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Localization of the Feline Sarcoma Virus *fgr* Gene Product ($P70^{gag\text{-}actin\text{-}fgr}$): Association with the Plasma Membrane and Detergent-Insoluble Matrix

RON MANGER,[1]* SURAIYA RASHEED,[2] AND LARRY ROHRSCHNEIDER[1]

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104,[1] and Department of Pathology, University of Southern California School of Medicine, Los Angeles, California 90033[2]

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The v-*fgr* oncogene codes for a unique transforming protein ($P70^{gag\text{-}actin\text{-}fgr}$) that contains virus-specific determinants and cell-derived sequences for both a tyrosine-specific kinase domain and an actin domain. We examined the subcellular distribution of the v-*fgr* protein by immunofluorescence microscopy and various cell fractionation techniques. By immunofluorescence, the v-*fgr* protein was localized in a diffuse cytoplasmic pattern within transformed cells. The v-*fgr* protein was not detectable at substratum adhesion sites. Crude membrane preparations (P100) obtained from *fgr*-transformed cells contained elevated levels of $P70^{gag\text{-}actin\text{-}fgr}$. Further analysis of membranes on discontinous sucrose gradients revealed that $P70^{gag\text{-}actin\text{-}fgr}$ cofractionated with plasma membranes. Using an alternate method of fractionation, we found that the majority of the v-*fgr* protein remained with the insoluble matrix obtained by treating cells with a buffer containing Triton X-100. When membranes were similarly treated with detergent, nearly all of v-*fgr* protein remained with the residual insoluble matrix. These results suggest that the transforming activity of $P70^{gag\text{-}actin\text{-}fgr}$ may be directed to subcellular cytoskeletal targets at or near the cytoplasmic face of the plasma membrane.

The primary translational product of the Gardner-Rasheed feline sarcoma virus (GR-FeSV) is a *gag-actin-onc* polyprotein ($P70^{gag\text{-}actin\text{-}fgr}$) possessing tyrosine-specific kinase activity in the *fgr*-specific portion (11, 13, 16). This *fgr*-specific portion has been shown to have considerable homology with other retroviral *onc* gene proteins with similar enzymatic activity (12). This homology that $P70^{gag\text{-}actin\text{-}fgr}$ shares with other *onc* gene proteins is most pronounced with the v-*yes* and v-*src* transforming proteins (8, 12). The most interesting feature of v-*fgr*, however, is the internal stretch of 128 amino acid residues that exhibits near 98% homology with mammalian γ-*actin* (12). These three unique domains of $P70^{gag\text{-}actin\text{-}fgr}$ suggest that GR-FeSV arose from a double recombination event involving feline leukemia virus and two distinct cellular genes, one a protein kinase and the other a cytostructural protein (12). Similar events may have generated viruses such as AEV and MH2; however, the unique incorporation of an actin-coding sequence into the v-*fgr* genome raises some intriguing questions regarding the possible relationship between this particular transforming protein and its intracellular target sites.

The present study was undertaken to determine the subcellular distribution of $P70^{gag\text{-}actin\text{-}fgr}$. Identification of potential cellular sites at which the v-*fgr* protein interacts should be useful in elucidating the mechanism of transformation induced by $P70^{gag\text{-}actin\text{-}fgr}$ in susceptible cells. To determine the intracellular distribution of $P70^{gag\text{-}actin\text{-}fgr}$, we used a number of independent but complimentary approaches, including indirect immunofluorescence microscopy, nonionic detergent extraction, and subcellular fractionation.

MATERIALS AND METHODS

Cell culture. The isolation and characterization of GR-FeSV-transformed Fischer rat embryo (GR-FRE) cells and GR-FeSV-transformed Mink (GR-Mink) cells have been described elsewhere (16). Transformed cells and their normal equivalents were grown and maintained in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine serum.

Antisera. Antibody generated to a synthetic peptide corresponding to regions 498 through 512 of Prague C strain Rous sarcoma virus $pp60^{src}$ (peptide 1) was affinity purified on peptide-bound Sepharose as described previously (6).

fgr-specific antiserum was prepared in (Wistar/Furth × Fisher)F_1 weanling rats. Animals were injected intramuscularly with 10^6 to 10^7 GR-FRE cells. All animals developed tumors at the site of injection and were bled at 6 to 8 weeks postinjection. A monoclonal antibody to feline leukemia virus $p15^{gag}$ was obtained from B. Chesebro (Rocky Mountain Laboratories, Hamilton, Mont.).

Immunoprecipitation and Western blot analysis. Radioactive labeling of cells and subsequent immunoprecipitations were carried out by methods described previously (6). Soluble cell extracts (100 μl) were reacted with 3 μg of peptide 1 antibody, 2.5 μl of tumor-bearing rat (TBR) serum, or 4 μl of diluted (1:150) anti-$p15^{gag}$ monoclonal ascites fluid. Immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis of total cellular extracts was done with peptide 1 antibody as described previously (6).

Protein kinase assays. Immunoprecipitates were prepared as described previously (6), absorbed with 60 μl of a 10% suspension of *Staphylococcus aureus*, washed extensively with a lysis buffer (10 mM Tris hydrochloride [pH 7.2], 50 mM NaCl, 0.5% Nonidet P-40), and then suspended in 25 μl of kinase buffer (20 mM Tris hydrochloride [pH 7.5], 10 mM $MnCl_2$); 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) was then added to this suspension. The reaction was allowed to proceed for 10 min at room temperature and was then stopped by the addition of 0.5 ml of lysis buffer containing 10 mM EDTA. Following three subsequent washes in lysis

* Corresponding author.

buffer, the immunecomplexes were analyzed by SDS-PAGE.

Immunofluorescence microscopy. Cells were plated at an initial density of 10^5 cells in 60-mm tissue culture dishes containing ethanol-sterilized glass cover slips and were cultured for at least 2 days prior to use. Cover slips with adherent cells were rinsed briefly in phosphate-buffered saline (containing Ca^{2+} and Mg^{2+}) at room temperature, drained, and fixed in methanol at −20°C for 5 min. Cells were immediately rehydrated in phosphate-buffered saline and stained for indirect immunofluorescence as described previously (8, 11).

Subcellular fractionation. All procedures were performed at 4°C. Confluent cell cultures were washed with phosphate-buffered saline (containing Ca^{2+} and Mg^{2+}), scraped from the dish with a rubber policeman, and pelleted by centrifugation at 1,000 × *g* for 5 min. The pellet was suspended in a lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.1], 5 mM KCl, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 200 Kallikrein inhibitor units of aprotinin (Trasylol) per ml), incubated for 10 min, and then Dounce homogenized until >80% of the nuclei were released, as observed by phase microscopy. The homogenate was centrifuged at 1,000 × *g* for 5 min to pellet nuclei, and the resultant supernatant was centrifuged at 100,000 × *g* for 30 min to obtain a high-gravity-force particulate fraction (P100) and a high-gravity-force soluble fraction (S100).

The P100 fraction was further separated by centrifugation through a discontinuous step gradient consisting of 20, 35, 40, and 50% sucrose. The P100 pellet was layered over the gradient and centrifuged at 40,000 rpm for 2 h in a Beckman SW41 rotor, and the resultant three fractions were collected at the interfaces. The purity of the subcellular fractions was monitored by the distribution of lactic dehydrogenase (24), NADH diaphorase (25), and 5'-nucleotidase (15, 27).

Nonionic detergent extraction of cells and membranes. Detergent extractions were based upon a method previously shown to preserve the integrity of the insoluble cytoskeletal matrix (5). Confluent cell cultures were treated with PHEM extraction buffer [0.15% Triton X-100, 60 mM piperazine-*N*, *N'*-bis(2-ethanesulfonic acid) (PIPES), 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$] (pH 6.9) for 5 min at 4°C. The soluble fraction was collected, and the adherent insoluble cytoskeletal matrix was rinsed twice with detergent-free PHEM extraction buffer and harvested by scraping with a rubber policeman. Soluble and insoluble fractions were adjusted to equal volumes in RIPAE buffer (6) for immunoprecipitation analysis. The effect of a high salt concentration upon detergent extraction was assayed by the addition of 500 mM NaCl to PHEM extraction buffer.

Crude membrane preparations (P100 pellet) were subjected to detergent treatment by suspension in PHEM extraction buffer for 15 min. The suspension was then centrifuged at 100,000 × *g* for 30 min, resulting in a translucent pellet and a soluble extract. Both fractions were adjusted to equal volumes in RIPAE buffer (6) prior to immunoprecipitation analysis.

RESULTS

Selection and characterization of P70$^{gag\text{-}actin\text{-}fgr}$-specific immune reagents. Because of the considerable amino acid sequence homology with both the v-*src* and v-*yes* transforming proteins (12), we examined the possibility of using site-specific anti-peptide antibodies prepared against pp60^{src} (6) for use in studies of the v-*fgr* protein. The peptide 1 region of v-*src*, consisting of residues 498 through 512, was found to be nearly identical to the corresponding region of v-*fgr*, consisting of residues 636 through 650: pp60^{src}, C-W-R-K-D-P-E-E-R-P-T-F-K-Y-L (22); P70$^{gag\text{-}actin\text{-}fgr}$, T-W-R-L-D-P-E-E-R-P-T-F-E-Y-L (12). Of the 15 amino acid residues in the peptide 1 region of v-*src*, only 3 are mismatched with the analogous region in v-*fgr* (differences underlined). Therefore, it seemed likely that antibodies selective for the peptide 1 domain of pp60^{src} would also cross-react with P70$^{gag\text{-}actin\text{-}fgr}$.

GR-Mink cells were metabolically labeled with $^{32}P_i$ and analyzed by immunoprecipitation (Fig. 1A). The peptide 1 antibody identified a diffuse band, possibly representing more than one protein species, at 70 kilodaltons (kDa) in detergent lysates from GR-Mink cells (Fig. 1, lane a). This band was not observed in immunoprecipitates prepared from normal mink cell lysates (Fig. 1, lane c). The specificity of this reaction was demonstrated by preabsorbing the antibody with excess peptide 1 prior to immunoprecipitation, which effectively eliminated identification of the 70-kDa band (Fig. 1, lane b).

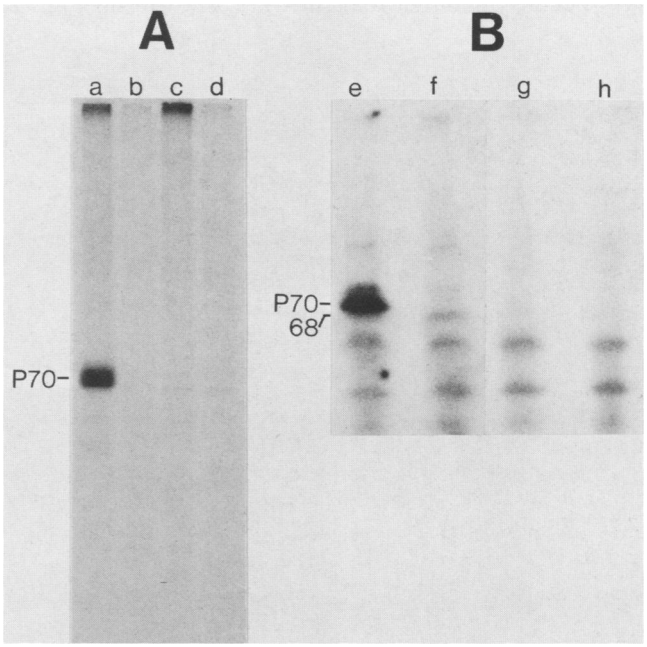

FIG. 1. Immunodetection of P70$^{gag\text{-}actin\text{-}fgr}$ by the peptide 1 antibody. (A) SDS-PAGE of immunoprecipitates prepared from $^{32}P_i$-labeled cells. RIPAE extracts from metabolically labeled GR-Mink cells (lanes a and b) and normal mink cells (lanes c and d) were immunoprecipitated with the peptide 1 antibody (lanes a and c) or with antibody that had been preabsorbed with excess peptide 1 (lanes b and d). The peptide 1 antibody identified a diffuse band, possibly representing more than one protein species, at 70 kDa in GR-Mink cell extracts (lane a). Preabsorption of antibody with peptide 1 eliminated the detection of the 70-kDa band (lane b). (B) Western blot immunodetection of P70$^{gag\text{-}actin\text{-}fgr}$ by the peptide 1 antibody. Whole cell extracts prepared from GR-Mink cells and normal mink cells were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was incubated with the peptide 1 antibody and then with ^{125}I-labeled protein A, and immune complexes were identified by autoradiography. Lanes: e and f, GR-Mink cells and normal mink cells reacted with the peptide 1 antibody, respectively; g and h, GR-Mink cells and normal mink cells reacted with antibody that had been preabsorbed with excess peptide 1, respectively.

Immunodetection of P70$^{gag\text{-}actin\text{-}fgr}$ by the peptide 1 antibody was further characterized by Western blotting (Fig. 1B). This technique revealed a closely spaced doublet, again in the 70-kDa region (Fig. 1, lane e). In addition, a weakly cross-reacting protein at 68 kDa was identified in normal cell lysates (Fig. 1, lane f). The 68-kDa protein may also have been present in transformed cell lysates; however, it would have been obscured by the proximity of the much more abundant 70-kDa v-*fgr* protein (Fig. 1, lane e). A weakly cross-reacting-protein at approximately 90 kDa was also identified by the peptide 1 antibody in both normal and transformed cell lysates (Fig. 1, lanes e and f). The specificity of these reactions was demonstrated by preabsorbing the antibody with excess peptide 1, which effectively eliminated the recognition of these proteins (Fig. 1, lanes g and h).

Antisera to the v-*fgr* transforming protein were also prepared in rodents. Weanling rats (Wistar/Furth × Fisher)F_1 were injected intramuscularly with GR-FRE cells, and tumors developed at the site of injection within 2 to 4 weeks. Sera obtained from tumor-bearing animals at 6 to 8 weeks after injection specifically immunoprecipitated at 70-kDa protein from both GR-Mink and GR-FRE cells but not from normal cells. Immunoprecipitations with the various *fgr*-specific immune reagents are shown in Fig. 2.

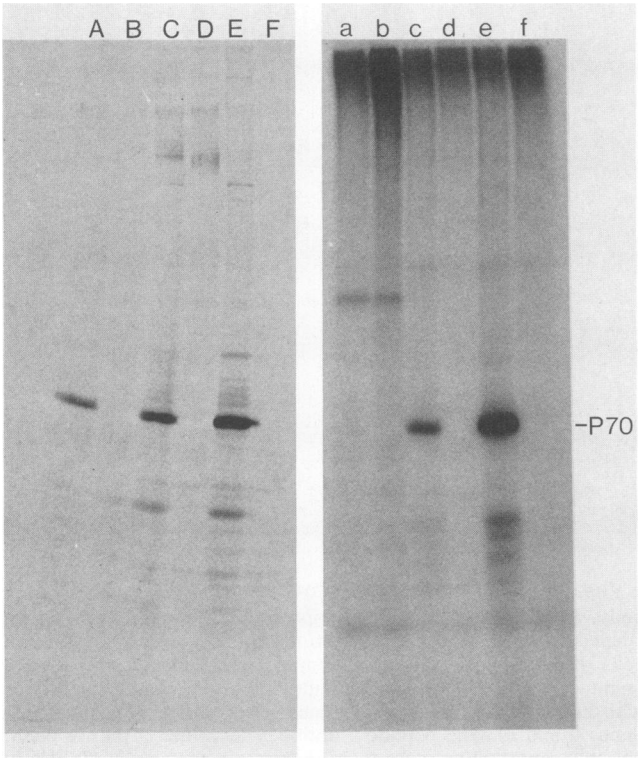

FIG. 2. Peptide 1 antibody inhibition of P70$^{gag\text{-}actin\text{-}fgr}$ kinase activity. Immunoprecipitates of [^{35}S]methionine-labeled GR-Mink cell lysate (lanes A, C, and E) and normal mink cell lysate (lanes B, D, and F) were prepared with the peptide 1 antibody (lanes A and B), TBR serum (lanes C and D), or the p15^{gag} monoclonal antibody (lanes E and F). Similar immunoprecipitates of GR-Mink cell lysate (lanes a, c, and e) and normal mink cell lysate (lanes b, d, and f) were assayed for in vitro kinase activity. These immunoprecipitates were prepared with the peptide 1 antibody (lanes a and b), TBR serum (lanes c and d), or the p15^{gag} monoclonal antibody (lanes e and f), incubated with [γ-^{32}P]ATP in kinase buffer, and resolved by SDS-PAGE and autoradiography.

[^{32}S]methionine-labeled normal and *fgr*-transformed cells were subjected to detergent extraction, and the lysates were reacted with the peptide 1 antibody, TBR serum, or a monoclonal antibody to p15^{gag}. The peptide 1 antibody and TBR serum specifically recognized a 70-kDa protein in *fgr*-transformed cell lysates (Fig. 2, lanes A and C) which was not present in normal cell lysates (Fig. 2, lanes B and D). Because P70$^{gag\text{-}actin\text{-}fgr}$ contains feline leukemia virus p15^{gag}-derived sequences (11–13, 16), the p15^{gag} monoclonal antibody precipitated a 70-kDa protein from transformed cell lysates but not from normal cell lysates (Fig. 2, lanes E and F). Thus, each of the immune reagents, the peptide 1 antibody, TBR serum, and the p15^{gag} monoclonal antibody, recognized p70$^{gag\text{-}actin\text{-}fgr}$.

The peptide 1 antibody had previously been shown by Gentry et al. to inhibit the kinase activity of pp60^{src} in the immune complex (6). Because of the similarities in the kinase domains of P70$^{gag\text{-}actin\text{-}fgr}$ and pp60^{src} (12), we examined the effect of the peptide 1 antibody on P70$^{gag\text{-}actin\text{-}fgr}$ kinase activity. Immunoprecipitates prepared from lysates of GR-Mink and normal cells with the peptide 1 antibody, TBR serum, or the p15^{gag} monoclonal antibody were incubated with [γ-^{32}P]ATP in Mn^{2+}-containing kinase buffer to detect autophosphorylation of the v-*fgr* protein. Both the p15^{gag} monoclonal antibody and TBR serum precipitated the *fgr* protein while allowing it to retain its kinase activity (Fig. 2, lanes c and e). However, the kinase activity of P70$^{gag\text{-}actin\text{-}fgr}$ was neutralized when P70$^{gag\text{-}actin\text{-}fgr}$ was immunoprecipitated with the peptide 1 antibody (Fig. 2, lane a). These results confirm that the kinase domains of v-*src* and v-*fgr* are very similar.

Labeling of P70$^{gag\text{-}actin\text{-}fgr}$ with [^{3}H]myristate. Although lipid addition through a myristate group is a rare form of fatty acid modification, it appears to be common for a number of mammalian retroviral *gag-onc* fusion proteins (21). Because v-*fgr* is a *gag-onc* fusion protein, we examined the possible incorporation of labeled myristic acid into P70$^{gag\text{-}actin\text{-}fgr}$.

GR-Mink cells and their normal equivalents were labeled in culture for 2 h with 0.5 mCi of [^{3}H]myristic acid per ml. As a positive control, Snyder-Theilen feline sarcoma virus-transformed FRE cells, which produce a myristylated *gag-onc* fusion protein (21), P85$^{gag\text{-}fes}$, were labeled identically. Immunoprecipitates prepared from labeled cells were analyzed by SDS-PAGE and autoradiography for the presence of [^{3}H]myristate-labeled P70$^{gag\text{-}actin\text{-}fgr}$ (Fig. 3). The p15^{gag} monoclonal antibody clearly detected a myristate-labeled 70-kDa protein in lysates from GR-Mink cells (Fig. 3, lane C) but not in lysates from normal mink cells (Fig. 3, lane B). As expected, the p15^{gag} monoclonal antibody immunoprecipitated an 85-kDa myristylated protein from Snyder-Theilen feline sarcoma virus-transformed FRE cells (Fig. 3, lane A). The p15^{gag} monoclonal antibody also identified a protein at approximately 58 kDa in GR-Mink cell lysates (Fig. 3, lane C). However, the 58-kDa protein was not detected under conditions in which the peptide 1 antibody immunoprecipitated the myristylated v-*fgr* protein (data not shown). These results are consistent with the idea that the 58-kDa protein may represent a degraded form of the v-*fgr* protein in which the terminal portion of the kinase domain has been lost. Alternatively, the 58-kDa species could represent a cross-reactive cellular protein or viral precursor product.

Indirect immunofluorescence staining of GR-FeSV-transformed cells. Having determined that the peptide 1 antibody recognizes P70$^{gag\text{-}actin\text{-}fgr}$ by immunoprecipitation and Western blot analysis, we chose this reagent for immunofluorescence studies. Although the cells in these studies

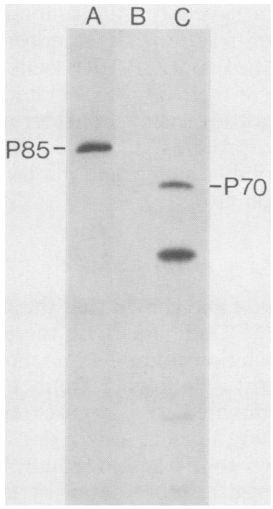

FIG. 3. Incorporation of [^{3}H]myristic acid into P70$^{gag\text{-}actin\text{-}fgr}$. GR-Mink cells and normal mink cells were metabolically labeled with [^{3}H]myristic acid and then extracted in RIPAE buffer. Lysates were immunoprecipitated with the p15^{gag} monoclonal antibody. Lanes: B, normal mink cells; C, GR-Mink cells. As a positive control, Snyder-Theilen feline sarcoma virus-transformed FRE cells, which contain the myristylated P85$^{gag\text{-}fes}$ protein, were similarly labeled and immunoprecipitated with the p15^{gag} monoclonal antibody (lane A).

were nonproductively transformed, the selection of the peptide 1 antibody reduced the possibility of localization through viral determinants other than the kinase domain.

Transformed cells and their normal equivalents were fixed and permeabilized in cold methanol and then incubated with the peptide 1 antibody followed by rhodamine-labeled goat anti-rabbit immunoglobulin G. A diffuse cytoplasmic staining pattern was observed in both GR-FRE and GR-Mink cells (Fig. 4A and B) but not in similarly prepared FRE or mink cells (Fig. 4a and b). This staining pattern was shown to be specific by preabsorbing the antibody with excess peptide 1, which abolished the cytoplasmic staining within transformed cells (data not shown). A nonspecific labeling of

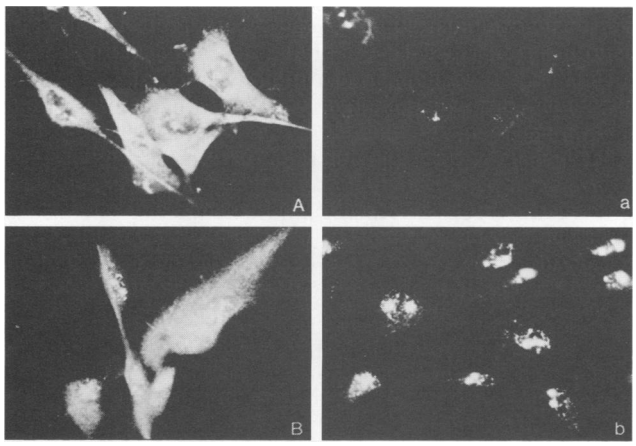

FIG. 4. Localization of P70$^{gag\text{-}actin\text{-}fgr}$ in transformed cells by indirect immunofluorescence microscopy. Cells were fixed and permeabilized in cold methanol and then stained with the peptide 1 antibody followed by a rhodamine-conjugated secondary antibody. A, GR-FRE cells; B, GR-Mink cells; a, FRE cells; b, normal mink cells.

nucleoli was noted in both transformed and normal mink cells; however, this was most likely due to nonspecific or carrier-specific antibodies (anti-keyhole limpet hemocyanin), since this pattern was not blocked by preabsorbtion with peptide 1. P70$^{gag\text{-}actin\text{-}fgr}$ was not seen at substratum adhesion sites. Similar staining patterns were observed with either TBR serum or the p15^{gag} monoclonal antibody (data not shown). Viable cell immunofluorescence (9) with the peptide 1 antibody, TBR serum, or the p15^{gag} monoclonal antibody did not detect *fgr*-specific determinants on the external cell surface.

It is interesting that the peptide 1 antibody detects two similar *onc* gene proteins, v-*yes* and v-*src*, at adhesion plaques and cell-cell junctions (5, 6), whereas the v-*fgr* protein appears to be cytoplasmic. The immunofluorescence pattern of P70$^{gag\text{-}actin\text{-}fgr}$ is in fact very similar to that of another *onc* gene protein with tyrosine-specific kinase activity, namely, P130$^{gag\text{-}fps}$ (4, 28).

Distribution of P70$^{gag\text{-}actin\text{-}fgr}$ among subcellular fractions. Immunofluorescence microscopy was insufficient in itself to assign a specific intracellular location to P70$^{gag\text{-}actin\text{-}fgr}$. To analyze the subcellular distribution of the v-*fgr* transforming protein in more detail, we made use of the quantitative cell fractionation techniques described by Courtneidge et al. (3). A nuclear pellet, a high-speed particulate fraction (P100), and a soluble fraction (S100) were obtained from GR-Mink cells. Each fraction was probed for the presence of P70$^{gag\text{-}actin\text{-}fgr}$ by assaying the autophosphorylation of immunoprecipitated v-*fgr* protein. Because the peptide 1 antibody and, to a lesser extent, TBR serum, inhibited the in vitro kinase activity of P70$^{gag\text{-}actin\text{-}fgr}$, we used the p15^{gag} monoclonal antibody to immunoprecipitate the v-*fgr* protein from cell fractions.

The largest concentration of the v-*fgr* protein, representing 42% of the total *fgr* kinase activity recovered, was contained within the high-speed particulate fraction (P100) (Table 1). Lesser amounts of *fgr* kinase activity, 30 and 27%, were detected in the S100 and nuclear fractions, respectively. The association of *fgr* kinase activity with the nuclear fraction may reflect the relative purity of this fraction, in that enzymatic markers of the endoplasmic reticulum (NADH diaphorase) and plasma membrane (5′-nucleotidase) showed that substantial amounts of these membranous components were retained with the crude nuclear pellet (Table 1). Furthermore, the levels of plasma membrane contamination (5′-nucleotidase activity) and *fgr* kinase activity were nearly identical in the nuclear fraction. A significant amount of P70$^{gag\text{-}actin\text{-}fgr}$, 30% of the total kinase activity, was detected in the soluble fraction (S100). There was little contamination of the S100 fraction with membranes, as indicated by activities of 7% for NADH diaphorase and 3% for 5′-

TABLE 1. Distribution of the v-*fgr* protein in subcellular fractions[a]

Fraction	% of total recovered[b]			
	Lactic dehydrogenase	NADH diaphorase	5′-Nucleotidase	v-*fgr*[c]
Nuclear	1	43	32	27
P100	2	50	65	42
S100	97	7	3	30

[a] Cells were homogenized and then fractionated by differential centrifugation.
[b] Average of three determinations.
[c] Kinase activity was detected by immunoprecipitation with excess p15^{gag} monoclonal antibody.

nucleotidase. These results suggested that there were two separate cellular compartments for the v-*fgr* protein; a minority (about 30%) associated with the cytosol, and the majority associated with membranes.

The distribution of P70$^{gag\text{-}actin\text{-}fgr}$ within crude membrane preparations (P100 pellet) were further analyzed by centrifugation of the P100 pellet through a four-step discontinuous sucrose gradient. v-*fgr* kinase activity cofractionated with the plasma membrane marker 5'-nucleotidase (Table 2). Light-density membranes, at the 20%/35% sucrose interface, contained the highest level of v-*fgr* kinase activity, 65% of total, along with 71% of the total 5'-nucleotidase activity. Thus, this fraction was enriched for both the plasma membrane and P70$^{gag\text{-}actin\text{-}fgr}$. Only low levels of P70$^{gag\text{-}actin\text{-}fgr}$ migrated at the 35%/40% and 40%/50% interfaces, whereas the marker for the endoplasmic reticulum, NADH diaphorase, was enriched at the 40%/50% sucrose boundary. Experiments with [^{35}S]methionine-labeled GR-Mink cells revealed a similar distribution of the v-*fgr* protein among the subcellular fractions (data not shown). Taken together, these results indicate that P70$^{gag\text{-}actin\text{-}fgr}$ preferentially associates with the plasma membrane, and the plasma membrane-bound component may represent the major intracellular pool of the v-*fgr* protein.

Association of P70$^{gag\text{-}actin\text{-}fgr}$ with the detergent-insoluble cytoskeletal matrix. The plasma membrane is known to retain a significant amount of protein in cytoskeletal matrix form following extraction with Triton X-100 (1, 10, 29). Because we had shown that the v-*fgr* protein cofractionates to a large degree with plasma membranes, we reasoned that this affiliation could also reflect an interaction with the detergent-insoluble cytoskeletal matrix. As an alternative to the subcellular fractionation methods described above, we extracted *fgr*-transformed cells in situ with 0.1% Triton X-100 in a buffer (PHEM) designed to retain the integrity of the cytoskeleton (20). Following treatment with PHEM extraction buffer, 58% of the [^{35}S]methionine-labeled v-*fgr* protein remained with the detergent-insoluble matrix. A similar proportion of the total v-*fgr* kinase activity was also detected with the insoluble matrix. However, if the salt concentration of the extraction buffer was raised to 500 mM NaCl, only 21% of the total cellular v-*fgr* protein remained with the insoluble matrix. Thus, there was evidence to suggest that the membrane-bound v-*fgr* protein was associated through weak ionic interactions with the cytoskeletal matrix.

To test if the membrane-associated v-*fgr* protein was in fact bound to cytoskeletal matrix proteins, we subjected crude membranes (P100 pellet) to extensive extraction with PHEM extraction buffer. P100 membranes were agitated for 15 min on ice in detergent buffer and then centrifuged at 100,000 × *g* for 30 min. The lysate and detergent-extracted pellet were then assayed for the amount of v-*fgr* kinase activity within each fraction. Detergent-treated P100 membranes from GR-Mink and GR-FRE cells retained, respectively, 90 and 96% of their v-*fgr*-associated kinase activities. These results are not surprising, considering that a few other plasma membrane proteins, including 5'-nucleotidase, remain with the insoluble membrane matrix following similar detergent extraction (10).

TABLE 2. Distribution of P70$^{gag\text{-}actin\text{-}fgr}$ following discontinuous sucrose gradient centrifugation[a]

Fraction	% of total recovered[b]		
	NADH diaphorase	5'-Nucleotidase	P70$^{gag\text{-}actin\text{-}fgr}$[c]
20%/35%	25	70	65
35%/40%	21	15	16
40%/50%	54	15	19

[a] Crude membranes (P100) obtained by homogenization and differential centrifugation were fractionated on discontinuous sucrose gradients.
[b] Average of three determinations.
[c] Kinase activity was detected by immunoprecipitation with excess p15^{gag} monoclonal antibody.

DISCUSSION

In this report, we have shown that the v-*fgr* transforming protein, P70$^{gag\text{-}actin\text{-}fgr}$, affiliates with the plasma membrane and that this association involves attachment to the detergent-insoluble matrix. Following Dounce homogenization, P70$^{gag\text{-}actin\text{-}fgr}$ fractionated to a large extent with the membrane fraction (P100), leaving only a third of the total v-*fgr* protein with the cytosolic fraction (S100). When membranes (P100) were analyzed in more detail by step gradient fractionation, P70$^{gag\text{-}actin\text{-}fgr}$ cofractionated with the plasma membrane marker 5'-nucleotidase but not with markers of the cytosol or endoplasmic reticulum. We have also detected modification of the v-*fgr* protein through the addition of myristic acid. Although myristylation is not a prerequisite for membrane association, all retroviral transforming proteins that contain this modification have propensities for membrane attachment (21, 23). The subcellular localization of P70$^{gag\text{-}actin\text{-}fgr}$ was also examined by an alternate method, the isolation of the detergent-insoluble cytoskeletal matrix. By this method, the majority of the v-*fgr* protein was shown to be bound to the insoluble matrix components. Furthermore, detergent extraction of membrane fractions left nearly all of the v-*fgr* protein with the residual membrane-associated insoluble matrix. These results demonstrate that the v-*fgr* protein is associated with the plasma membrane and that this association may be mediated through the cytoskeletal matrix.

All oncogenes that possess a tyrosine kinase activity have been found associated in some form or other with the cellular plasma membrane. Such oncogenes can be classified into three distinct groups based on the type of plasma membrane association. One group represents integral transmembrane proteins most likely derived from normal growth factor receptors (e.g., *erbB*, *neu*, *fms*, and probably *ros*). Another group represents proteins associated with the cytoplasmic surface of the plasma membrane and notably sublocalized in cell-substratum adhesion sites (e.g., *abl*, *src*, and *yes*). The third group also exhibits an association with the cytoplasmic surface of the plasma membrane but much less avidly and is not found in cell-substratum adhesion sites (e.g., *fps/fes*). The results we have presented suggest that *fgr* also belongs to this third group of tyrosine kinase-containing oncogenes. Determined by a number of criteria and techniques, the intracellular distribution of P70$^{gag\text{-}actin\text{-}fgr}$ appears to resemble most closely that of P130$^{gag\text{-}fps}$, the transforming protein encoded by Fujinami sarcoma virus. Crude membrane preparations of Fujinami sarcoma virus-transformed cells prepared in low-ionic-strength buffers contain the majority of P130$^{gag\text{-}fps}$ (4, 28). Our fractionations of GR-FeSV-transformed cells prepared under similar ionic conditions demonstrated that the majority of P70$^{gag\text{-}actin\text{-}fgr}$ also resided in the crude membrane fraction. Furthermore, P130$^{gag\text{-}fps}$ cofractionates with plasma membranes (28), a result we found in the present studies for P70$^{gag\text{-}actin\text{-}fgr}$.

Another prominent similarity between P130$^{gag\text{-}fps}$ and P70$^{gag\text{-}actin\text{-}fgr}$ is found in their intracellular patterns observed

by immunofluorescence microscopy. By this technique, $P130^{gag\text{-}fps}$ appears to be predominantly cytoplasmic and not localized at cellular adhesion areas (4, 28). Our immunofluorescence results for $P70^{gag\text{-}actin\text{-}fgr}$ revealed a nearly identical pattern. It is interesting that many other *onc* gene proteins with tyrosine-specific kinase activity, such as *src*, *abl*, and *yes*, localize at cell-cell junctions as well as substratum adhesion sites (5, 17, 19); however, *fps/fes* transforming proteins are not detected at adhesion plaques and have not been reported in cell-cell junctions (4, 18, 28). The similar fluorescence patterns of both $P70^{gag\text{-}actin\text{-}fgr}$ and $P130^{gag\text{-}fps}$ may reflect the salt-dependent association of both transforming proteins with the detergent-insoluble matrix. Like $P70^{gag\text{-}actin\text{-}fgr}$, $P130^{gag\text{-}fps}$ could be released from whole cells with detergent buffers containing 0.5 M salt (4).

In our studies of $P70^{gag\text{-}actin\text{-}fgr}$, we noted that myristylation was a prominent modification. This is not surprising, since a number of viral *onc* gene proteins that are plasma membrane associated are also similarly modified (2, 21, 23). In our search for other protein modifications, we did not detect glycosylated forms of $P70^{gag\text{-}actin\text{-}fgr}$; however, several highly phosphorylated forms of the v-*fgr* protein were detected when cells were metabolically labeled in the presence of the phosphatase inhibitor *ortho*-vanadate (R. Manger, unpublished data). We are presently investigating whether these modifications of the v-*fgr* protein reflect compartmentalization within transformed cells between bound or cytosolic components and whether these modifications affect the transforming activity of $P70^{gag\text{-}actin\text{-}fgr}$.

It is unclear from the present studies if the actin domain of the v-*fgr* protein has any role in cellular location or transformation. If the actin moiety were involved in targeting of this *onc* gene protein, it seems reasonable that $P70^{gag\text{-}actin\text{-}fgr}$ would be incorporated into the actin-containing cytoskeletal network. Both GR-Mink and GR-FRE cells contain well-formed actin stress fibers (Manger, unpublished data) yet $P70^{gag\text{-}actin\text{-}fgr}$ does not localize within these structures as determined by immunofluorescence microscopy. However, the direct association of $P70^{gag\text{-}actin\text{-}fgr}$ with the detergent-insoluble matrix implies some interaction involving cytostructural elements. Thus, we cannot rule out the possible interaction of the v-*fgr* protein with the cortical actin meshwork. Elucidation of the function of the v-*fgr* actin domain in both intracellular targeting and transformation would be best done by transfection experiments in which the actin region is deleted.

The association of $P70^{gag\text{-}actin\text{-}fgr}$ with the plasma membrane, as determined by the fractionation studies, may appear to be in contradiction to the immunofluorescence pattern of a diffuse cytoplasmic locality. However, these results most likely reflect the distribution of $P70^{gag\text{-}actin\text{-}fgr}$ in the membrane-associated detergent-insoluble matrix. For example, fluorescence studies of cytoplasmic actin have shown a variable amount of diffuse fluorescence often attributed to unpolymerized cytosolic actin (7, 14). However, electron-microscopic immunocytochemical studies by Willingham et al. (26) have indicated that very little actin is in the cytosol but is localized, for the most part, in the microfilament mat attached to the cytoplasmic face of the plasma membrane at both the upper and lower surfaces. Thus, the resultant image in immunofluorescence microscopy is one of a diffuse background with only bundles of actin filaments appearing in detail. Our immunofluorescence studies of the v-*fgr* protein also revealed a cytoplasmic pattern. In addition, the actin matrix attached to purified plasma membranes remains intact in membranes which have been extracted with detergents (24). In the case of the v-*fgr* protein, it also remained with the extracted membrane-associated matrix. It should also be mentioned that the v-*fgr*-transformed cells used in this study have a flattened morphology, which makes any discrete membrane fluorescence difficult to observe.

The actual mechanism by which the related tyrosine-specific retroviral kinases, such as v-*src*, v-*yes*, v-*abl*, and v-*fgr*, transform susceptible cells is still not clear. However, the expression of these transforming proteins at the membrane and matrix localities correlates with drastic alterations in normal cell growth control. It will be of interest in future studies to modify either the v-*fgr* protein or components of the cytoskeletal matrix to see if we can perturb the targeting of this unique *onc* gene protein and perhaps alter its transforming abilities.

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