Cytoplasmic Localization of the Transforming Protein of Fujinami Sarcoma Virus: Salt-Sensitive Association with Subcellular Components

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Received 17 August 1982/Accepted 14 October 1982

Fujinami sarcoma virus (FSV) encodes a transforming protein of 130,000 daltons (P130) which is associated with a tyrosine-specific protein kinase activity. To elucidate mechanisms involved in cell transformation by FSV, we have studied the intracellular location of P130 in rat cells nonproductively infected with FSV. Immunofluorescent staining of several FSV-transformed rat cell lines with a tumor regressor antiserum specific against the *fps* sequences of P130 showed that the major staining was localized in the cytoplasm. Staining was also seen in cell ruffles and in some cases at areas of cell contact. The cytoplasmic location of P130 staining in cells infected with temperature-sensitive mutants of FSV was unchanged when they were grown at permissive or nonpermissive temperature. Cell fractionation of FSV-transformed cells under various conditions showed that the ionic strength used during cell fractionation had a striking effect on the distribution of P130. At 10 mM NaCl, 70% of P130 sedimented in the large granule fraction, whereas at 500 mM NaCl 70 to 90% of P130 was recovered in the cytosol fraction. Furthermore, a combination of ionic and nonionic detergents that effectively solubilized subcellular membranes was insufficient to solubilize P130 unless the salt concentration was raised. We conclude that the majority of P130 and its associated protein kinase activity are localized in the cytoplasm and that P130 is not an integral membrane protein.

Fujinami sarcoma virus (FSV) is a defective avian sarcoma virus that causes rapid transformation both in vivo and in vitro (17, 24). The genome of FSV consists of viral *gag* sequences fused to unique, cell-derived sequences called *fps*. The *gag-fps* genome of FSV encodes a transforming protein of 130,000 daltons (P130) that contains *gag*-coded sequences at its N terminus and *fps*-coded sequences at its C terminus (17, 24). P130 is associated with a distinct tyrosine-specific protein kinase activity (13, 30) which has been implicated as essential to the mechanism of cell transformation by FSV (16, 30). A cellular homolog of P130 has recently been identified by immunoprecipitation with antibody directed against *fps*-coded sequences. This protein, NCP98, has been shown to be antigenically, structurally, and enzymatically related to FSV P130 (26).

It is believed that interaction of P130 with subcellular targets as yet unidentified triggers the process of cellular alteration to the transformed state. As a first step to identify these subcellular targets of P130 and to elucidate the mechanism of transformation by FSV, we undertook a study to determine the subcellular location of P130 and the nature of its association

with subcellular components. These questions were examined by a combination of two different approaches: (i) indirect immunofluorescent staining, using a specific antibody directed against P130; and (ii) quantitative cell fractionation. To simplify the analysis, we chose rat fibroblasts transformed by FSV, because in these cells the only detectable viral protein being expressed is P130.

The results of our study appear to indicate that P130 is primarily a cytoplasmic protein, but also present at some areas of the plasma membrane, and that it is not an integral membrane protein.

MATERIALS AND METHODS

Cells and viruses. Fisher rat embryo fibroblasts, 3Y1, infected with wild-type FSV were maintained as described previously (26). 3Y1 cells transformed with Schmidt-Ruppin Rous sarcoma virus (RSV), subgroup A (19), were provided by S. Kawai, University of Tokyo. 3Y1 cells were also transformed by temperature-sensitive FSV mutants, NY225 and NY240 (16), and clonal lines were selected for each transformant. The permissive and nonpermissive temperatures for these cultures were 32 and 38°C, respectively. The preparation of chicken embryo fibroblasts and their infection with FSV were described previously (17).

Antisera. FSV-specific regressing tumor antisera (anti-FST) were obtained by injection of FSV-transformed 3Y1 cells into syngeneic rats as described before (26). Anti-FST and rat preimmune sera were absorbed with disrupted Rous-associated virus-2 virions as described previously (13). Rabbit antisera against Rous-associated virus-2 virion proteins and sera from rabbits bearing tumors induced by the Schmidt-Ruppin strain of RSV, subgroup D, were prepared as described previously (13).

Indirect-immunofluorescence microscopy. Cells grown on glass cover slips were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.2) for 20 min at room temperature. All subsequent operations were carried out at room temperature. After rinsing thoroughly with PBS, cover slip specimens were treated with 0.05% Triton X-100 in PBS for 10 min. Specimens were then incubated for 20 min with 50 μl of a 1:10 dilution of either anti-FST or preimmune sera that had been previously absorbed with disrupted Rous-associated virus-2 particles and dialyzed against PBS. After incubation, cells in cover slips were thoroughly rinsed in PBS and incubated for 20 min with 50 μl of 0.05-mg/ml rhodamine-conjugated rabbit antirat immunoglobulin G (Cappel Laboratories, Cochranville, Pa.), used as secondary antibody. Before use, the secondary antibody was preabsorbed with formaldehyde-fixed and Triton-extracted FSV-transformed rat cells. After incubation, cell specimens were rinsed in PBS and mounted in glycerol-PBS (1:1). Cover slips were then examined with a Zeiss photomicroscope III with epifluorescence illumination, using a barrier filter of 530 nm for rhodamine isothiocyanate.

Isotopic labeling of cells. (i) [^{35}S]methionine labeling. Subconfluent cultures of FSV-infected rat cells were labeled for 8 h as described previously (13).

(ii) [^3H]choline labeling. FSV-transformed cells grown in 100- or 35-mm plates were incubated for 24 h in 5 or 0.75 ml, respectively, of Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% calf serum and 10 μCi of choline-[methyl- ^3H]chloride (80 Ci/mmol; New England Nuclear, Boston, Mass.) per ml. At the end of incubation, cell cultures were processed as described in the text.

(iii) [^3H]fucose labeling. FSV-transformed cell cultures were labeled with 50 μCi of L-[5,6- ^3H]fucose (56 Ci/mmol; New England Nuclear) per ml and processed as described above for [^3H]choline labeling.

Cell fractionation. FSV-infected rat or chicken cells to be used for cell fractionation were grown in 100-mm plates. Subconfluent cultures were washed three times with ice-cold isotonic Tris buffer (25 mM Tris-hydrochloride [pH 7.4], 138 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 5.6 mM glucose) and placed immediately on ice. All subsequent operations were carried out at 4°C. After the isotonic buffer was completely removed, each plate received 1.5 ml of homogenizing medium (10 or 500 mM NaCl as indicated, 10 mM Tris-hydrochloride [pH 7.4], 2% Trasytol [FBA Pharmaceuticals, New York]), and cells were let stand for 5 min. After this, cells were collected by scraping with a rubber policeman, transferred to a hand-operated, tight-fitting Dounce homogenizer, and homogenized by 25 strokes. Immediately after homogenization sucrose was added to a final concentration of 0.25 M.

Cell homogenates were centrifuged at $10,000 \times g$ for 10 min in a Sorvall SE-12 rotor (Sorvall Instruments, Dupont Co., Newton, Conn.) to obtain a P10 pellet (nuclear and mitochondrial fraction) and an S10 supernatant (post-mitochondrial supernatant). The S10 supernatant was then centrifuged at $200,000 \times g$ for 1 h in an SW50.1 rotor (Beckman Instruments, Palo Alto, Calif.) to obtain a P200 pellet (crude microsomal fraction) and an S200 supernatant (cytosol fraction). The P10 and P200 pellets were resuspended in appropriate volumes of a buffer containing 0.25 M sucrose–10 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–2% Trasytol.

Alternatively, cell homogenates were prepared as above in low-NaCl-containing homogenizing buffer, the sucrose concentration was then adjusted to 0.25 M, and the NaCl concentration of appropriate aliquots was adjusted as indicated in the text. The treated homogenates were then centrifuged directly at $200,000 \times g$ for 1 h. The pellet thus obtained was called P'200 because this fraction contained the subcellular components present in both P10 and P200 described above. The supernatant obtained was equivalent to the S200 cytosol fraction described above.

Samples to be immunoprecipitated were diluted fivefold with modified RIPA buffer (0.05 M Tris-hydrochloride [pH 7.4], 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate [DOC], 0.1% sodium dodecyl sulfate [SDS], 25 mM EDTA, 2% Trasytol), mixed in a Vortex mixer for 30 to 45 s, and clarified by centrifugation at $10,000 \times g$ for 5 min. Immunoprecipitation was as described below.

Extraction of cell cultures by ionic and nonionic detergents. Subconfluent cultures of FSV-transformed rat cells grown in 35-mm plates were washed three times with ice-cold isotonic Tris buffer. All subsequent operations were carried out at 4°C. After the isotonic buffer was completely removed, the cultures were incubated for 2.5 min with 0.4 ml of extraction buffer (10 mM Tris-hydrochloride [pH 7.4], 0.25 M sucrose, 2% Trasytol, 0.5% Triton X-100, 0.1% DOC [when indicated], and the indicated concentrations of KCl). After incubation, the supernatant containing the detergent-soluble material was collected. The detergent-insoluble material remaining in the plates was then incubated for 2.5 min in 0.4 ml of modified RIPA buffer and then collected by scraping with a rubber policeman. Samples to be immunoprecipitated were processed as described above.

Protein analysis. Immunoprecipitation, the protein kinase assay, SDS-polyacrylamide gel electrophoresis (PAGE), and quantitation of ^{35}S or ^{32}P radioactivity in gel bands were carried out as described previously (13).

Biochemical determinations. Protein content was determined according to Bradford (5). 5'-Nucleotidase was assayed according to Widnell and Unkeless (39); cytochrome *c* oxidase, according to Smith (36); lactate dehydrogenase (LDH), according to Stolzenbach (37); and NADH diaphorase, according to Avruch and Wallach (3). Total cold trichloroacetic acid-insoluble radioactivity in [^3H]choline- and [^3H]fucose-labeled samples was measured as described before (2, 31).

RESULTS

Indirect immunofluorescent staining of rat cells transformed by FSV. Indirect immunofluores-

cent staining of FSV-transformed rat cells was carried out with an antiserum obtained from syngeneic rats injected with FSV-transformed rat cells (26). Although this antiserum (anti-FST) recognized primarily the *fps*-coded sequences in P130 and no *gag* proteins were detected in these cells (26, 34), we routinely treated the anti-FST serum with detergent-disrupted Rous-associated virus-2 virions to absorb any anti-*gag* antibody that could be present. Figure 1 shows a representative staining pattern of four FSV-transformed cell lines that were tested. In all cases, the reactive antiserum primarily stained diffusely the entire cytoplasm. Staining at cell ruffles (Fig. 1A and B) and in some cases at areas of cell contact (Fig. 1C) was also observed. By contrast, little fluorescence was observed with transformed cells treated with preimmune serum (Fig. 1E) or with uninfected cells treated with immune serum (Fig. 1F). From these results we conclude that P130 is a cytoplasmic protein that is also present at some areas of the plasma membrane.

We also studied the subcellular location of P130 in rat cells infected with two temperature-sensitive mutants of FSV. Cells infected with these mutants express P130 to the same extent at

permissive and nonpermissive temperatures, but at nonpermissive temperature cells are not transformed and P130 kinase is inactive (16). In cells grown at either permissive or nonpermissive temperatures (Fig. 2), P130 staining was localized in the cytoplasm. We have noted, however, that at 38°C the cytoplasm had a more granular appearance than at 32°C. The intensity of staining was lower at the nonpermissive temperature, presumably because cells were flattened out at this temperature.

Distribution of P130 in subcellular fractions: effect of salt concentration. To investigate whether P130 molecules were associated with sedimentable cellular components, we carried out quantitative cell fractionation at two different ionic strengths. FSV-transformed rat cells were lysed in a buffer containing either 10 or 500 mM NaCl and centrifuged at increasing centrifugal forces to obtain four cell fractions: P10, S10, P200, and S200. In these studies, acid-insoluble [³H]choline label was used to follow the distribution of membrane phospholipids (31); acid-insoluble [³H]fucose label, as a marker of plasma membrane glycoproteins (2, 29); 5'-nucleotidase, as another marker of plasma membrane (38); cytochrome *c* oxidase, as a marker of

FIG. 1. Indirect-immunofluorescence staining pattern of rat cells transformed by FSV. Uninfected (F) or FSV-infected rat cell cultures (A and E, clone 2; B, clone 8; C, clone 3; D, clone 12) were stained with the indicated antisera: (A–D and F) absorbed anti-FST; (E) preimmune serum.

FIG. 2. Effect of growth temperature on immunofluorescence staining pattern of rat cells infected with temperature-sensitive FSV mutants. Rat 3Y1 cells infected with temperature-sensitive FSV NY225 (A and B) and NY240 (C and D) grown at either 32°C (A and C) or 38°C (B and D) were stained with absorbed anti-FST.

mitochondria (38); NADH diaphorase, as a marker of endoplasmic reticulum (3); and LDH, as a marker of soluble proteins in the cytosol (11). The distribution of markers of different subcellular components was not affected by the salt concentrations used during cell fractionation (Table 1). In both cases, about 75% of the total phospholipids in membranes were sedimented into the P10 pellet. In addition to nuclei, this pellet contained the majority of the mitochondria and about half of the membranes derived from the plasma membrane and the endoplasmic reticulum. The postmitochondrial supernatant,

S10, contained nearly all of the LDH and about half of the plasma membrane and endoplasmic reticulum markers. P200 was a crude microsomal fraction consisting of elements derived from the endoplasmic reticulum, plasma membrane, and residual mitochondrial material. The cytosol fraction, S200, was nearly depleted of subcellular membranes and consisted primarily of soluble material (Table 1). More than 95% of the LDH was recovered in this fraction. Figure 3 shows the SDS-PAGE analysis of immunoprecipitates from each fraction by antiserum against *gag*-coded proteins. From comparison of lanes

TABLE 1. Distribution (percent) of [35 S]methionine-labeled P130, P130 kinase, and subcellular markers in cell fractions prepared at different ionic strengths^a

Ionic strength	Fraction	Total protein	[3 H]choline	[3 H]fucose	5'-Nucleotidase	Cytochrome c oxidase	LDH	NADH diaphorase	35 S-labeled P130	P130 kinase
10 mM NaCl	Homogenate	100	100	100	100	100	100	100	100	100
	S10	63.2	17.7	46.6	43.9	10.1	103.8	48.8	28.0	23.1
	P10	33.8	76.0	51.5	45.3	95.2	2.8	46.3	68.9	64.7
	S200	52.6	3.9	12.0	10.6	<1.0	95.6	14.5	8.6	6.4
	P200	7.7	9.0	32.4	28.1	6.4	1.0	30.1	7.9	8.6
500 mM NaCl	Homogenate	100	100	100	100	100	100	100	100	100
	S10	66.4	20.7	49.2	48.6	14.5	96.3	53.4	89.2	85.6
	P10	31.4	78.0	48.6	44.2	88.9	2.5	43.6	6.1	8.2
	S200	60.5	7.5	17.5	12.8	<1.0	98.1	14.3	77.4	71.9
	P200	3.7	7.1	29.7	32.4	7.0	1.3	33.4	6.9	4.4

^a Unlabeled, [3 H]choline-, [3 H]fucose-, or [35 S]methionine-labeled rat cells transformed by FSV were fractionated into S10, P10, S200, and P200 fractions in buffer containing either 10 or 500 mM NaCl. Appropriate portions of each fraction were then immunoprecipitated with an excess of antiviral antiserum. Immunoprecipitates from [35 S]methionine-labeled samples were analyzed directly by SDS-PAGE in an 8.5% gel followed by autoradiography. Immunoprecipitates from unlabeled samples were assayed for protein kinase activity and then analyzed by SDS-PAGE as above. 35 S and 32 P radioactivity in P130 gel bands and [3 H]choline and [3 H]fucose radioactivity in each fraction were measured. Unlabeled fractions were assayed for total protein and enzyme markers as described in the text. Values are percentages of the total in the cell homogenate.

A to E with lanes K to O and lanes F to J with lanes P to T in Fig. 3, it is apparent that the distribution of [35 S]methionine-labeled P130 and that of P130 protein kinase activity are identical. It is also apparent that the soluble form of P130 is fully active as a protein kinase activity (Fig. 3, lane T; Table 1). The ionic strength used during fractionation had a striking effect on the distribution of P130 (Fig. 3). At low ionic strength about 70% of P130 sedimented at low speed, appearing in the P10 pellet (Fig. 3, lanes B and L; Table 1). By contrast, at 500 mM NaCl, 70 to 90% of P130 was not sedimentable at either low speed or high speed, appearing first in the S10 fraction (Fig. 3, lanes H and R; Table 1) and then in the S200 cytosol fraction (Fig. 3, lanes J and T; Table 1). Replacing NaCl by KCl or including 2 mM EDTA had no effect on this distribution (data not shown).

To examine the effect of ionic strength on the distribution of P130 in more detail, we prepared a cell homogenate in low-salt-containing buffer and appropriate aliquots were then adjusted to various salt concentrations. The treated homogenates were then directly centrifuged at 200,000 $\times g$ to obtain a P'200 pellet and an S200 cytosol fraction. The increase in NaCl concentration caused a specific release of P130 into the cytosol fraction (Fig. 4). This effect was observed with two different clonal lines of FSV-transformed rat cells as well as with FSV-transformed chicken cells. This indicates that the salt effect observed is not restricted to rat cells but that it reflects a general property of the transforming protein of

FSV. The results presented in Fig. 4 were obtained by quantitation of P130 kinase in each fraction, but essentially the same ion dependence was observed for the release of [35 S]methionine-labeled P130 (data not shown). Under the same conditions, the majority of p60^{src} of RSV-infected rat cells was sedimented in the P200 fraction even at high salt concentrations (Fig. 4), suggesting that RSV p60^{src} is more tightly bound to sedimentable components than is FSV P130. It can also be seen in Fig. 4 that after centrifugation very little of the total membrane and plasma membrane markers remained in the cytosol fraction throughout the entire range of NaCl concentration. This ruled out the possibility that the effect observed was due to incomplete sedimentation of membranes with increasing salt concentration. By contrast, under the same conditions, virtually all of the LDH was recovered in the cytosol fraction (Fig. 4). Since the increase in salt concentration had little effect on the distribution of total protein between P'200 and S200 (data not shown), the increase in P130 in the S200 fraction represents an enrichment of P130 molecules in this fraction. The specific release of P130 by salts in the absence of detergents observed in Fig. 4 strongly suggests that P130 is not an integral membrane protein (35).

It should be noted that 50% release of P130 occurred below 200 mM NaCl, which corresponds to an ionic strength that is not too far from that of physiological conditions. This brings a degree of uncertainty as to what fraction

FIG. 3. Distribution of FSV P130 in subcellular fractions prepared at different ionic strengths. Unlabeled or [35 S]methionine-labeled rat cells transformed by FSV were fractionated into P10, S10, P200, and S200 fractions in buffer containing either 10 or 500 mM NaCl. Appropriate portions of each fraction were then immunoprecipitated with an excess of antiviral antiserum. Immunoprecipitates from [35 S]methionine-labeled samples were analyzed directly by SDS-PAGE in an 8.5% gel followed by autoradiography. Immunoprecipitates from unlabeled samples were assayed for protein kinase activity and then analyzed by SDS-PAGE analysis as above. (A to E) [35 S]methionine-labeled cells, 10 mM NaCl fractionation: A, total cell homogenate; B, P10; C, S10; D, P200; E, S200. (F to J) [35 S]methionine-labeled cells, 500 mM NaCl fractionation: F, total cell homogenate; G, P10; H, S10; I, P200; J, S200. (K to O) Unlabeled cells, 10 mM NaCl fractionation: K, total cell homogenate; L, P10; M, S10; N, P200; O, S200. (P to T) Unlabeled cells, 500 mM NaCl fractionation: P, total cell homogenate; Q, P10; R, S10; S, P200; T, S200. (A to J) and (K to T) correspond to two separate gels.

of P130 exists in nonsedimentable form *in vivo* and also cautions that the results of cell fractionation experiments could be strongly dependent on the composition of the buffers used.

Salt dependence of P130 extraction by detergents. Most integral membrane proteins are solubilized by ionic detergents (35). To further examine the possibility that P130 is an integral membrane protein, we determined the effect of ionic and nonionic detergents on the extraction of P130. Cultures of rat cells transformed by FSV were extracted with buffers of increasing ionic strength containing 0.5% Triton X-100 in the presence or in the absence of 0.1% DOC. The pattern of extraction of P130 in the presence of detergents was salt dependent (Fig. 5). Triton-DOC was slightly more effective than Triton alone in solubilizing P130. Without salts, Triton-DOC treatment by itself was insufficient to extract P130 and a higher salt concentration was required. On the other hand, Triton-DOC treatment was very effective in solubilizing the protein and phospholipid components of membranes regardless of the salt concentration. This was evidenced by the solubilization of nearly 80% of the plasma membrane fucose-labeled glycoproteins and 70 to 75% of the choline-labeled total membrane phospholipids (Fig. 5). This differential behavior of detergents with

respect to the solubilization of membrane components and P130 suggests that the increased solubility of P130 with increasing ionic strength is not a secondary effect due to increased solubility of membrane components but a direct effect of salt on P130 extraction. That detergent solubilization of membranes was insufficient to solubilize P130 and that increased salt concentration was not only necessary but also sufficient to extract P130 strongly suggest that at low ionic strength P130 is not associating with lipids of a lipid bilayer but is interacting with proteins primarily through ionic interactions.

Effect of growth temperature on salt-dependent release of temperature-sensitive P130. To analyze the possibility that the binding of P130 to sedimentable components has any biological significance in cell transformation by FSV, we studied the effect of growth temperature on the salt-dependent release of P130 of temperature-sensitive mutants. The susceptibility of temperature-sensitive P130 to salt extraction was not changed when cells were grown at either permissive or nonpermissive temperatures, and this behavior was observed when extraction was carried out both in the presence (Fig. 6B) and in the absence (Fig. 6A) of detergents. Thus, there was no correlation between the affinity of P130 for sedimentable components and ability to transform.

FIG. 4. Salt-dependent release of FSV P130 into the cytosol fraction. Subconfluent cultures of FSV-infected chicken (○) or rat (●, Δ, ▼, ▽, □, ▲) fibroblasts were labeled with either [3 H]choline or [3 H]fucose. Labeled cultures were fractionated in buffers containing the indicated concentrations of NaCl into a P'200 total membrane fraction and an S200 cytosol fraction in one step, as described in the text. After cell fractionation, [3 H]choline and [3 H]fucose radioactivity in each fraction and protein kinase activity in immunoprecipitates from each fraction were measured. Subconfluent cultures of unlabeled RSV-transformed rat cells (■) were fractionated as above, and protein kinase activity in immunoprecipitates from each fraction was measured. Immunoprecipitation of cell fractions from FSV- or RSV-transformed cells was carried out with an excess of antivirion or RSV tumor-bearing rabbit antisera, respectively. After the immunoprecipitates were assayed for protein kinase activity, they were analyzed by SDS-PAGE followed by autoradiography. P130 and p60^{src} kinase activities were quantitated by counting 32 P radioactivity in P130 and immunoglobulin G gel bands, respectively. Symbols: (Δ) P130 protein kinase activity from FSV-infected rat cells, clone 3; (●) P130 protein kinase activity from FSV-infected rat cells, clone 12; (○) P140 protein kinase activity from FSV-infected chicken cells (the FSV stock used to infect chicken cells codes for a 140,000-dalton species); (■) p60^{src} protein kinase activity from RSV-transformed rat cells; (▲) [3 H]choline; (▼) [3 H]fucose; (□) 5'-nucleotidase; (▼) LDH. Percentage of total in S200 represents radioactivity in the S200 fraction as a percentage of total radioactivity in the cell homogenate.

DISCUSSION

In this paper we present evidence that the transforming protein of FSV is predominantly distributed in the cytoplasm of infected cells and that FSV P130 is not an integral membrane protein.

The intracellular location of P130 was examined by indirect immunofluorescent staining of transformed cells. To optimize specific staining of P130, we used nonproducer rat cells that do not express any viral proteins other than P130, and we used an absorbed tumor regressor antiserum that recognizes exclusively FSV-specific sequences in P130 (26). The results obtained with several independently derived clones of rat transformed cells and antisera from different rats consistently showed that P130 has a predominant cytoplasmic location and that it is also present at some areas of the plasma membrane. Although it is conceivable that only a small fraction of P130 interacts with cellular targets at very defined subcellular sites, the wide distribution of P130 in the cytoplasm may suggest that primary targets of P130 are also widely distributed in the cytoplasm. The cytoplasmic distribution of FSV P130 was not changed in cells infected with temperature-sensitive mutants of

FIG. 5. Salt dependence of FSV P130 extraction by detergents. Subconfluent cultures of unlabeled FSV-transformed rat cells were fractionated into detergent-soluble and detergent-insoluble fractions as described in the text. Extraction buffers contained the indicated concentrations of KCl and did not contain DOC. Appropriate aliquots from each fraction were immunoprecipitated with an excess of antivirion antiserum and assayed for protein kinase activity (●). Alternatively, FSV-transformed cell cultures labeled with either [3 H]choline or [3 H]fucose were extracted as above, except that extraction buffers also contained 0.1% DOC. After detergent fractionation, [3 H]choline (□) and [3 H]fucose (■) radioactivity in each fraction and protein kinase activity (○) in immunoprecipitates from each fraction were measured as described in the legend to Fig. 4. Percentage of total extracted represents radioactivity in the detergent-soluble fraction as a percentage of the total radioactivity extracted from a control culture in modified RIPA buffer.

FIG. 6. Effect of growth temperature on salt-dependent release of temperature-sensitive P130 in the presence and in the absence of detergents. Subconfluent cultures of rat cells infected with temperature-sensitive FSV NY225 were grown at either 32°C (□, Δ) or 38°C (■, ▲) and were labeled with [35 S]methionine. Labeled cultures were fractionated in buffers containing the indicated concentrations of NaCl into a P'200 and an S200 fraction (A) or they were fractionated in buffers containing the indicated concentrations of KCl and 0.5% Triton X-100 into detergent-soluble and detergent-insoluble fractions (B). Appropriate portions of each fraction were immunoprecipitated with an excess of antiviral antiserum. Immunoprecipitates were analyzed by SDS-PAGE in 8.5% gels followed by autoradiography, and 35 S radioactivity in P130 gel bands was measured. Percentage of total in S200 and percentage of total extracted have the same meaning as in Fig. 4 and 5, respectively.

FSV at either permissive or nonpermissive temperature. We have noted, however, that a more granular fluorescent staining is seen at 38°C. It is possible that this enhanced granular staining of P130 molecules at nonpermissive temperature represents accumulations of inactive P130. However, the granular staining is not present in every cell, and it may be the result of nonspecific cytopathic changes sometimes seen in cultures kept at nonpermissive temperature (cells have more granules and vacuoles by observation with phase microscopy).

To investigate the nature of the association of P130 with subcellular components, we carried out quantitative cell fractionation of FSV-transformed rat cells. At low ionic strength a large fraction of P130 and its protein kinase activity sedimented with large particles. However, increase in the ionic strength resulted in the release of the majority of P130 and its protein kinase activity into a nonsedimentable form. Fifty percent release of P130 into the cytosol fraction occurred at an ionic strength near 0.2, not very far from that of physiological conditions, which is 0.15. Since the salt concentration at local compartments can vary within the cell, it is difficult to conclude what is the physiological state of P130 *in vivo*. In addition to the specific release of P130 by salts in the absence of detergents, P130 was not solubilized by a combination of ionic and nonionic detergents. An integral membrane protein is expected to behave in

the opposite way; i.e., it would have been solubilized by this combination of detergents even at low ionic strength and it would not have been extracted by high-salt-containing buffers in the absence of detergents (35). Therefore, these results strongly suggest that P130 is not an integral membrane protein and that its association to subcellular components is mediated by salt-sensitive protein-protein interactions.

Although we cannot rule out that the associations of P130 with subcellular components at low ionic strength represent an aggregation artifact that takes place after cell homogenization, this binding of P130 may be one of the steps involved in the mechanism of cell transformation by FSV. Our studies with temperature-sensitive mutants of FSV (Fig. 6) showed that the binding itself was not temperature sensitive. Preliminary characterization of P130-containing fractions obtained at low ionic strength, by isopycnic sucrose density gradient centrifugation, shows that these large P130-containing complexes do not cofractionate with membranes (unpublished data). This observation and the fact that P130 was not extracted by a combination of ionic and nonionic detergents suggest that P130 may be interacting with a structural protein matrix. In this connection, it has been suggested that the transforming proteins of PRC II, a closely related avian sarcoma virus, RSV, and Abelson murine leukemia virus, a mammalian retrovirus, may be associated with cytoskeletal elements (1,

4, 7). The transforming proteins of these viruses, like FSV P130, are all associated with tyrosine-specific protein kinase activities (8, 9, 12, 13, 16, 18, 25, 27, 30, 41). This raises the possibility that interaction with a structural protein matrix may be a general property of transforming proteins associated with tyrosine-specific protein kinase activities.

Comparison of the amino acid sequences of FSV P130 and RSV p60^{src} shows that 40% sequence homology exists in a region comprising 280 amino acids at the C termini of both proteins (33a). On this basis it was proposed that the *src* and the *fps* genes were derived from a common ancestor gene. Although their protein kinase activities are similar to each other in their specificity for tyrosine residues, some differences in their enzymatic properties such as their nucleotide and divalent cation specificities have been observed (14). In this study we present evidence that FSV P130 may also differ from p60^{src} in the nature of the binding to subcellular components, which is salt sensitive for FSV P130 and salt resistant for p60^{src} (Fig. 4; 20, 22, 23). Comparisons of the intracellular location of FSV P130 and p60^{src} is more difficult because several different subcellular locations of p60^{src} have been observed (6, 7, 10, 15, 20–23, 28, 32, 33, 40). Whether FSV P130 and p60^{src} cause cell transformation by acting on similar or different targets remains to be determined.

ACKNOWLEDGMENTS

We thank T. Hanafusa, S. Kawai, and B. Mathey-Prevot for providing cell cultures infected with FSV mutants and RSV and one of the anti-FST sera used in this study, respectively. We thank F. Cross, B. Mathey-Prevot, and M. Shibuya for reading the manuscript. Excellent technical assistance by Rosemary Williams, Doris Gundersen, and Susan Nornes is greatly appreciated.

This work was supported by Public Health Service grant CA14935 from the National Cancer Institute and American Cancer Society grant MV128. E.W. is a recipient of Public Health Service grant PHS AG03020. R.A.F. is a recipient of Public Health Service training grant T32AI07233 from the National Institutes of Health.

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