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Cellular Localization of the Transforming Protein of Wild-Type and Temperature-Sensitive Fujinami Sarcoma Virus

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Fujinami sarcoma virus (FSV) encodes a 140,000-dalton transforming protein, P140, which contains *gag*- and *fps*-specific sequences. The cellular localization of this protein was examined by fractionation of [^{35}S]methionine-labeled, FSV-infected chicken embryo fibroblasts. In homogenates of cells infected by wild-type, temperature-resistant FSV prepared in either hypotonic or isotonic buffer, 60 to 80% of the P140 was particulate. Isopycnic separation on discontinuous sucrose gradients indicated that the majority of the particulate P140 was present in a light membrane fraction enriched for plasma membranes. Much of the particulate P140 could be solubilized by the addition of 0.6 M salt to a postnuclear supernatant, suggesting that P140 is not an integral membrane protein. Particulate P140 may be asociated with membranes either directly as a peripheral membrane protein or indirectly via cytoskeletal elements. In cells infected by mutants of FSV temperature sensitive for cellular transformation, most of the P140 is particulate at the permissive temperature, whereas most is soluble at the nonpermissive temperature; this change in distribution is not a secondary consequence of the change in cellular phenotype, since it also occurs in nonconditionally transformed cells doubly infected with temperature-sensitive FSV and wild-type Rous sarcoma virus. The movement of P140 from the particulate to the soluble fraction occurs rapidly when cells infected by temperature-sensitive FSV are shifted from the permissive to the nonpermissive temperature. Furthermore, P140 moves from the soluble to the particulate fraction, although somewhat more slowly, when cells are shifted from the nonpermissive to the permissive temperature. These observations suggest that the association of P140 with plasma membranes or the cytoskeleton may play a role in transformation by FSV.

The genome of Fujinami sarcoma virus (FSV) encodes a 140,000-dalton protein containing N-terminal *gag*-related sequences and C-terminal FSV-specific or *fps* sequences (23, 33, 36). In vivo, P140 is phosphorylated at serine within the *gag* portion of the protein and at both serine and tyrosine in the *fps* portion (14, 36). In vitro, in the immune complex kinase assay P140 becomes phosphorylated at tyrosine, and it or an associated kinase catalyzes the phosphorylation of exogenously added substrates (14, 23, 36). FSV infection results in the phosphorylation of a number of cellular polypeptides at tyrosine residues (4, 8, 36). In cells infected by mutants of FSV temperature sensitive for transformation (*ts*FSV), the in vivo phosphorylation of sites within the *fps* region, the in vitro phosphorylation of P140 at tyrosine and the in vivo phosphorylation of cellular polypeptides at tyrosine are all temperature dependent (36). These findings indicate that P140-dependent phosphorylation of cellular polypeptides at tyrosine residues is involved in transformation by this virus.

The properties of P140 described above are in many respects similar to those previously reported for the Rous sarcoma virus (RSV) transforming protein, pp60^{src} (8, 9, 24, 38, 42). Furthermore, the phenotype of FSV-transformed cells is similar to that of RSV-transformed cells, although the changes observed are in many cases less marked than those observed with RSV (22). These observations suggest that RSV and FSV may transform by similar mechanisms. Experiments with cell fractionation have shown that most of pp60^{src} in RSV-transformed chicken embryo fibroblasts is recovered in particulate fractions enriched for plasma membranes and can be solubilized only with detergent (12, 30, 31). Immunocytochemical analyses have shown that pp60^{src} is exposed on the cytoplasmic face of the plasma membrane and is concentrated in areas of cell-substrate or cell-cell contact (40, 44). We have used cell fractionation techniques to compare the cellular location of FSV P140 with that of pp60^{src} in chicken embryo fibroblasts. We report here that the particulate fraction of P140 is membrane associated, either directly as a peripheral membrane protein or indirectly via cytoskeletal elements, and this association is temperature dependent in cells infected by *ts*FSV. Feldman et al. (15) and Woolford and Beemon (45) have recently reported the results of similar studies on the P130 encoded by a related strain of FSV.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEF) were infected with a *ts*FSV (36) or with a wild-type, temperature-resistant strain (*tr*FSV) isolated by Wen-Hwa Lee from a chicken tumor induced with the *ts*FSV (33). Virus stocks were concentrated by ultrafiltration to achieve high multiplicities of infection. The infected cells were grown at 38°C for 3 days, subcultured once at 2.5 × 10^6 cells per 100-mm dish, and labeled 2 days later.

Radiolabeling. Cells were preincubated for 10 min in methionine-free Dulbecco modified Eagle medium containing 4% calf serum and 1% chicken serum. The preincubation medium was replaced with the same medium supplemented with 15% of the normal amount of methionine and 200 μCi of [^{35}S]methionine (Amersham Corp.) per plate. The cells were labeled for 16 to 18 h at either 36 or 41°C. Chase experiments were performed by removing the labeling medium, rinsing

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the culture twice in complete medium (Dulbecco modified Eagle medium containing 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum), and then incubating the cells in complete medium for 30 min before another change to complete medium for the duration of the chase period.

Preparation of homogenates. The preparation of homogenates and subsequent fractionation procedures were performed essentially as described by Radke et al. (37). All procedures were performed at 0 to 4°C. Two to five 100-mm plates containing a total of 2.0×10^7 to 7.5×10^7 cells were rinsed twice with phosphate-buffered saline (PBS), scraped into PBS, and pelleted for 1 min at $1,000 \times g$ in a clinical centrifuge. After the pellet was rinsed once with hypotonic TKM buffer (20 mm Tris [pH 7.1], 5 mM KCl, 1 mM $MgCl_2$, 1% Aprotinin), the cells were dispersed and swollen in TKM for 15 min. The cell suspension was homogenized by 20 strokes in a tight-fitting Dounce homogenizer. The volume was adjusted to exactly 3 ml with TKM buffer, and samples were removed for analysis by immunoprecipitation or for marker enzyme assays.

Fractionation. In the initial series of experiments a nuclear pellet was generated by low-speed centrifugation, and the supernatant from this initial centrifugation was subjected to high-speed centrifugation to obtain a particulate and a soluble fraction. To obtain a nuclear pellet, the homogenate was centrifuged at 1,500 rpm in a Sorvall HB4 rotor ($375 \times g$) for 10 min at 0°C, and the crude nuclear pellet was suspended in 1 ml of TKM. This pellet was then homogenized five times in the Dounce homogenizer and aspirated three times through a 1-ml syringe fitted with a 25-gauge needle. The suspension was then pelleted as described above, suspended in TKM buffer, and aspirated again five times through the same syringe. After a final centrifugation, the nuclear pellet was suspended in TKM buffer and analyzed for P140 content and subcellular markers as described below. The original postnuclear supernatant (PNS) and the supernatants from the nuclear pellet washes were pooled and centrifuged in thick-walled polyallomer tubes in a Beckman SW50.1 rotor at 38,000 rpm ($150,000 \times g$) for 90 min at 0°C to generate particulate (P_{150}) and soluble (S_{150}) fractions.

In experiments designed to compare the solubility of P140 under different conditions, the homogenates were (i) centrifuged directly at $150,000 \times g$ as described above to generate a soluble fraction and a crude particulate fraction or (ii) used to prepare a PNS, which was then loaded directly onto a sucrose gradient as described below.

Sucrose gradients. Further fractionation of the P_{150} fraction or PNS was accomplished by sucrose density centrifugation as described by Courtneidge et al. (12), with the following modifications. The P_{150} fraction was suspended in approximately 3 ml of gradient buffer (5 mM KCl, 5 mM NaCl, 0.1 mM EDTA, 20 mM Tris [pH 7.1], and 1% Aprotinin). The pellet was dispersed by aspirating it five times through a 25-gauge syringe needle, followed by five strokes in a tight Dounce homogenizer. The dispersed material was loaded on top of a discontinuous sucrose gradient consisting of successive 2.5-ml layers of 50, 40, 35, and 20% (wt/vol) sucrose, all in gradient buffer, in a 12-ml polyallomer tube. In experiments designed to fractionate the PNS directly, a measured sample of this material was loaded directly onto the gradient (37). After centrifugation for 2 h at 40,000 rpm in a SW41 rotor at −2°C, the material included in each interface and the layer above the interface was collected. The fractions were then adjusted to equal volumes with sucrose and gradient buffer and assayed directly for P140 and subcellular markers.

Immunoprecipitation and gel analysis of subcellular fractions. An equal percentage of each fraction to be analyzed (usually the material from approximately 10^6 cells) was treated with a mixture of DNase I (Worthington Diagnostics) and RNase A (Worthington) for 10 min at 0°C and then extracted with RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% Aprotinin, 20 mM Tris [pH 7.4]) for 20 min at 0°C. The lysates were centrifuged for 10 min at 10,000 rpm in a Sorvall SS34 rotor ($39,000 \times g$) at 0°C. Clarified lysates were cleared of non-specifically binding proteins by preprecipitation with normal goat serum and fixed *Staphylococcus aureus* (IgGsorb; Enzyme Center). The preprecipitated lysates were then incubated with anti-*gag* serum (goat anti-p19/p27; provided by the National Cancer Institute) for 45 to 60 min, and the immune complexes were adsorbed with IgGsorb. The bound immune complexes were washed and isolated as described by Gilmore et al. (18) and analyzed by electrophoresis on a 7.5% sodium dodecyl sulfate–polyacrylamide gel. An equal percentage of the homogenate and each fraction was analyzed with appropriate corrections for samples removed during prior steps; thus if the recovery were 100%, the sum of P140 in the subcellular fractions would equal the amount in the homogenate. The recovery of P140 in these experiments averaged over 80%. The gels were fixed, stained, dried, and fluorographed, and the band corresponding to P140 was excised and counted as previously described (37).

Marker assays. Plasma membrane content was determined by measuring 5′ nucleotidase (3, 37). The samples were taken up in TKM buffer and incubated in an assay mixture containing 10 mM $MgCl_2$, 0.1 mM AMP, 100 mM glycine (pH 9.0), and 2 μCi of [^{3}H]AMP (Amersham) per ml. After 30 min at 37°C, unhydrolyzed AMP was precipitated at room temperature with freshly prepared solutions of $Ba(OH)_2$ and $ZnSO_4$, and the amount of free [^{3}H]adenosine in the supernatant was determined by liquid scintillation counting (27). The amount of free adenosine corresponded to measurements of free phosphate, but the former measurement proved to be the most reliable and was used as the standard measurement of enzyme activity (P. Moss, unpublished data). The soluble protein in each fraction was determined by assaying for lactate dehydrogenase activity (26), and the endoplasmic reticulum content was measured with an assay for NADH diaphorase (3). Protein was measured with the Bradford assay (6).

Immunofluorescence. Immunofluorescent staining was carried out as previously described (37). Briefly, uninfected or FSV-transformed CEF (10^4 cells) were seeded onto 12-mm glass cover slips and used 18 h later for immunofluorescent staining. The cells were washed once with PHEM buffer [0.06 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 0.025 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 0.01 M ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 0.002 M $MgCl_2$ (pH 6.9)] (41) and fixed with 2% paraformaldehyde in PHEM buffer for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in PHEM buffer for 20 min at room temperature. Fixed and permeabilized cells were washed once in PHEM buffer and three times in PBS. Each cover slip was incubated with 12 μl of a 1:80 dilution of a rat anti-P140 serum, which has been shown to be specific for the *fps* protein (J. Young and G. S. Martin, submitted for publication), or preimmune serum in a moist chamber for 45 min at room temperature. After three washes in PBS, the cover slips were incubated with 12 μl (25 μg/ml) of fluorescein

isothiocyanate-conjugated rabbit anti-rat immunoglobulin G (Sigma Chemical Co.) for 45 min at room temperature. The cover slips were again washed extensively in PBS and incubated at room temperature for 45 min with rhodamine-conjugated phalloidin (20 μg/ml), which was kindly provided by T. Wieland (Max Planck Institute for Medicine, Heidelberg, Federal Republic of Germany) (46). Stained preparations were mounted in PBS-glycerol (1:9) containing the anti-bleaching agent *p*-phenylenediamine (25) and viewed with a Zeiss photomicroscope III with a Planachromat 40×/0.65 objective with appropriate barrier filters. Photographs were made with Kodak Plus-X film that was developed with Ilford microphen developer.

RESULTS

Distribution of P140 in *tr*FSV transformed cells. A homogenate prepared in hypotonic buffer as described above was fractionated to obtain a nuclear pellet, a high-speed pellet (P_{150}), and a high-speed supernatant (S_{150}). The bulk of [^{35}S]methionine-labeled P140 from *tr*FSV-transformed cells fractionated by this procedure was found in the P_{150} fraction (Fig. 1); similar results were obtained with the immune complex kinase assay (data not shown). Phase-contrast microscopy of the nuclear fraction revealed that over 90% of the nuclei were intact, a conclusion that was supported by experiments demonstrating that in cells prelabeled with [^{3}H]thymidine, 94% of the isotope was recovered in the final nuclear pellet (Table 1). The nuclear fraction, however, routinely contained 20 to 25% of the plasma membrane marker, 5′ nucleotidase, as well as a small percentage of the cytosol marker lactate dehydrogenase (Table 1) and p27 (Fig. 1). The small amount of membrane contamination in the nuclear pellet may account for the small percentage of P140 in this fraction. Similarly, the S_{150} fraction contained low levels of 5′ nucleotidase and NADH diaphorase activity, corresponding roughly to the amount of P140 found in this fraction (Table 1). These results suggested that P140 was primarily associated with the membranous material of the P_{150} fraction.

Further fractionation of the P_{150} on discontinuous sucrose gradients revealed that temperature-resistant P140 (*tr*P140) was recovered primarily from the 20%–35% interface, which was greatly enriched for 5′ nucleotidase activity (Fig. 2, Table 2). The 20%–35% interface also contained 26% of the marker for endoplasmic reticulum, NADH diaphorase, but most of this activity was found at the 40%–50% interface, where only 11% of the P140 was recovered. These results demonstrate that particulate P140 fractionates predominantly with plasma membranes or associated cytoskeletal components.

To test the possibility that the inital pelleting step might result in an artifactual binding of P140 to plasma membranes, a PNS from a hypotonic homogenate of FSV-transformed cells was analyzed by directly layering it onto a discontinuous sucrose gradient. Under these conditions, virtually all of the soluble marker (lactate dehydrogenase) remained in the PNS, whereas the plasma membrane marker (5′ nucleotidase) was retained at the 20%–35% and 35%–40% interfaces; similarly, only 26% of P140 remained in the PNS, and 64% was recovered from the 20%–35% and 35%–40% interfaces (Table 3). Thus a substantial portion of the membrane association of P140 is not dependent on prior pelleting, suggesting that it is not artifactually induced by this pelleting step.

After the experiments described above had been completed, we learned of similar experiments carried out by Feldman et al. (15), whose results indicated that the subcellular association of P140 with particulate components was salt sensitive. To examine the effects of salt concentrations on the association of P140 with cellular membranes, increasing quantities of NaCl were added to samples of a PNS prepared from a hypotonic homogenate of FSV-transformed cells, and the samples were centrifuged to obtain particulate and soluble fractions (Fig. 3). As the salt concentration was raised, increasing quantities of P140 were found in the soluble fraction. At physiological salt concentration, however, P140 was still largely associated with particulate material. When a homogenate was brought to isotonic salt concentration and loaded onto a discontinuous sucrose gradient, the bulk of recovered P140 migrated with the light-density membranes (data not shown). The possibility that Mg^{2+} might cause aggregation of P140 to membranes (20) was eliminated by separate experiments, which demonstrated that the presence or absence of 1 mM Mg^{2+} in the homogenization buffer did not significantly affect the results (data not shown). We conclude that P140 is associated with membranes, perhaps as a peripheral membrane protein attached to membranes via other structural elements such as the cytoskeleton.

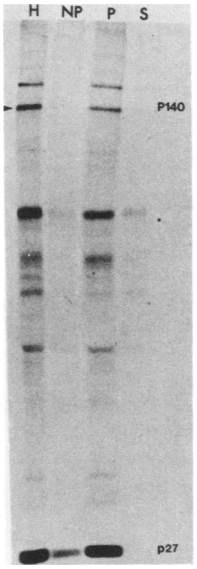

FIG. 1. Distribution of P140 in subcellular fractions of *tr*FSV-transformed cells homogenized in hypotonic buffer. Infected CEF were labeled with [^{35}S]methionine, homogenized, and fractionated by differential centrifugation as described in the text. P140 (arrow) was immunoprecipitated from each fraction with anti-*gag* serum, and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Abbreviations: H, homogenate; NP, nuclear pellet; P, P_{150}; S, S_{150}. The distribution of the [^{35}S]methionine counts in P140 recovered in each fraction was as follows: nuclear pellet, 14%; P_{150}, 68%; S_{150}, 18%.

Immunofluorescent localization of the *fps* protein. To visualize the location of P140$^{gag\text{-}fps}$, we used a rat anti-*fps* serum (Young and Martin, submitted for publication) for indirect immunofluorescence staining of paraformaldehyde-fixed, *tr*FSV-transformed CEF. The anti-P140 serum preabsorbed with disrupted FSV virions to remove anti-*gag* reactivity yielded an immunofluorescent staining pattern identical to that of unabsorbed serum (data not shown), indicating that the observed fluorescence was specific for *fps* sequences of the P140 molecule. No staining of cells was observed unless the fixed cells were permeabilized, suggesting that the P140

TABLE 1. Distribution of *tr*FSV P140 and marker enzymes in subcellular fractions prepared in hypotonic buffer

Fraction	Mean % in fraction (range)[a]					
	P140	Protein	[^{3}H]thymidine	Lactate dehydrogenase	5′ Nucleotidase	NADH diaphorase
NP	17 (11–23)	22 (14–26)	94	5 (3–11)	21 (18–25)	10 (9–12)
P_{150}	65 (59–68)	25 (21–32)	5	7 (5–18)	63 (61–70)	79 (75–83)
S_{150}	18 (15–23)	53 (41–56)	1	89 (86–93)	16 (12–20)	11 (8–13)

[a] Actual recoveries relative to the amount in the homogenate: P140, 69%; protein, 76%; [^{3}H]thymidine, 84%; lactate dehydrogenase, 87%; 5′ nucleotidase, 71%; NADH diaphorase, 68%.

protein was not located on the outer cell surface. In fixed FSV-transformed cells permeabilized with 0.1% Triton X-100, a mottled, nonnuclear pattern of fluorescence was observed (Fig. 4A). One striking feature of the pattern of fluorescence was an exclusion of staining from areas resembling vacuoles. To ensure that the cells we examined in the FSV-transformed population were fully transformed, we stained with rhodamine-conjugated phalloidin in a double-label experiment. Phalloidin binds exclusively to polymerized forms of actin (46), and the loss of actin cables is a more sensitive criterion of transformation in FSV-transformed cells than is overall cellular morphology (5). The decoration of actin filaments with phalloidin is shown (Fig. 4B) in the same cells that are stained with anti-*fps* serum. Extensive loss of actin microfilament bundles was evident in only one of the cells pictured, and this same cell exhibited a strong pattern of fluorescence with the rat anti-*fps* serum. Conversely, the cell with extensive actin microfilament bundles showed only very faint staining with the rat anti-*fps* serum, indicating that this cell was expressing only low levels of P140 and was probably not transformed. Incubation of cells with preimmune rat serum did not give significant levels of fluorescence (data not shown). However, because we were unable to detect a specific plane of fluorescence, we were not able to distinguish between a peripheral or cortical location and an intracytoplasmic location.

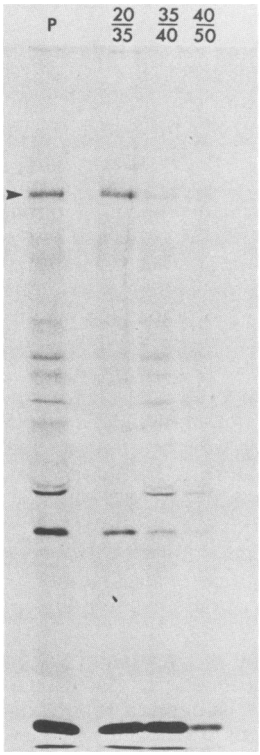

FIG. 2. Discontinuous sucrose gradient fractionation of a P_{150} fraction derived from a hypotonic postnuclear supernatant of *tr*FSV-transformed cells. The suspended P_{150} was fractionated on a step gradient and analyzed for P140 content by immunoprecipitation as described in the text and in the legend for Fig. 1. The percentage of P140 (arrow) at each interface was as follows: 20%–35%, 69%; 35%–40%, 23%; 40%–50%, 8%.

Distribution of P140 in *ts*FSV-transformed cells. To determine whether transformation could be correlated with the subcellular location of P140, we used a mutant of FSV that is temperature-sensitive for transformation (36). Fractionation of *ts*FSV cells grown at the permissive temperature of 35°C demonstrated that, like *tr*P140, most of the *ts*P140 partitioned with the P_{150} fraction, and that the bulk of the particulate material comigrated with plasma membranes after sucrose gradient centrifugation (data not shown). However, direct fractionations of either hypotonic or isotonic homogenates prepared from *ts*FSV-infected cells grown and labeled at 35 or 41°C demonstrated that the relative amount of *ts*P140 in the S_{150} fraction increased approximately twofold in infected, nontransformed cells grown at 41°C (Fig. 5A). In comparison, P140 from *tr*FSV-transformed cells remained predominantly in the P_{150} fraction in cells cultivated at either temperature (Fig. 5B). Since the distribution of the subcellular markers did not change significantly in *tr*FSV- or *ts*FSV-infected cells grown at either temperature (data not shown), it appears that *tr*P140 and *ts*P140 are associated primarily with the plasma membrane or membrane-associated structures only under conditions in which the cells are transformed.

To examine the kinetics of movement of P140 to the soluble fraction after a temperature shift, the following experiment was performed. *ts*FSV-transformed cells were prelabeled for 18 h at 35°C, shifted in unlabeled medium to 41°C, and analyzed for P140 content in the P_{150} and S_{150} fractions. The results showed that *ts*P140 was recovered predominantly in the S_{150} fraction within 30 min after the temperature shift, suggesting that previously synthesized P140 that had been membrane associated became soluble

TABLE 2. Distribution of *tr*FSV P140 and subcellular markers after fractionation of the P_{150} on discontinuous sucrose gradients[a]

Sucrose interface	% at interface[b]			
	P140	Protein	5′ Nucleotidase	NaDH diaphorase
20%–35%	66	47	61	26
35%–40%	23	24	23	31
40%–50%	11	29	16	43

[a] The percentages are based on the total recovered in the fractions assayed.
[b] Actual recoveries relative to the amount in the P_{150} loaded onto the gradient: P140, 48%; protein, 49%; 5′ nucleotidase, 94%; NADH diaphorase, 56%

TABLE 3. Distribution of *tr*FSV P140 in discontinuous sucrose gradients after direct fractionation of the PNS

Fraction	% in fraction[a]				
	P140	Protein	Lactate dehydrogenase	5′ Nucleotidase	NADH diaphorase
PNS[b]	28	53	93	6	36
20%–35%	35	31	6	58	24
35%–40%	29	13	<1	31	27
40%–50%	8	3	<1	4	13

[a] Recovery relative to amount loaded onto gradient: P140, 43%; protein, 87%; lactate dehydrogenase, 96%; 5′ nucleotidase, 59%; NADH diaphorase, 41%.

[b] PNS after gradient centrifugation. Gradient fractions include the interface and the layer immediately above (e.g., 20%–35% includes the 20% layer as well as the 20%–35% interface).

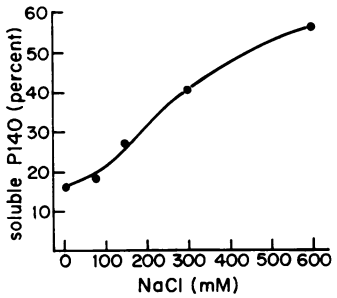

FIG. 3. Solubilization of *tr*FSV P140 by NaCl. *tr*FSV-transformed CEF were homogenized in TKM buffer. The homogenate was then immediately divided into 5 samples, each of which was adjusted with NaCl to the concentration indicated on the abscissa. P_{150} and S_{150} fractions were generated directly and analyzed by immunoprecipitation for P140 as described in the legend for Fig. 1. The P140 band (arrow) was excised, and the radioactivity determined as described in the text. The recovery of P140 in the particulate and soluble fractions was normalized to 100%.

after the shift (Fig. 6A). *ts*FSV-transformed cells maintained and chased at 35°C did not show any change in P140 location. Identical experiments with *tr*FSV-infected cells showed only a small change in the distribution of *tr*P140 after the shift to the nonpermissive temperature (Fig. 6B). These results suggest that there is a rapid movement of *ts*P140 to the soluble fraction which is not due to a transient response to the temperature change and which appears to be a specific property of the mutant protein. However, it should be noted that although Lee et al. (32) reported that *ts*P140 turns over at the same rate at permissive and nonpermissive temperatures, under the conditions of these temperature shift experiments there appears to be enhanced turnover of *ts*P140 (Fig. 6A); thus we cannot exclude the possibility that the temperature shift induces selective degradation of membrane-associated P140 in *ts*FSV-infected cells.

To determine whether soluble P140 synthesized in *ts*FSV-infected cells grown at the nonpermissive temperature could associate with the plasma membrane after a shift to the permissive temperature, *ts*FSV-infected cells were grown and labeled for 18 h at 41°C and then chased at 35°C for 2 h. The association of P140 with the plasma membrane occurred after the temperature downshift (Fig. 7). This association was slow by comparison with the dissociation that occurred on a shift up to the nonpermissive temperature (Fig. 6). Virtually identical results were obtained when cycloheximide (5 μg/ml) was added to the chase medium, suggesting that label reutilization was not contributing to the increase in the amount of P140 in the P_{150} fraction after the shift to 35°C (data not shown).

To demonstrate that the temperature-sensitive association of P140 with the particulate fraction is a property of the temperature-sensitive mutant protein and not a secondary consequence of a change in cellular phenotype or altered functional properties of the transformed cell, the following experiment was performed. Cells transformed by *ts*FSV were superinfected with Schmidt-Ruppin-D RSV to generate doubly infected cells. These cells synthesized both $pp60^{src}$ and P140, but acquired the rounded morphology of SR-D-transformed cells at both 35 and 41°C. When these doubly infected cells were fractionated into P_{150} and S_{150} fractions, *ts*P140 was predominantly particulate at 35°C and soluble at 41°C as in cells transformed with *ts*FSV alone, whereas $pp60^{src}$ remained in the particulate fraction at both temperatures (Fig. 8). These results also demonstrate that the temperature-dependent behavior of *ts*P140 is a specific property of the mutant FSV protein.

DISCUSSION

Our results indicate that when FSV-transformed chicken embryo fibroblasts are homogenized and subjected to conventional cell fractionation procedures, the majority of the FSV-encoded P140 is particulate and can be recovered in a light-density membrane fraction enriched for plasma membranes. RSV $pp60^{src}$ is also recovered in plasma membrane fractions (12, 30, 31), and immunocytochemical studies have shown that $pp60^{src}$ is present on the cytoplasmic face of the plasma membrane, particularly in regions of cell-cell and cell-substrate contact where the cytoskeletal protein vinculin is localized (17, 40, 44). However, $pp60^{src}$ behaves as an integral membrane protein in that it can be solubilized only by nonionic or ionic detergents (11, 30, 31); although $pp60^{src}$ does not contain any extensive hydrophobic sequences, it is covalently modified by the attachment of lipid, and this may contribute to its tight association with membranes (16, 43). In contrast, FSV P140 can be solubilized by high concentrations of salt, as is also the case for the P130 encoded by a related strain of FSV in transformed rat cells (15). Thus P140 does not behave as an integral membrane protein. In addition, P140 does not appear to contain covalently bound lipid (B. Sefton, personal communication).

The initial question raised by these observations is whether the fractionation behavior of P140 reflects its intracellular location in vivo, or whether P140 adventitiously associates with cytoplasmic structures during or after homogenization. It seems likely that the cofractionation of P140 with membranes is physiological rather than an artifactual association induced during homogenization. P140 fractionates with membranes under a variety of conditions: centrifugation of homogenates prepared under either hypotonic or isotonic conditions (Table 1, Fig. 5), direct fractionation of a PNS on discontinuous sucrose gradients (Table 3), and homogenization of the cells in the presence or absence of Mg^{2+} (data not shown). However it should be noted that the salt sensitivity of the membrane association as reported here differs slightly from that reported by others. Thus Feldman et al. (15) report that about 30 to 40% of P130 is soluble at isotonic salt, with about 60 to 80% soluble at 200 to 250 mM salt, whereas Woolford and Beemon (45) report that about 60% is soluble at isotonic salt. Thus the precise distribution of P140 or P130 in vivo between soluble cytosolic and membrane-associated

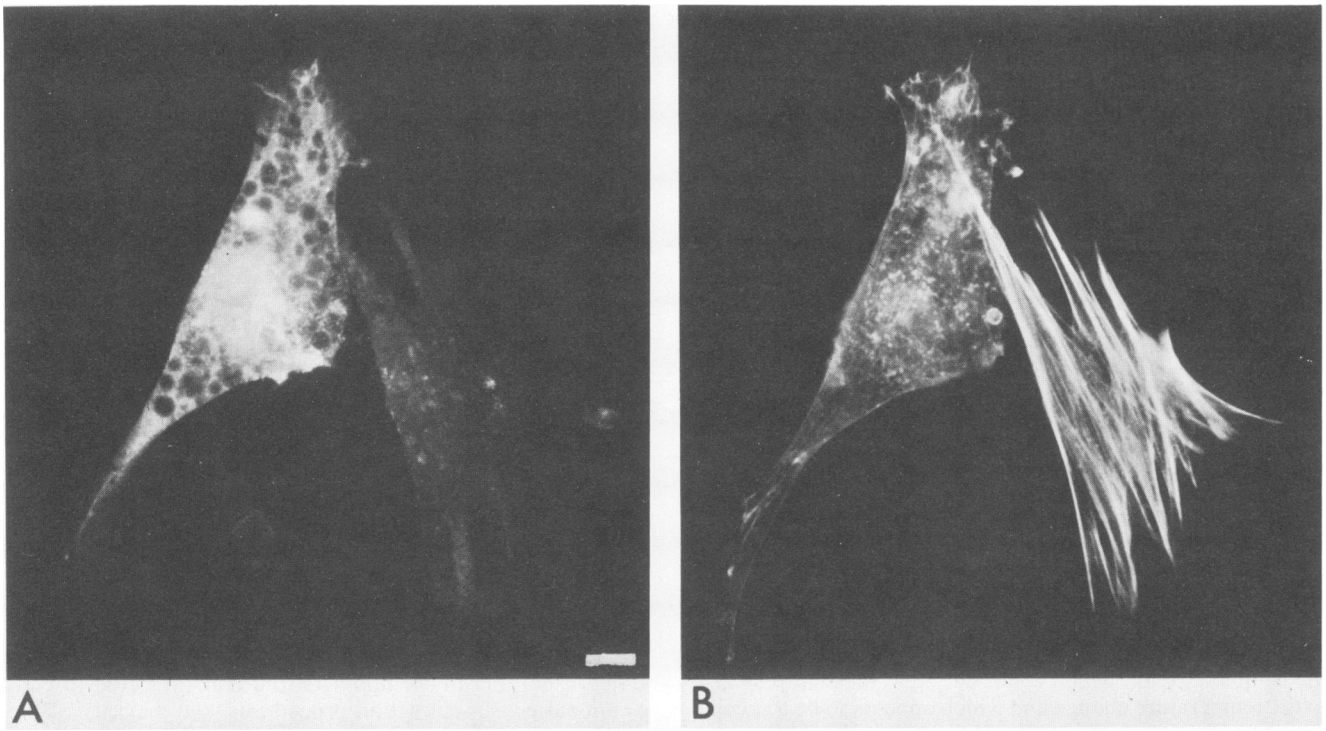

FIG. 4. Immunofluorescence staining of P140 in *tr*FSV-transformed cells. *tr*FSV-transformed CEF growing on glass cover slips were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were treated sequentially with rat anti-*fps* serum, with fluorescein isothiocyanate-conjugated second antibody, and then with rhodamine-conjugated phalloidin to stain actin cables. Cells in the same field were photographed in the same focal plane with barrier filters appropriate to show (A) P140 staining and (B) F-actin staining. Photographs were taken with a 40× objective. Bar, 10 μm.

forms remains uncertain. P91, the transforming protein of the recombinant virus F36, which contains *fps*-specific sequences but no *gag*-derived amino acid sequences, is also particulate; this suggests that the membrane association of the *fps* gene product is not due to the *gag*-encoded portion of P140 (Young and Martin, submitted for publication).

It is likely that membrane fractions isolated by isopycnic banding contain cytoskeletal and other subcellular components, and thus it is not yet possible to define the precise cellular component with which P140 interacts. The data reported here indicate that P140 is recovered in light-membrane fractions, and similar findings have been reported by Woolford and Beemon (45), whereas Feldman et al. found that sedimentable cytoplasmic complexes containing P140 do not cofractionate with membranes (15). It is possible that P140 does not interact with membranes directly, but with a structural protein matrix that, under the conditions used here, cofractionates with membranes. The latter possibility is consistent with the finding that the related protein $P105^{gag\text{-}fps}$ encoded by PRC II is recovered in the detergent insoluble matrix when PRC II-transformed cells are briefly exposed to low concentrations of nonionic detergents (1), and that P130 is also detergent insoluble except at high salt concentrations (15).

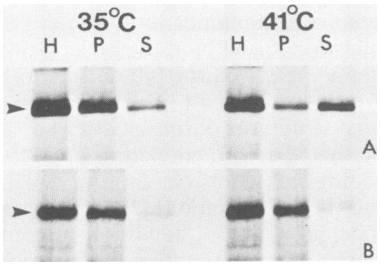

FIG. 5. Comparison of the distribution of P140 in *ts*FSV- and *tr*FSV-infected cells at 35 and 41°C. (A) Distribution of P140 in fractions of *ts*FSV-transformed cells grown at temperatures permissive (35°C) and nonpermissive (41°C) for transformation. Homogenates were centrifuged directly into P_{150} and S_{150} fractions, and P140 was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described in the legend to Fig. 1. Only the P140 region of the gel is shown. The percentage of P140 (arrow) in the pellet (P) was 74% at 35°C and 39% at 41°C. (B) Distribution of P140 in *tr*FSV-transformed cells grown at 35 and 41°C. The percentage of P140 in the pellet (P) was 82% at 35°C and 79% at 41°C.

It is clearly desirable to confirm the results of cell fractionation with immunochemical studies. Our immunofluorescence micrographs reveal a diffuse background staining (possibly soluble P140) and a coarse or patchy staining pattern that may be either intracytoplasmic or cortical. Feldman et al. (15) concluded on the basis of similar data that P130 was largely cytoplasmic, with some localization in areas of cell contact and cell ruffles. However, the absence of distinct peripheral staining does not exclude a cortical or plasma membrane association. In flattened cells the cell margins are quite thin; for example, staining of spectrin or the 36K substrate for tyrosine phosphorylation two proteins known to be plasma membrane associated, reveals a patchy network without marked peripheral staining (7, 11, 19, 37). Furthermore, the proportions of soluble P140 reported here and by Feldman et al. (15) may be sufficiently high to make it impossible to resolve by immunofluorescence any plasma

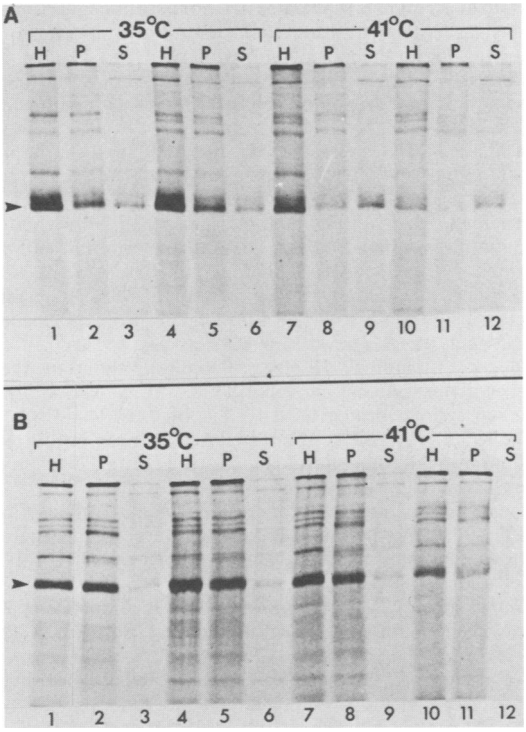

FIG. 6. (A) Distribution of P140 in *ts*FSV-infected cells shifted from 35 to 41°C. Each set of cells was labeled with [^{35}S]methionine for 18 h at 35°C, chased with cold methionine-containing medium for 30 min at 35°C, and then either shifted to 41°C for 30 min or 2 h or maintained at 35°C for 2 h. Cells were fractionated into P_{150} and S_{150} fractions and analyzed for P140 content as described in the legend for Fig. 1. Only the P140 region of the gel is shown. Tracks: 1 through 3, after 30-min chase at 35°C; 4 through 6, after 2-h chase at 35°C; 7 through 9, after 30-min chase at 41°C; 10 through 12, after 2-h chase at 41°C. Abbreviations: H, homogenate; P, P_{150}; S, S_{150}. The percentage of particulate P140 was 69% in track 2, 71% in track 5, 40% in track 8, and 31% in track 11. Only the upper one-third of the fluorograph is shown. (B) Distribution of P140 in *tr*FSV-transformed cells prelabeled at 35°C and chased at 35 or 41°C. Tracks and abbreviations are as in A. The percentage of particulate P140 was 84% in track 2, 83% in track 5, 79% in track 8, and 80% in track 11.

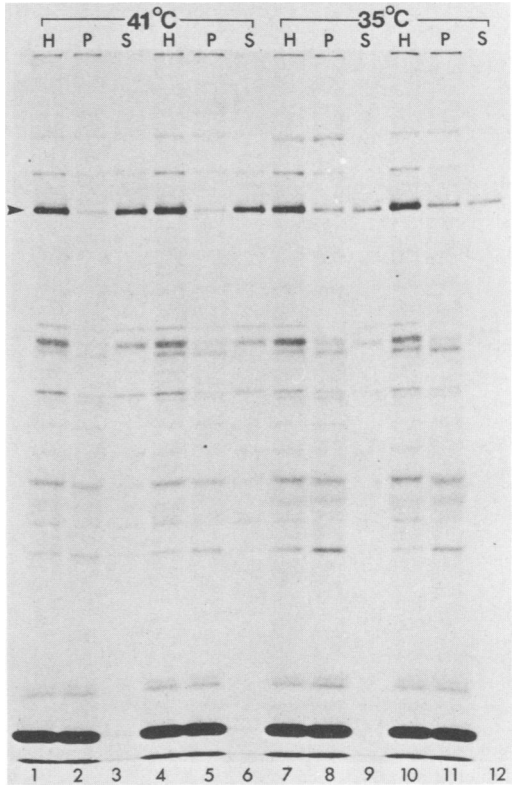

FIG. 7. Distribution of P140 in *ts*FSV-infected cells prelabeled at 41°C and shifted to 35°C. Each set of cells was labeled with [^{35}S]methionine for 18 h at 41°C, chased for 30 min at 41°C, and then shifted to 35°C for 30 min or 2 h or maintained at 41°C for an additional 2 h. Cells were fractionated and analyzed for P140 content (arrow) as described in the legend for Fig. 1. Tracks 1 through 3, after 30-min chase at 41°C; 4 through 6, after 2-h chase at 41°C; 7 through 9, after 30-min chase at 35°C; 10 through 12, after 2-h chase at 35°C. Abbreviations: H, homogenate; P, P_{150}; S, S_{150}. The percentage of particulate P140 was 31% in track 2, 29% in track 5, 43% in track 8, and 52% in track 11.

membrane association of the particulate P140. Immunoelectron microscopy will be necessary to determine the precise intracellular location of the particulate fraction of P140 in these cells.

The finding that more than 60% of *ts*P140 is solubilized when *ts*FSV-transformed cells are shifted to the nonpermissive temperature raises the possibility that the association of P140 with membranes or the cytoskeleton (or both) is required for transformation. A similar temperature-sensitive association with plasma membranes has been reported for *ts*pp60^{src} (10a). In cells infected with recovered avian sarcoma viruses, mutants in which pp60^{src} is predominantly soluble show a decreased tumorigenicity in vivo (28). Furthermore, the normal cell homolog of P140, NCP98 (34), is primarily soluble at physiological salt (Young and Martin, submitted for publication), suggesting that particulate association may correlate with the transforming activity of P140. However, soluble P140 appears to function as a phosphoacceptor in the immune complex kinase assay (15; Gilmore and Moss, unpublished observations), and if this represents a valid assay of the functional state of the protein these observations would suggest that membrane association is not required for the activation of the protein. Furthermore, approximately 30% of *ts*P140 remains particulate at the nonpermissive temperature, whereas cells revert to a nearly normal phenotype and the level of phosphorylation of cellular polypeptides at tyrosine falls to a level comparable to that of uninfected cells (36). It therefore seems unlikely that dissociation of *ts*P140 from a structural matrix is sufficient in itself to explain the phenotypic reversion of *ts*FSV-infected cells at the nonpermissive temperature. Furthermore, Feldman et al. (15) have reported that two other *ts*FSV mutants, *ts*NY225 and *ts*NY240, do not show a temperature-dependent association with cytoplasmic structures. Nevertheless, association with the plasma membrane or other cytoplasmic structure may be necessary for P140 to induce the phosphorylation of cellular substrates which may be important in transformation. The identity of such target polypeptides is as yet uncertain. However, a number of cellular substrates for transformation-specific phosphorylation have been identified, and the spectrum of polypeptides phosphorylated by FSV is similar to that observed in RSV-infected cells (8). At least one of these polypeptides, the 36K protein (13, 38, 39), is now known to be associated with the plasma membrane (2, 10, 11, 21, 35, 37). Which, if any, of these cellular substrates for tyrosine phosphorylation plays a role in viral transforma-

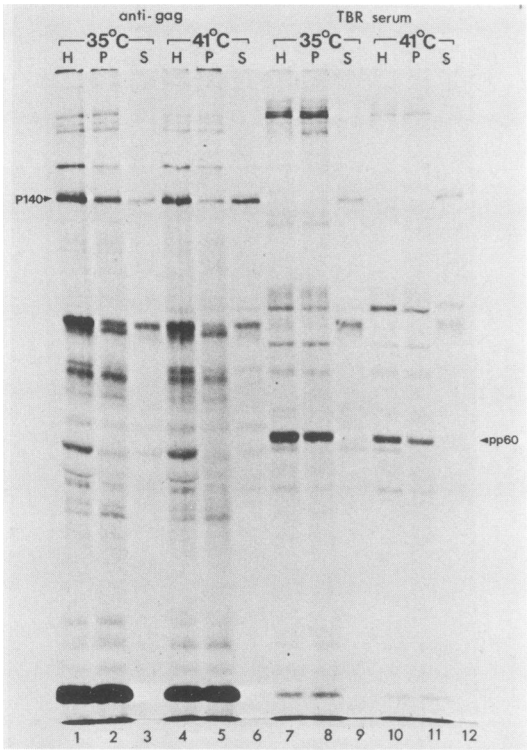

FIG. 8. Subcellular distribution of P140 and pp60^{src} in cells doubly infected by *ts*FSV and RSV SR-D. CEF were infected with *ts*FSV and superinfected with SR-D 3 days later. Two days later these cells were labeled (18 h) at either 35°C (tracks 1 through 3 and 7 through 9) or 41°C (tracks 4 through 6 and 10 through 12). Fractionation and analysis of P140 or pp60^{src} content were performed as described in the legend for Fig. 1, except that the fractions were split into two sets and each set was separately immunoprecipitated with either anti-*gag* serum (tracks 1 through 6) or tumor-bearing rabbit (TBR) serum (39) (tracks 7 through 12). Abbreviations: H, homogenate; P, P_{150}; S, S_{150}. The percentage of particulate P140 (left arrow) was 67% in track 2 and 37% in track 5. The percentage of particulate pp60^{src} (right arrow) was 76% in track 8 and 76% in track 11.

tion is not yet known. However, these observations suggest that the association of P140 with membranes or the cytoskeleton may be necessary for P140 to effect phosphorylation of cellular substrates important in the transformation process.

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