ASV-infected vole cells and CEF.** Subcellular fractionation by differential centrifugation was employed as an initial assay to determine whether the loss of morphological transformation in revertant vole cells was associated with a difference in the intracellular localization of pp60$^{src}$. SR-ASV-infected CEF were also analyzed by this procedure to determine whether nonpermissive vole cells behaved similarly to permissive cells with respect to distribution of pp60$^{src}$ and its associated protein kinase activity. Revertant and transformed vole cells and SR-ASV-infected CEF were disrupted in hypotonic solution by Dounce homogenization, and the cell extracts were fractionated into cytosol ($S_{200}$) and particulate ($P_{200}$) fractions by differential centrifugation. The cytosol fraction contained soluble cell sap material, and the particulate fraction consisted of insoluble protein complexes and subcellular organelles. Greater than 95% of the cells were disrupted by this procedure, with nuclei remaining morphologically intact as observed by phase-contrast microscopy. In addition, greater than 92% of external (plasma membrane) and internal membranes (endoplasmic reticulum) were preserved as structures that could be pelleted by centrifugation as indicated by the membrane enzyme markers 5'-nucleotidase and NADH diaphorase, respectively (Table 1). The detection of less than 4% of the total lactate dehydrogenase (a soluble enzyme) activity in the particulate fraction (Table 1) suggested that nonspecific trapping of proteins during differential centrifugation was unlikely. The cytosol and particulate fractions were then subjected to immunoprecipitation with TBR serum to determine the distribution of both pp60$^{src}$ and *src* protein kinase activity among these cellular fractions. It was apparent that in all three vole cell types as well as in ASV-infected CEF that the majority of pp60$^{src}$ sedimented with particulate cell components (Fig. 1). In addition, in both cellular fractions of the cell types analyzed, a 52K (52,000-molecular-weight) polypeptide was also immunoprecipitated by TBR serum. This protein was present in substantially lower amounts than pp60$^{src}$ and in most instances was proportionate to the concentration of pp60$^{src}$ in that fraction. One-dimensional peptide analysis performed on the 52K polypeptide from both permissive and nonpermissive cells with *S. aureus* V8 protease indicated that this protein is related to pp60$^{src}$ (A. Lau, unpublished data) and is most probably generated from pp60$^{src}$ by proteolysis. The proteolytic generation of this polypeptide from pp60$^{src}$ has recently been reported by Krueger et al. (16).

To quantitate the cytosol and particulate distributions of pp60$^{src}$, the protein band corresponding to pp60$^{src}$ shown in Fig. 1 was excised, and the amount of $^{35}S$ radioactivity was determined. These results and the distribution of the corresponding *src* protein kinase activity are summarized in Table 1. Transformed, revertant, and retransformed vole cells all demonstrated a similar enrichment of pp60$^{src}$ in the particulate fraction which varied from 83 to 91%. Although there was also a preferable association of pp60$^{src}$ with the particulate fraction of ASV-infected CEF, it was moderately reduced (60 to 70%) compared with that observed for vole cells (83 to 91%). The distribution of *src* protein kinase activity in all cell types examined was essentially identical to that of pp60$^{src}$ (Table 1), suggesting that the particulate-associated pp60$^{src}$ protein

kinase activity was active in all of the cells analyzed and that no differences in this activity could be demonstrated between transformed and revertant vole cells.

The intracellular distribution of both pp60$^{src}$ and *src* protein kinase activity was dependent upon the method of cell disruption. If ASV-infected cells were disrupted by sonication, a procedure which not only resulted in the complete disruption of nuclei but also in a large reduction in the amount of plasma membrane and endoplasmic reticulum that could be pelleted under our centrifugation conditions, the majority of pp60$^{src}$ and an equivalent amount of *src* protein kinase activity were released from the particulate fraction (Fig. 2 and Table 2). The complete release of pp60$^{src}$ was achieved by incubation of the disrupted cell suspension in RIPA buffer before centrifugation (Fig. 3).

**Examination of purified nuclei for the presence of pp60$^{src}$ and *src* protein kinase activity.** Since nuclei were present in the par-

TABLE 1. *Percent distribution of pp60$^{src}$, src protein kinase activity, and marker enzymes in the cytosol ($S_{200}$) and particulate ($P_{200}$) fractions of Dounce-homogenized ASV-infected vole cells and CEF*[a]

| Cell type | Fraction | Distribution (%) | | | | |
|---|---|---|---|---|---|---|
| | | pp60$^{src}$ | *src* protein kinase | 5'-Nucleotidase | NADH diaphorase | Lactate dehydrogenase |
| Revertant (4R) | $S_{200}$ | 11 (12) | 14 (15) | | | |
| | $P_{200}$ | 89 (88) | 86 (85) | | | |
| Revertant (866R) | $S_{200}$ | 6 (9) | 5 (13) | | 3.6 | |
| | $P_{200}$ | 94 (91) | 95 (87) | | 96.4 | |
| Retransformed (866RT) | $S_{200}$ | 12 (10) | 20 (12) | 0.8 | 7.9 | 97.6 |
| | $P_{200}$ | 88 (90) | 80 (88) | 99.2 | 92.1 | 2.4 |
| Transformed (1T) | $S_{200}$ | 25 (17) | 20 (18) | 2.6 | 5.4 | 97.2 |
| | $P_{200}$ | 75 (83) | 80 (82) | 97.3 | 94.6 | 2.7 |
| ASV-infected CEF | $S_{200}$ | 30 (35) | 22 (24) | | 3.6 | 96.3 |
| | $P_{200}$ | 70 (65) | 78 (76) | | 96.4 | 3.6 |

[a] $S_{200}$ and $P_{200}$ fractions were prepared from Dounce-homogenized [$^{35}$S]methionine-labeled revertant (4R and 866R), retransformed (866RT), and transformed (1T) vole cells and ASV-infected CEF and were assayed for pp60$^{src}$, *src* protein kinase activity, 5'-nucleotidase, NADH diaphorase, and lactate dehydrogenase as described in the text. pp60$^{src}$ was quantitated from the experiment shown in Fig. 1. The distribution of pp60$^{src}$ and enzymatic activities in the $S_{200}$ and $P_{200}$ fractions are expressed as percentages of total recovery. The total recovery of pp60$^{src}$ and *src* protein kinase activity and cellular enzyme activities were greater than 80 and 85%, respectively. Percentages within parentheses are the average of two and of five or more determinations of pp60$^{src}$ and *src* protein kinase activity, respectively.

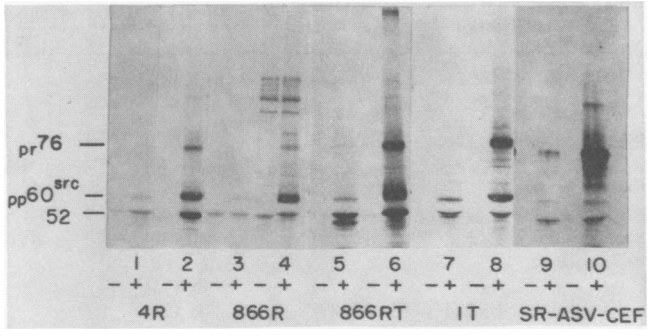


FIG. 1. *Distribution of pp60$^{src}$ between the cytosol and particulate fractions of Dounce-homogenized ASV-infected vole cells and CEF. Cytosol and particulate fractions prepared from Dounce-homogenized [$^{35}$S]-methionine-labeled revertant (4R and 866R), retransformed (866RT), and transformed (1T) vole cells and ASV-infected CEF were immunoprecipitated with either normal rabbit serum (−) or TBR serum (+), and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. 1, 3, 5, 7, and 9, Cytosol fractions; 2, 4, 6, 8, and 10, particulate fractions.*

ticulate fractions of Dounce-disrupted ASV-infected vole cells and CEF, an attempt was made to determine whether any pp60$^{src}$ could be demonstrated in cell nuclei. In these studies, nuclei were prepared from detergent-lysed ASV-infected vole cells and CEF, and both purified nuclei and the postnuclear fraction were analyzed for pp60$^{src}$. Less than 3 and 1% of total pp60$^{src}$ (Fig. 4) and its associated protein kinase activity (data not shown) could be detected in purified nuclei prepared from vole cells and CEF, respectively. Although nuclei appeared to be free of cytoplasmic contamination by phase-contrast microscopy, a faint protein band corresponding to Pr76 could be detected in the preparation of nuclei from ASV-infected CEF (Fig. 4, lane 6), suggesting that the low level of pp60$^{src}$ found associated with the nuclei of these cells as well as with vole cell types may result from cytoplasmic contamination. Nevertheless, it appears that the vast majority of particulate-bound pp60$^{src}$ is associated with a cell component(s) other than nuclei.

**Association of pp60$^{src}$ and *src* kinase ac-**

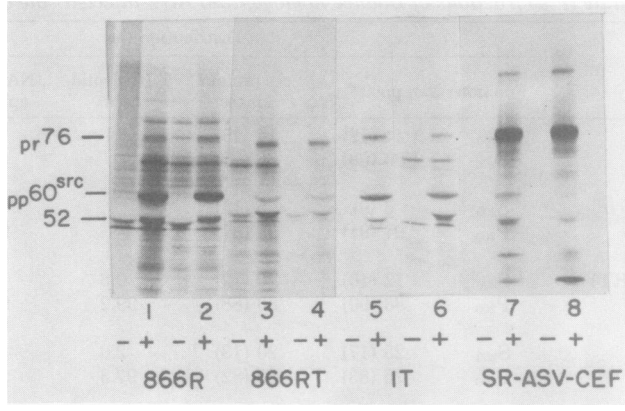


FIG. 2. *Distribution of pp60$^{src}$ between the cytosol and particulate fractions of sonicated ASV-infected vole cells and CEF. Cytosol and particulate fractions prepared from sonicated [$^{35}$S]methionine-labeled revertant (866R), retransformed (866RT), and transformed (1T) vole cells and ASV-infected CEF were immunoprecipitated with their normal rabbit serum (−) or TBR serum (+), and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. 1, 3, 5, and 7, Cytosol fractions; 2, 4, 6, and 8, particulate fractions.*

TABLE 2. *Percent distribution of pp60$^{src}$, src protein kinase activity, and marker enzymes in the cytosol ($S_{200}$) and particulate ($P_{200}$) fractions of sonicated ASV-infected vole cells and CEF*[a]

| Cell type | Distribution (%) | | | | |
|---|---|---|---|---|---|
| | Fraction | pp60$^{src}$ | *src* protein kinase | 5′-Nucleotidase | NADH diaphorase |
| Revertant (866R) | $S_{200}$ | 53 | 55 (60) | | 31 |
| | $P_{200}$ | 47 | 45 (40) | | 69 |
| Retransformed (866RT) | $S_{200}$ | 56 | 63 (64) | 35 | 36 |
| | $P_{200}$ | 43 | 37 (36) | 65 | 64 |
| Transformed (1T) | $S_{200}$ | 63 (61) | 54 (63) | 35 | 32 |
| | $P_{200}$ | 37 (39) | 46 (37) | 65 | 68 |
| ASV-infected CEF | $S_{200}$ | 67 (71) | 69 (71) | | 26 |
| | $P_{200}$ | 32 (29) | 31 (29) | | 74 |

[a] $S_{200}$ and $P_{200}$ fractions prepared from sonicated [$^{35}$S]methionine-labeled revertant (866R), retransformed (866RT) and transformed (1T) vole cells and ASV-infected CEF were assayed for pp60$^{src}$, *src* protein kinase, 5′-nucleotidase, and NADH diaphorase as described in the text. pp60$^{src}$ was quantitated from the experiment shown in Fig. 2. The distribution of pp60$^{src}$ and enzymatic activities in the $S_{200}$ and $P_{200}$ fractions are expressed as percentages of total recovery. The total recovery of pp60$^{src}$ and *src* protein kinase activity and cellular enzyme activities were greater than 80 and 85%, respectively. Percentages within parentheses are the average of two and of three to five determinations of pp60$^{src}$ and *src* protein kinase activity, respectively.

**tivity with plasma membrane.** To identify the specific particulate cell component(s) associated with the SR-ASV transforming protein, postnuclear particulate fractions prepared from ASV-infected vole cell types and ASV-infected CEF were subjected to centrifugation on discontinuous sucrose gradients to fractionate subcellular organelles. The gradient localization of the various subcellular organelles in the particulate fraction and their purity were determined by assaying the appropriate organelle markers. The degree of separation of subcellular organelles (plasma membrane, endoplasmic reticulum, lysosomes, and mitochondria) present in a particulate fraction prepared from a revertant vole cell line is shown in Fig. 5. The majority of plasma membranes (>60%), as identified by the amount of bound $^{125}$I-labeled WGA, was present in two discrete gradient regions containing 23 to 33% sucrose. A similar distribution of plasma membrane was also observed for transformed and retransformed vole cells as well as for ASV-infected CEF (see Fig. 7). Membranes corresponding to endoplasmic reticulum were identified by

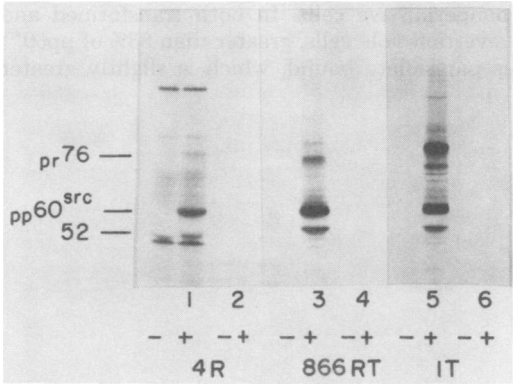


FIG. 3. *Total release of pp60$^{src}$ from the particulate fraction of ASV-infected vole cells. [$^{35}$S]methionine-labeled revertant (4R), retransformed (866RT), and transformed (1T) vole cells were disrupted by sonication, treated with detergent, and then processed to obtain cytosol and particulate fractions. The subcellular fractions were immunoprecipitated with either normal rabbit serum (-) or TBR serum (+), and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. 1, 3, and 5, Cytosol fractions; 2, 4, and 6, particulate fractions.*

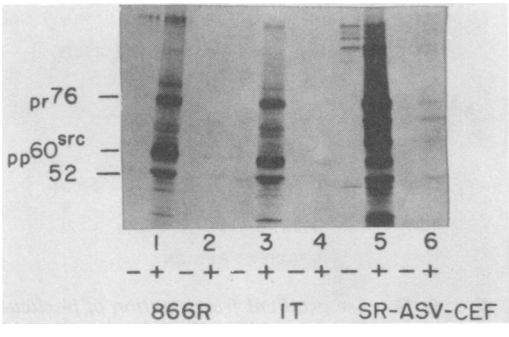


FIG. 4. *Examination of purified nuclei for the presence of pp60$^{src}$. Purified nuclei were prepared from [$^{35}$S]methionine-labeled revertant (866R) and transformed (1T) vole cells and ASV-infected CEF as described in the text. Both purified nuclei and the postnuclear supernatants were then analyzed for pp60$^{src}$ by immunoprecipitation with either normal rabbit serum (-) or TBR serum (+). 1, 3, and 5, Postnuclear supernatants; 2, 4, and 6, purified nuclei.*

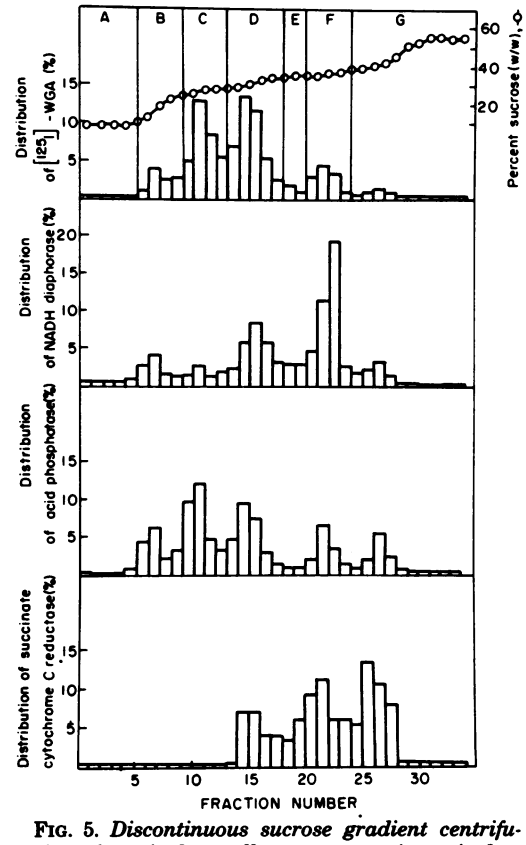


FIG. 5. *Discontinuous sucrose gradient centrifugation of particulate cell components. A particulate fraction prepared from revertant (866R) vole cells was fractionated on a discontinuous sucrose gradient as described in the text. The localization of subcellular organelles was determined by assaying each fraction for appropriate markers: [$^{125}$I]WGA, plasma membrane; NADH diaphorase, endoplasmic reticulum; acid phosphatase, lysosomes; and cytochrome c-succinate reductase, mitochondria. The distribution of organelle markers is expressed as percentages of total recovery which were greater than 70%. Regions of the gradient labeled A through G represent fractions which were pooled and analyzed for pp60$^{src}$ and src protein kinase activity (Fig. 6 and 7).*

NADH diaphorase activity, and greater than 60% were present in gradient regions containing a larger amount of sucrose (30 to 37%). The majority of endoplasmic reticulum was present in the gradient fractions containing 35 to 37% sucrose. For all ASV-infected vole cell types analyzed, the endoplasmic reticulum showed a similar distribution, with approximately 30% of the recovered endoplasmic reticulum present in the plasma membrane region. A slightly greater contamination of plasma membrane with endoplasmic reticulum was reproducibly observed for ASV-infected CEF (see Fig. 7d). The distribution of lysosomes as determined by acid phosphatase activity was essentially identical to that of plasma membranes. The majority of mitochondria (80%) as identified by a cytochrome *c*-succinate reductase assay were present in the denser portion of the gradient, and less than 15% contamination of plasma membranes with mitochondria was observed. The gradient distribution of mitochondria and lysosomes in the particulate fraction of retransformed and transformed vole cells as well as ASV-infected CEF was similar to that shown for the revertant vole cell line (data not shown).

The sucrose gradients containing the fractionated particulate components of infected vole cell types and CEF were divided into regions A through G as shown in Fig. 5, and the appropriate fractions were pooled and then immunoprecipitated with TBR serum. In all three vole cell types, as well as ASV-infected CEF, the majority of pp60$^{src}$ was localized in gradient regions C and D which were not only enriched for, but also contained, the majority of the plasma membranes (Fig. 6). The 52K polypeptide showed a distribution similar to pp60$^{src}$ and, in most instances, was present in proportionately lower amounts compared with that of pp60$^{src}$ in the same gradient region. The copurification of the plasma membrane and the SR-ASV transforming protein is more apparent in Fig. 7, which shows the percent distribution of pp60$^{src}$, *src* kinase activity, plasma membrane, and endoplasmic reticulum. The percentages of pp60$^{src}$ and *src* protein kinase activity were identical in distribution to that of the plasma membranes.

To determine the nature of the association of pp60$^{src}$ with the plasma membrane in transformed and revertant vole cells, the ability of high salt concentrations to extract pp60$^{src}$ from crude plasma membrane fractions prepared from vole cells and ASV-infected CEF was examined. No significant release of pp60$^{src}$ from the membrane fractions was observed for any of the cell types examined when incubation was performed in the presence of 1 M NaCl (Table 3). However, partial extraction was achieved by treatment with nonionic detergent. Thus, in both transformed and revertant vole cells, pp60$^{src}$ appears to be intimately associated with the plasma membrane and not merely nonspecifically absorbed to it.

## DISCUSSION

The results of our study demonstrate that pp60$^{src}$ is localized primarily in the particulate cell fraction and is directly associated with the cell plasma membrane in both permissive and nonpermissive cells. In both transformed and revertant vole cells, greater than 83% of pp60$^{src}$ is particulate bound, which is slightly greater

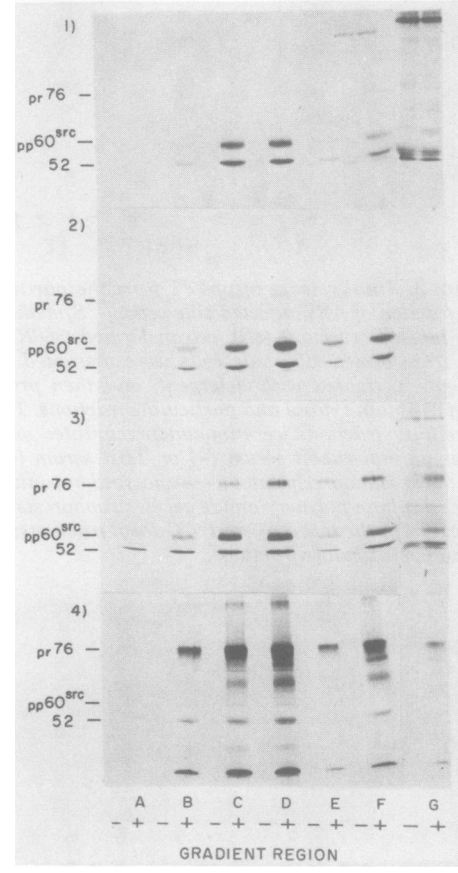


FIG. 6. *Sucrose gradient fractionation of particulate-bound pp60$^{src}$ from ASV-infected vole cells and CEF. Particulate fractions prepared from ASV-infected vole cells and CEF were centrifuged through a discontinuous sucrose gradient. Each gradient was divided into regions A through G as shown in Fig. 5, and the fractions were pooled and immunoprecipitated with either normal rabbit serum (–) or TBR serum (+). (1) Revertant (866R); (2) retransformed (866RT); (3) transformed (1T); and (4) ASV-infected CEF.*

than the 60 to 70% found in the particulate fraction of permissive ASV-infected CEF. In all cell types analyzed, the distribution of *src* protein kinase activity in the cytosol and particulate fractions was identical with pp60$^{src}$, suggesting

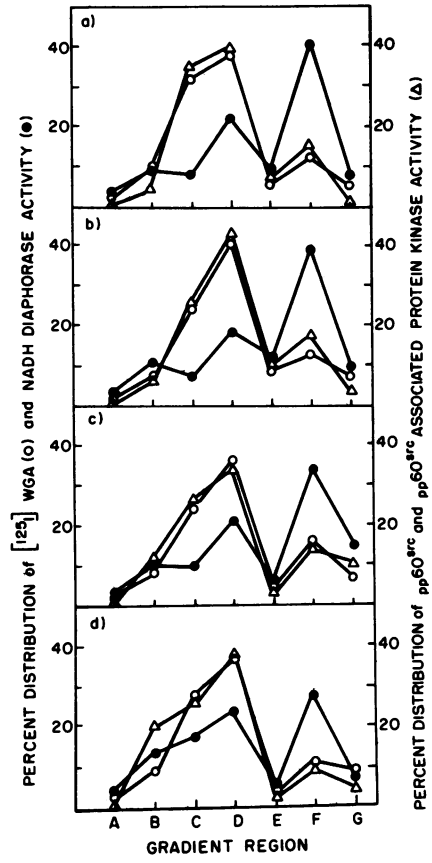


FIG. 7. *Discontinuous sucrose gradient distribution of pp60$^{src}$, src protein kinase, plasma membrane, and endoplasmic reticulum. Particulate fractions prepared from ASV-infected vole cells and CEF were fractionated on a discontinuous sucrose gradient. Localizations of plasma membranes and endoplasmic reticulums were determined by assaying each gradient fraction for $^{125}$I-labeled WGA and NADH diaphorase activity, respectively. Gradient fractions were then pooled as shown in Fig. 5 and assayed for pp60$^{src}$ and src protein kinase by immunoprecipitation. pp60$^{src}$ was quantitated from the experiment shown in Fig. 6. The distribution of pp60$^{src}$ and enzymatic activities in the pooled gradient fractions (A through G) are expressed as percent total recovery. The total recovery of pp60$^{src}$ and src protein kinase activity varied from 65 to 74%, whereas those for $^{125}$I-labeled WGA and NADH diaphorase were 72 to 80% and 79 to 82%, respectively. a, Revertant (866R); b, retransformed (866RT); c, transformed (1T); and d, ASV-infected CEF. Symbols: ○, $^{125}$I-labeled WGA; ●, NADH diaphorase; and △, pp60$^{src}$ and src protein kinase.*

TABLE 3. *Ability of various agents to elute src protein kinase activity from plasma membranes*[a]

| Cell type (strain) | Fraction | Distribution (%) of *src* protein kinase activity in the following solution:[b] | | |
|---|---|---|---|---|
| | | A | B | C |
| Transformed (1T) | $S_{50}$ | 3 | 4 | 33 |
| | $P_{50}$ | 97 | 96 | 67 |
| Retransformed (866RT) | $S_{50}$ | 5 | 7 | 48 |
| | $P_{50}$ | 95 | 93 | 52 |
| Revertant (866R) | $S_{50}$ | 4 | 9 | 38 |
| | $P_{50}$ | 96 | 91 | 62 |
| Revertant (4R) | $S_{50}$ | 14 | 13 | 51 |
| | $P_{50}$ | 86 | 87 | 49 |
| ASV-infected CEF | $S_{50}$ | 14 | 16 | 68 |
| | $P_{50}$ | 86 | 84 | 32 |

[a] ASV-infected vole cells and CEF were disrupted by Dounce homogenization as described in method 3 in the text. Immediately after cell breakage, the suspension was adjusted to 0.25 M sucrose and 1 mM EDTA, and nuclei were removed by low-speed centrifugation. The postnuclear supernatant was then centrifuged at 50,000 × *g* for 30 min, and the crude plasma membrane pellet was washed twice in buffer containing 10 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 0.25 M sucrose, and 1% Trasylol and suspended by Dounce homogenization in 10 mM Tris-hydrochloride (pH 7.4) containing 1% Trasylol. The crude plasma membrane suspension was then divided into three aliquots, adjusted to the indicated solutions, and after 15 min on ice centrifuged at 50,000 × *g* for 30 min. The pellet fraction was resuspended in the same solution employed for the elution assay, and both the pellet ($P_{50}$) and supernatant ($S_{50}$) fractions were then sonicated, adjusted to RIPA buffer, and subjected to immunoprecipitation, and the *src* protein kinase was assayed as described in the text. *src* protein kinase activity is expressed as the percentage of total recovered activity associated with the $P_{50}$ and $S_{50}$ fractions. The total recovery of *src* protein kinase activity ranged from 70 to 80%.

[b] A, 10 mM Tris-hydrochloride (pH 7.4)–1% Trasylol; B, 10 mM Tris-hydrochloride (pH 7.4)–1 M NaCl–1% Trasylol; C, 10 mM Tris-hydrochloride (pH 7.4)–10 mM KCl–150 mM NaCl–1.5 mM $MgCl_2$–10% glycerol–1% Triton X-100–1% Trasylol.

that detectable differences do not exist in the kinase activity of cytosol- and particulate-bound pp60$^{src}$. While these data were being assimilated for publication, we learned that other investigators had obtained similar observations with the permissive ASV-transformed CEF system (16; J. M. Bishop, personal communication). However, in contrast to the observations of one of these studies (16), we found that the distribution of pp60$^{src}$ was dependent upon the method of cell disruption. If sonication was em-

ployed instead of Dounce homogenization, the majority of pp60$^{src}$ could be released from the particulate fraction. The disruption of cells by sonication also resulted in a significant reduction in the amount of plasma membrane and endoplasmic reticulum that could be pelleted by our conditions of centrifugation, which was consistent with the release of pp60$^{src}$ from the particulate fraction. Since this group of investigators did not monitor the distribution of membranes by marker enzymes, it is conceivable that their sonication procedure had a less disruptive effect on membranes than our method. Our results are in contrast with that of Brugge et al. (4), who reported that only 10 to 20% of pp60$^{src}$ is associated with the particulate fraction of Dounce-disrupted ASV-transformed CEF. The reason for this discrepancy is unclear.

Further support for the association of pp60$^{src}$ and *src* protein kinase activity with the plasma membrane was demonstrated by discontinuous sucrose gradient centrifugation of the particulate fraction. Although the gradient fractions containing plasma membranes also contained a significant amount of endoplasmic reticulum, the percent distribution of pp60$^{src}$ and *src* protein kinase activity was identical with that of plasma membrane and distinct from that of endoplasmic reticulum. These results are in agreement with recent reports which have demonstrated the association of pp60$^{src}$ with plasma membranes of permissive and nonpermissive cells by employing either subcellular fraction methods (16) or electron microscopic immunocytochemistry techniques (28). The electron microscopic immunocytochemistry studies of Willingham et al. (28) indicate that pp60$^{src}$ is concentrated on the inner surface of the plasma membrane of ASV-infected NRK cells, primarily in regions under ruffles and near junctions connecting adjacent cells. This observation is consistent with our inability to radiolabel pp60$^{src}$ in intact cells by lactoperoxidase-catalyzed iodination (Krzyzek et al., unpublished data).

In addition to the finding that the amount of pp60$^{src}$ associated with the plasma membrane is similar for both transformed and morphologically reverted vole cells, it also appears that in both vole cell types as well as in ASV-infected CEF pp60$^{src}$ appears to be directly associated with the plasma membrane, possibly as an intrinsic membrane protein, and not merely absorbed to it during cell fractionation. This is indicated by the inability to extract pp60$^{src}$ from plasma membranes with high salt concentrations, a condition which removes both absorbed and peripheral membrane proteins (11, 26, 29). Partial extraction of pp60$^{src}$ could be achieved with nonionic detergent, and the extent of solubilization was somewhat greater for SR-ASV-infected CEF than for vole cells. Krueger et al. (16) recently reported a similar effect of high salt concentrations and nonionic detergent on the extraction of pp60$^{src}$ from plasma membranes of SR-ASV-infected CEF. Our results tentatively suggest that in both transformed and reverted mammalian cells and ASV-infected CEF pp60$^{src}$ is localized in a similar membrane environment.

The intimate association of the majority of pp60$^{src}$ with the plasma membrane suggests that some or all of the numerous cellular changes accompanying ASV infection could result from the interaction of pp60$^{src}$ with cell membrane proteins. Because these changes are pleiotropic, it is conceivable that pp60$^{src}$ interacts with several distinct plasma membrane proteins. Although revertant cells no longer exhibit the morphologically transformed phenotype, they are nevertheless partial revertants since they express several other parameters of the transformed phenotype, such as tumorigenicity, growth in agar, and secretion of high levels of plasminogen activator (20, 21). Moreover, it appears that the loss of morphological transformation is not associated with a functional defect in pp60$^{src}$ itself, because ASV rescued from revertant cells can morphologically transform CEF (17). In addition, our studies do not indicate any significant differences in either the amount or the nature of association of pp60$^{src}$ from transformed or revertant vole cells with the plasma membrane. Together, these observations suggest that morphological reversion results from a defect in some cell protein, possibly membrane associated, with which pp60$^{src}$ must interact to induce morphological transformation. Studies are currently in progress to identify this protein.

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