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Subcellular Localization of Glycoproteins Encoded by the Viral Oncogene v-*fms*

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The McDonough strain of feline sarcoma virus encodes a polyprotein that is cotranslationally glycosylated and proteolytically cleaved to yield transforming glycoproteins specified by the viral oncogene v-*fms*. The major form of the glycoprotein (gp120^{fms}) contains endoglycosidase H-sensitive, N-linked oligosaccharide chains lacking fucose and sialic acid, characteristic of glycoproteins in the endoplasmic reticulum. Kinetic and steady-state measurements showed that most gp120^{fms} molecules were not converted to mature forms containing complex carbohydrate moieties. Fixed-cell immunofluorescence confirmed that the majority of v-*fms*-coded antigens were internally sequestered in transformed cells. Dual-antibody fluorescence performed with antibodies to intermediate filaments (IFs) showed that the IFs of transformed cells were rearranged, and their distribution coincided with that of v-*fms*-coded antigens. No specific disruption of actin cables was observed. The v-*fms* gene products cofractionated with IFs isolated from virus-transformed cells and reassociated with IFs self-assembled in vitro. A minor population of v-*fms*-coded molecules (gp140^{fms}) acquired endoglycosidase H-resistant, N-linked oligosaccharide chains containing fucose and sialic acid residues, characteristic of molecules processed in the Golgi complex. Some gp140^{fms} molecules were detected at the plasma membrane and were radiolabeled by lactoperoxidase-catalyzed iodination of live transformed cells. We suggest that v-*fms*-coded molecules are translated as integral transmembrane glycoproteins, most of which are inhibited in transport through the Golgi complex to the plasma membrane.

The retroviral oncogene v-*fms* differs from most known oncogenes in encoding a transforming glycoprotein (2, 36). This oncogene, represented exclusively in the McDonough strain of feline sarcoma virus (SM-FeSV), was acquired by recombination between feline leukemia virus (FeLV) and proto-oncogene sequences (c-*fms*) present in the cellular DNA of domestic cats (8, 13). Substitution of the v-*fms* gene for portions of the FeLV *gag* and *pol* genes renders the SM-FeSV genome replication defective. The truncated *gag* gene (Δ*gag*) and the v-*fms* sequences form an open reading frame that encodes a polyprotein containing amino-terminal and carboxyl-terminal portions specified by the Δ*gag* and v-*fms* sequences, respectively (4, 34, 43). Glycosylation and proteolysis of the primary translational product generate a *gag*-coded amino-terminal peptide and a carboxyl-terminal v-*fms*-coded glycoprotein (gp120^{fms}) that accumulates in transformed cells. A higher-molecular-weight form of the glycoprotein (gp140^{fms}) has also been detected and was presumed to result from further posttranslational processing of gp120^{fms} molecules. Immunofluorescence of fixed transformed cells with either polyvalent antisera or monoclonal antibodies specific for v-*fms*-coded antigens demonstrated a granular perinuclear staining pattern, whereas v-*fms*-coded antigens were not previously detected at the cell surface (2).

A prerequisite for glycosylation is the translocation of proteins from the cytoplasm into the cisternae of the endoplasmic reticulum (ER) (for review, see reference 24). Translocation is mediated by signal peptides located near the amino terminus of nascent polypeptide chains that bind to signal recognition particles on the ER membrane (1, 28, 45). The proteins synthesized on membrane-bound polyribosomes are transferred across the ER membrane and are either targeted to membranous organelles or secreted from the cell. In the case of most integral transmembrane proteins, translocation across the ER membrane is interrupted by hydrophobic stop transfer signals in the nascent chain that immobilize the protein in the membrane and position carboxyl-terminal amino acid residues in the cytoplasm. Once the proteins are positioned in the ER membrane, their orientation is maintained regardless of their final destination in the cell (6, 50). Nucleotide sequence analysis of SM-FeSV proviral DNA revealed characteristic signal sequences in the polyprotein (18). In addition to an amino-terminal signal peptide, a 26-amino-acid stop transfer sequence was predicted to occur near the middle of the glycoprotein ca. 400 amino acids from the carboxyl terminus, suggesting that a substantial portion of the molecule is oriented at the cytoplasmic side of the ER membrane. The terminal 200 amino acids of the predicted cytoplasmic domain show a high degree of amino acid homology to a family of retroviral-transforming proteins that specify tyrosine-specific protein kinases (18).

Based on the perinuclear localization of the v-*fms*-coded glycoproteins in transformed cells, most molecules appeared not to be targeted to the plasma membrane. An analysis of oligosaccharide processing now shows that most v-*fms*-coded glycoproteins are retarded in their transport within the ER-Golgi complex. These events are associated with the rearrangement of intermediate filaments (IFs) in the cytoplasm of transformed cells and their codistribution with internally sequestered v-*fms*-coded antigens. A minority of v-*fms*-coded molecules acquire complex N-oligosaccharide chains and can be detected on the cell surface.

MATERIALS AND METHODS

Cells. Mv 1 Lu mink embryonic lung cells were originally obtained from the American Type Culture Collection (CCL 64). NRK cells (10) were obtained from George Todaro (Oncogene, Seattle, Wash.). The derivation and properties

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of clonal lines of these cells nonproductively transformed by SM-FeSV were described in previous studies (8, 13, 34). Cells for immunofluorescence were grown on 12-mm^2 glass cover slips in humidified 60-mm petri dishes and were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum for 48 h before fixation. Cells propagated in roller bottles or flasks were maintained in the same medium.

Antisera and monoclonal antibodies. Rabbit antisera to keratin and vimentin were generously provided by T. T. Sun and R. O. Hynes, respectively, and have been extensively characterized (21, 39, 40). These antibodies were also used to stain frozen sections of rat skin and intestine by one of us (M.A.G.); the keratin antibodies were specific for epithelial layers, whereas vimentin antibodies decorated only subcutaneous regions. Affinity-purified, fluorescein-conjugated goat antirabbit immunoglobulin G (IgG) and rhodamine-conjugated goat anti-rat IgG were obtained commercially (Kirkegaard and Perry, Inc.) and preadsorbed against fixed Mv 1 Lu and NRK cells as described below. Polyvalent antisera to v-*fms*-coded antigens were prepared in tumored rats as previously described (34). Monoclonal antibodies to v-*fms*-coded epitopes were prepared by fusion of spleen cells from a tumored rat with an established rat myeloma cell line (2). Two monoclonal IgG preparations (SM 1.32.6 and SM 3.19.4) were mixed at a 1:1 ratio before radioiodination. Sodium dodecyl sulfate (SDS)-denatured v-*fms*-specific proteins retain antigenic sites reactive with these antibodies after electrophoresis and transfer to nitrocellulose.

Immunofluorescence. Cells on cover slips were exposed for 10 s to hypotonic buffer containing 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.9), 5 mM $MgSO_4$, 2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid], 1 mM dithiothreitol, 1 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100, followed by a 10-s rinse in the same buffer without the detergent and fixation in methanol for 6 min at −10°C. Antisera were diluted in buffer containing 10 mM Tris-hydrochloride (pH 7.2), 2 mg of bovine serum albumin per ml, 0.1% Triton X-100, and 0.02% saponin (TBTS). The rat antiserum to v-*fms*-coded antigens was sequentially preadsorbed against methanol-fixed Mv 1 Lu and NRK cells at 37°C for 45 min. This serum could also be adsorbed with disrupted feline leukemia viral antigens without altering the nature of its immunofluorescence reactivity. All antisera were clarified by centrifugation at 15,000 × *g* for 10 min at 4°C and used at a final dilution of 1/50 (rat anti-*fms*), 1/25 to 1/50 (rabbit antivimentin), and 1/100 (rabbit antikeratin). In dual labeling experiments, equal volumes of rat and rabbit sera were mixed before reaction with fixed cells. Affinity-purified fluorescein and rhodamine conjugates were diluted in TBTS buffer and mixed 1:1 to a final concentration of 50 μg/ml before use.

Cells were mounted in Aqua-Mount (Lerner) containing 0.25 mg of ρ-phenylenediamine to reduce fading during dual fluorescence microscopy (22, 30). Slides were viewed and photographed within 48 h after mounting by using epifluorescence illumination at 546 nm with a TK495 (Leitz/Opto-Metric Div. of E. Leitz Inc.) beam-splitting mirror for blue excitation (fluorescein) and a TK580 beam-splitting mirror for green excitation (rhodamine) with a K580 suppression filter. Cover slips were examined with a 100× oil immersion objective on a Leitz Orthoplan microscope. Matched exposure and developing times were used for recording all paired negatives and prints.

Electron microscopy. Purified IFs for thin-section electron microscopy were pelleted by centrifugation at 3,000 × *g* for 10 min. Pellets were fixed in 2.5% glutaraldehyde, followed by 1% osmium tetroxide, ethanol dehydration, and embedding in Epon 812. Thin sections were cut and double stained with uranyl acetate and lead citrate. For negative staining, purified IFs in suspension were adsorbed for 5 min to a glow-discharged, carbon-coated Parlodion-covered 300-mesh copper grid. The residual fluid was blotted, and a drop of 2% phosphotungstic acid (pH 4.2) was applied briefly. Again, the residual fluid was blotted, and the grid was allowed to air dry. All photographs of electron microscopic samples were made with a Hitachi HU-12A electron microscope operated at 75 kV.

Subcellular filament fractionation. IFs were isolated by a slightly modified, previously published protocol (16). Subconfluent cultured cells were scraped from 850-cm^2 plastic roller bottles into phosphate-buffered saline (PBS) and pelleted by low-speed centrifugation. The pelleted cells from 12 culture bottles were suspended in 15 ml of 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.01 M NaCl and 2 mM $MgCl_2$. Water (30 ml) was added rapidly with vortex mixing, and the cells were allowed to swell for 15 min on ice. The cells were disrupted in a Dounce homogenizer as visualized by phase-contrast microscopy, and the nuclei were collected by centrifugation at 1,000 × *g* for 10 min. Each of the following extractions was followed by centrifugation at 8,000 × *g* for 10 min to collect insoluble material. The nuclear and perinuclear material was extracted in four 10-ml volumes of 10 mM Tris-hydrochloride (pH 7.5)–2 mM EGTA–140 mM KCl, followed by two 10-ml volumes of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EGTA. The pelleted material was next incubated on ice at 4°C for 15 h in a 10-ml volume of 10 mM Tris-hydrochloride (pH 7.5)–10 mM EGTA. The insoluble pellet was suspended for 10 min at 37°C in a 10-ml volume of 20 mM Tris-hydrochloride (pH 8.0)–50 μg of DNase I (Worthington Diagnostics) per ml–5 mM MgCl–50 mM NaCl–0.1 mM phenylmethylsulfonyl fluoride and incubated again in a 10-ml volume of 10 mM Tris-hydrochloride (pH 7.5)–10 mM EGTA for 15 h on ice at 4°C. The insoluble material was extracted four times by incubation on ice at 4°C for 6 h in 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM EGTA, 600 mM KI, 10 mM $Na_2S_2O_3$, and 10 mM 2-mercaptoethanol. The final pellet was washed in five 10-ml volumes of ice-cold water.

In vitro self-assembly of IFs. IFs purified as described above were suspended in a 10-ml volume of 0.01 M Tris-hydrochloride (pH 7.5) containing 1% SDS and 8 M urea and incubated for 15 h at 25°C. The denatured filaments were renatured after chromatography over a 5-ml packed volume of Dowex anion-exchange resin (acetate form, AG 1-X2, 200-400 mesh, Bio-Rad Laboratories) (47). The eluate was dialyzed against 2 liters of 5 mM Tris-hydrochloride (pH 7.5)–25 mM 2-mercaptoethanol–0.1 mM EDTA (38) for 2 h at 25°C and then for 15 h at 4°C. The reassembled filaments were pelleted for 10 min at 2,000 × *g* and washed in ice-cold water.

Metabolic radiolabeling, preparation of cell lysates, and immunoprecipitation. Metabolic radiolabeling medium consisted of DMEM supplemented with 2% dialyzed fetal calf serum. Subconfluent cultures of SM-FeSV-transformed cells in 75-cm^2 tissue culture flasks were incubated at 37°C in either methionine-free medium containing 0.5 mCi of L-[^{35}S]methionine (1,200 Ci/mmol) per ml or DMEM containing D-[6-^{3}H]glucosamine hydrochloride (1.0 mCi/ml; 40 Ci/mmol), D-[2-^{3}H]mannose (1.0 mCi/ml; 15 Ci/mmol), or L-[6-^{3}H]fucose (0.5 mCi/ml; 84 Ci/mmol). After 2 h, 5 ml of fresh

DMEM containing 10% fetal calf serum was added, and the incubation was continued for an additional 6 h. Monolayers were lysed in RIPA buffer (50 mM Tris-hydrochloride [pH 7.4] containing 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) with 2% aprotinin (Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors. Immunoprecipitation was carried out as previously described (8), using protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Inc.) as immunoadsorbant.

For pulse-chase experiments, parallel cultures of SM-FeSV-transformed Mv 1 Lu cells grown in 60-mm tissue culture dishes were incubated for 1 h in methionine-free radiolabeling medium and then labeled for 15 min in this medium containing 200 μCi of L-[^{35}S]methionine per ml. The labeling medium was removed, and the cultures were incubated in 2 ml of complete DMEM containing 10% fetal calf serum and a 100-fold excess of nonradioactive L-methionine. At the indicated time points during the chase interval, the medium was removed, and cells were lysed in RIPA buffer containing protease inhibitors. Immunoprecipitation of both the detergent lysate and chase medium was carried out as described above.

Radioiodination of cell surface proteins. Cells grown to confluence in 25-cm^2 flasks were rinsed extensively with PBS and iodinated by the addition of 1 mCi of Na^{125}I and 20 μg of lactoperoxidase (79 U/mg; Sigma) in 1 ml of PBS per flask. Two separate 10-μl samples of fresh 30% hydrogen peroxide per flask were added at 60-s intervals. After a 10-min reaction at room temperature, the reaction mixture was removed, and the cell sheets were rinsed extensively with PBS. Polyclonal or monoclonal antibodies at a ratio of 20 μl in 500 μl of PBS (vol/vol) were added to the cell sheets and reacted for 15 min at room temperature. The solution was then removed, and the cells were lysed and immunoprecipitation was continued as described above.

Enzymatic digestions of immunoprecipitates. Metabolically radiolabeled v-*fms* glycoproteins were immunoprecipitated from 0.5-ml lysates with 20 μl of polyvalent rat serum and protein A-Sepharose CL-4B (40 μl of a 50% [vol/vol] slurry in RIPA buffer) as immunoadsorbant. Immune complexes were washed three times in RIPA buffer and then for three additional times in RIPA buffer lacking SDS and EDTA. The resulting precipitates were divided and incubated for 20 h at 37°C in 20 μl of either (i) 0.3 U of *Streptomyces griseus* endo-β-*N*-acetylglucosaminidase H (endo H) (EC 3.2.1.96) (Sigma) per ml in 150 mM sodium citrate (pH 5.3) or (ii) 1.0 IU of *Vibrio cholerae* neuraminidase (EC 3.2.1.18) (Calbiochem-Behring) per ml in 50 mM sodium acetate (pH 5.5) containing 150 mM NaCl and 4 mM $CaCl_2$. Parallel control incubations were done by using the indicated buffer solutions without enzymes. Reactions were terminated by the addition of 60 μl of sample buffer (0.0625 M Tris-hydrochloride [pH 6.8] containing 3% SDS, 10% glycerol, and 50 mM dithiothreitol) and heating to 100°C for 2 min. Products were analyzed by SDS-gel electrophoresis and autoradiography or fluorography.

Polyacrylamide gel electrophoresis and immunoblotting. The SDS-polyacrylamide disc gel electrophoresis technique was essentially that of Laemmli (25), except that the sample was buffered at pH 8.0 and contained 5% glycerol. Apparent molecular weights of proteins were estimated from their electrophoretic mobility relative to that of the following marker proteins: myosin (200 kilodaltons [kd]), phosphorylase *b* (92.5 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), and α-chymotrypsinogen (25.7 kd). Highly purified keratin preparations were treated with 1% SDS at 25°C until fully solubilized before boiling in sample buffer for electrophoresis. Samples were separated on continuous-gradient 6 to 12% acrylamide slab gels at a concentration of 30 μg of protein per lane and electrophoretically transferred (42) to 0.20-μm nitrocellulose membrane filters (Schleicher & Schuell, Inc.) in transfer buffer containing 25 mM Tris, 200 mM glycine, and 20% methanol. The transferred blot was rinsed in TNE-NP40 buffer, containing 50 mM Tris-hydrochloride (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40, followed by preadsorption in the same buffer containing 3% bovine serum albumin and 1% bovine gamma globulin for 3 h at 37°C. Preadsorbed blots were reacted with 2×10^6 cpm per lane of ^{125}I-labeled monoclonal antibody in preadsorb buffer for 16 h at 4°C. Immunoblots were then rinsed extensively in TNE-NP40 buffer, dried, and exposed to Kodak XRP-1 X-ray film.

Peptide mapping. Metabolically radiolabeled polypeptides in polyacrylamide gel slices were oxidized with performic acid and digested with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated trypsin as previously described (32). Eluted peptides were separated in two dimensions on cellulose-coated, thin-layer plates (20 by 20 cm; EM Laboratories) by electrophoresis at 1,000 V for 20 min in acetic acid-formic acid-water (15:5:80, pH 1.9), followed by ascending chromatography in 1-butanol-pyridine-acetic acid-water (65:50:10:40) containing 7% (wt/vol) 2,5-diphenyloxazole. Dried plates were exposed to Kodak XAR-5 film for fluorography.

RESULTS

Kinetics of formation of v-*fms*-coded polypeptides. Figure 1 shows the results of kinetic studies in which parallel cultures of mink cells transformed by SM-FeSV were labeled with [^{35}S]methionine for 15 min and then incubated in fresh medium containing nonradioactive methionine. Cell lysates were prepared at various intervals, and SM-FeSV polypep-

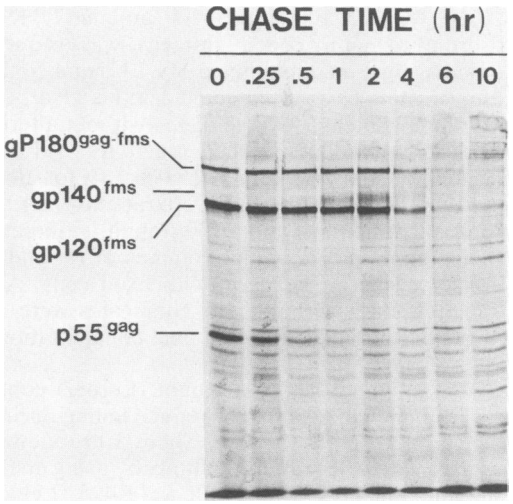

FIG. 1. Kinetics of formation of gp140^{fms}. Parallel cultures of SM-FeSV-transformed cells were labeled with [^{35}S]methionine for 15 min at 37°C and then chased for the indicated times in complete medium containing a 100-fold excess of nonradioactive methionine. Detergent lysates were prepared, and SM-FeSV polypeptides were immunoprecipitated. Samples were analyzed by SDS-gel electrophoresis in a 7.5% polyacrylamide slab. SM-FeSV polypeptides are indicated at the left margin.

tides were precipitated with antisera, electrophoretically separated in polyacrylamide, and visualized by autoradiography of the dried slab gel. After labeling for 15 min, gP180$^{gag\text{-}fms}$ and its cleavage products, gp120^{fms} and p55^{gag}, were readily apparent. At chase intervals between 1 and 4 h after labeling, an additional polypeptide of 140 kd was observed; like gp120^{fms}, the 140-kd polypeptide was precipitated with polyvalent or monoclonal antibodies directed to v-*fms*-coded antigens but did not react with antisera to *gag* gene-coded determinants (hence, the designation gp140^{fms}). Immunoprecipitation of the harvested medium from the experiment shown in Fig. 1 showed no evidence for secretion of v-*fms* glycoproteins during the chase interval.

Cleavage of gP180$^{gag\text{-}fms}$ to p55^{gag} and gp120^{fms} does not appear to represent artifactual proteolysis in vitro since all three molecular species are observed in detergent lysates prepared in the presence of protease inhibitors. Moreover, the various products are seen in SM-FeSV-transformed rat and mouse nonproducer cells, including those derived by transfection with molecularly cloned SM-FeSV proviral DNA (8). These three polypeptides can be detected after labeling intervals as brief as 5 min, suggesting that proteolytic cleavage might be a cotranslational event. However, gP180$^{gag\text{-}fms}$ was not completely processed to p55^{gag} and gp120^{fms} (Fig. 1), nor were either of these rapidly labeled v-*fms*-coded glycoproteins quantitatively converted to gp140^{fms} molecules during the chase interval. Immunoblotting experiments confirm that gp120^{fms} is the major glycoprotein species detected under steady-state conditions in transformed cells (2; see below).

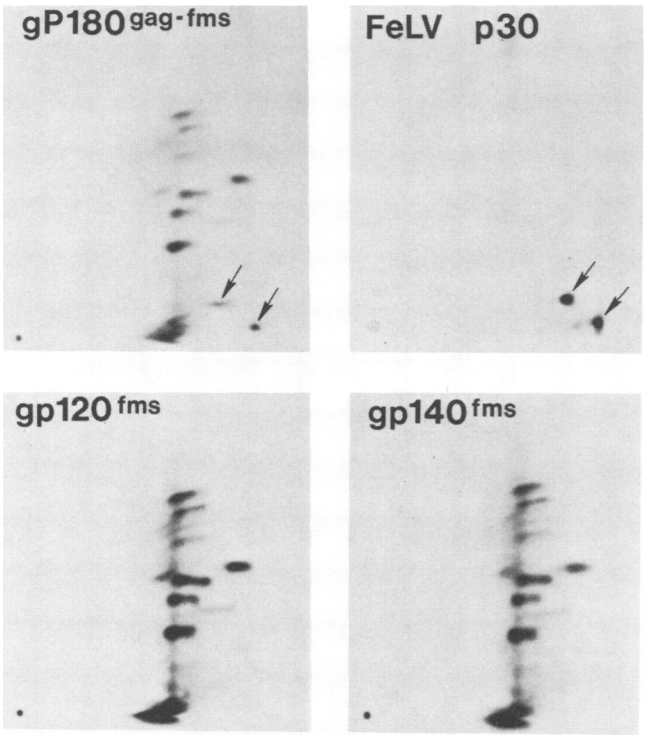

FIG. 2. Two-dimensional tryptic peptide analysis of [^{35}S]methionine-labeled v-*fms*-coded glycoproteins. The indicated polypeptides were oxidized with performic acid and digested with trypsin. Peptides were applied at the origin in the lower left corner of each panel and were separated by electrophoresis at pH 1.9 from left (anode) to right (cathode), followed by thin-layer chromatography (bottom to top). Radiolabeled tryptic peptides were visualized by fluorography. For comparison, methionine-containing tryptic peptides of the FeLV p30 structural protein are also shown. Peptides common to gP180$^{gag\text{-}fms}$ and FeLV p30 are marked with arrows in the upper panels.

Carbohydrate moieties of gp120^{fms} and gp140^{fms}. Tryptic peptide analyses indicate that gp120^{fms} and gp140^{fms} have very similar sets of [^{35}S]methionine-labeled peptides (Fig. 2). Both molecules lack two methionine-containing tryptic peptides characteristic of the *gag* gene-coded p30 sequence of gP180$^{gag\text{-}fms}$; these two peptides were present in tryptic digests of the p55^{gag} cleavage product from SM-FeSV-transformed cells. The fingerprints of gP180$^{gag\text{-}fms}$, gp120^{fms}, and gp140^{fms} all contained unresolved peptides that failed to migrate in the organic chromatography solvent and were therefore presumed to be glycopeptides.

Carbohydrate addition to the v-*fms* polypeptides is sensitive to the antibiotic tunicamycin (2), showing that the oligosaccharides are N-linked to asparagine residues. Biosynthesis of N-linked carbohydrate chains involves a series of sugar transfer reactions that are catalyzed by enzymes distributed in defined order throughout the ER-Golgi system (24). In particular, protein-linked oligosaccharides in the ER cisternae contain both N-acetylglucosamine and mannose; remodeling of these mannose-rich components takes place in the Golgi system, in which some mannose is removed and the chains acquire additional N-acetylglucosamine as well as galactose, fucose, and sialic acid residues characteristic of complex carbohydrate chains. To assay for the presence of these different classes of oligosaccharides in v-*fms*-coded products, SM-FeSV-transformed mink cells were radiolabeled for 6 h with either L-[^{35}S]methionine, D-[^{3}H]glucosamine, or L-[^{3}H]fucose. SM-FeSV polypeptides were immunoprecipitated from detergent lysates, and the different v-*fms* glycoprotein species were electrophoretically separated. gP180$^{gag\text{-}fms}$, gp120^{fms}, and gp140^{fms} were each metabolically labeled with [^{35}S]methionine and [^{3}H]glucosamine (Fig. 3A). However, only gp140^{fms} was labeled with [^{3}H]fucose, suggesting that this species of v-*fms* glycoprotein contains complex carbohydrate chains, whereas gP180$^{gag\text{-}fms}$ and gp120^{fms} do not. Identical results were obtained with other subclones of SM-FeSV-transformed cells. In agreement with the kinetic studies shown in Fig. 1, the quantity of gp120^{fms} exceeded that of gp140^{fms} even after continuous labeling for 6 h in the presence of the amino acid precursor.

These conclusions were verified with specific glycosidic enzymes. Endo H cleaves mannose-rich, N-linked oligosaccharide chains, leaving a single N-acetylglucosamine attached to the corresponding asparagine residue of the polypeptide chain (23). However, endo H does not attack complex carbohydrate chains formed in the Golgi apparatus. By contrast, neuraminidase selectively removes the terminal sialic acid residues found in complex chains but does not digest high-mannose chains characteristic of glycoproteins in the ER. Digestion of the immunoprecipitated proteins with these enzymes (Fig. 3B and C) demonstrated that both gP180$^{gag\text{-}fms}$ and gp120^{fms} were sensitive to endo H but resistant to neuraminidase. Conversely, gp140^{fms} was resistant to endo H but showed a decrease in apparent molecular weight after neuraminidase treatment.

The apparent molecular weights of unglycosylated SM-FeSV gene products detected in the presence of the antibiotic tunicamycin are 155 kd (P155$^{gag\text{-}fms}$) and 95 kd (p95^{fms}) (2). Incubation of gP180$^{gag\text{-}fms}$ and gp120^{fms} with endo H yielded products only slightly larger than the corresponding polypeptides labeled metabolically with [^{35}S]methionine in the presence of tunicamycin (Fig. 3B). Endo H treatment of

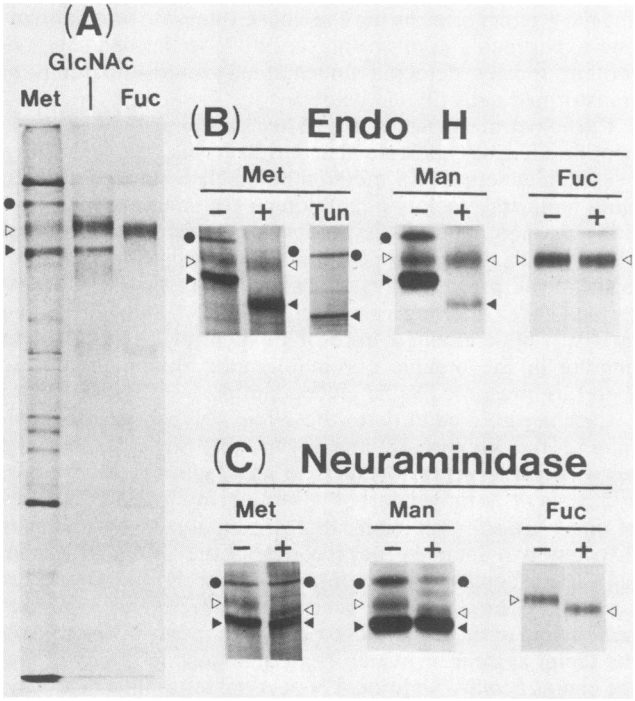

FIG. 3. Analysis of the carbohydrate moieties of v-*fms*-encoded glycoproteins. SM-FeSV-transformed mink cells were incubated for 6 h in the presence of the indicated radioactive precursors: L-[^{35}S]methionine (Met), D-[^{3}H]glucosamine (GlcNAc), D-[^{3}H]mannose (Man), or L-[^{3}H]fucose (Fuc). SM-FeSV proteins were immunoprecipitated, separated by SDS-gel electrophoresis in 7.5% polyacrylamide slabs, and detected by fluorography of the dried gel. (A) Immunoprecipitates of SM-FeSV glycoproteins metabolically radiolabeled for 6 h with the indicated precursors. (B) Immunoprecipitates were incubated at 37°C for 20 h in the absence (−) or presence (+) of endo H before electrophoresis. For comparison of [^{35}S]methionine-labeled digestion products, unglycosylated v-*fms*-coded polypeptides metabolically radiolabeled in the presence of tunicamycin (Tun) were included in the adjacent lane of the gel. (C) Immunoprecipitates were incubated at 37°C for 20 h in the absence (−) or presence (+) of neuraminidase before electrophoresis. In each panel, the mobilities of gP180$^{gag\text{-}fms}$ (●), gp 120^{fms} (▶), and gp140^{fms} (▷) are noted in the left margin. In B and C, the corresponding products after incubation with enzyme are marked in the right margin, and only the relevant portions of the fluorograms are shown.

[^{3}H]mannose-labeled glycoproteins removed all detectable radioactivity from gP180$^{gag\text{-}fms}$ and ca. 90% of the label from gp120^{fms}. The residual radioactivity in gp120^{fms} (seen in Fig. 3B as a protein of ca. 100 kd after endo H treatment) may represent mannose-rich chains protected from digestion in immune complexes by steric effects, partially remodeled chains having an endo H-resistant structure (41), or [^{3}H]mannose incorporated into a minor population of O-linked oligosaccharides. Taken together with the kinetic experiments, these biochemical studies indicate that gp140^{fms} is derived from a limited subset of gp120^{fms} molecules undergoing conversion of mannose-rich, N-linked oligosaccharides to complex carbohydrate chains.

gp140^{fms} molecules at the cell surface. Integral transmembrane glycoproteins acquiring complex N-oligosaccharide chains within the Golgi complex are generally transported to membranous organelles and the plasma membrane. If gp140^{fms} molecules were transported to the cell surface, they would be oriented with their amino-terminal portions protruding from the plasma membrane (18), potentially accessible to lactoperoxidase-catalyzed iodination of live cells. SM-FeSV-transformed and -untransformed control mink cells were therefore radiolabeled with ^{125}I and lysed with detergent, and immune precipitates containing iodinated molecules from the cell surface were examined for the presence of v-*fms*-coded molecules.

Radioiodinated gp140^{fms} molecules were precipitated with a monoclonal antibody that reacts with all molecular forms of the v-*fms*-coded glycoprotein (Fig. 4, lane 2), including gP180$^{gag\text{-}fms}$, gp120^{fms}, and gp140^{fms} (2). No radiolabeled protein was precipitated from iodinated transformed cells with nonimmune rat serum (Fig. 4, lane 1), nor were iodinated v-*fms* proteins from untransformed control cells precipitated with monoclonal antibodies to v-*fms*-coded epitopes (Fig. 4, lane 3). An additional control experiment in which unlabeled transformed cells were lysed with detergent and the proteins were subsequently iodinated confirmed that all forms of the v-*fms*-coded glycoprotein can be labeled with lactoperoxidase (data not shown). The selective detection at the cell surface of gp140^{fms}, the least predominant form of the glycoprotein in transformed cells, shows that the targeting of v-*fms*-coded molecules to the plasma membrane is specific for those glycoproteins containing complex oligosaccharide chains.

Immunofluorescent localization of v-*fms*-coded antigens. In a previous study, fixed-cell immunofluorescence with either polyvalent antisera or monoclonal antibodies specific for v-

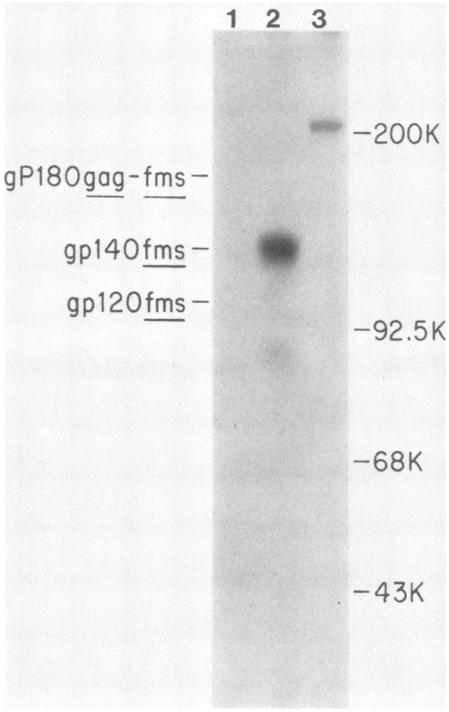

FIG. 4. Analysis of ^{125}I-labeled v-*fms*-coded proteins from the plasma membrane of transformed cells. Immune precipitates of surface-labeled, transformed (lanes 1 and 2) and control (lane 3) mink cells were prepared by using monoclonal antibody to v-*fms*-coded epitopes (lanes 2 and 3) and nonimmune rat serum (lane 1). The positions of gP180$^{gag\text{-}fms}$, gp140^{fms}, and gp120^{fms} (shown at left) were those of the ^{35}S-methionine-labeled proteins run in the same gel. Only gp140^{fms} was detected at the surface of transformed cells. The band at ca. 200 kd detected in normal cells was not further characterized.

fms-coded antigens showed a granular staining pattern concentrated in the perinuclear region of transformed cells (2). This characteristic distribution of v-*fms* glycoproteins is compatible with the sequestration of most of the v-*fms*-coded glycoprotein within the ER-Golgi complex. Ultrastructural examination of SM-FeSV-transformed mink and rat cells showed that the perinuclear cytoplasm contained a condensed network of IFs. To determine whether there was a correlation between the localization of v-*fms*-coded glycoproteins and IF antigens, dual immunofluorescence studies of the major IF subunit proteins and v-*fms*-coded antigens were undertaken.

These experiments were performed with established cell lines from normal rat kidney (NRK) and mink lung epithelium (Mv 1 Lu) and with transformed subclones, each of which contains a single proviral DNA copy of the SM-FeSV genome. Both parental cell lines express the IF subunits keratin and vimentin, consistent with their epithelial origin (for review, see reference 26). In the present studies, the IF antigens were assayed with well-characterized rabbit antisera which specifically decorate keratin or vimentin filaments (21, 39, 40). Results similar to those shown below were obtained with the two different antisera with both cell systems. For brevity, only the results of antikeratin immunofluorescence in NRK cells are presented.

Figure 5 illustrates immunofluorescent staining of control NRK cells (A and B) compared with an SM-FeSV-transformed subclone (C and D). The cells were exposed to nonionic detergent before fixation and staining to facilitate in situ extraction of cellular lipids and soluble cytoplasmic proteins, leaving the insoluble cytoskeletal elements intact (17, 20). The cells were then simultaneously reacted with the rabbit antikeratin serum (Fig. 5A and C) and a specific rat antiserum to the v-*fms*-coded glycoproteins (Fig. 5B and D). Cells in the same viewing field were examined for both classes of antigens with rhodamine- and fluorescein-conjugated, affinity-purified antibodies specific for rat and rabbit immunoglobulins, respectively.

The control NRK cells (Fig. 5A) demonstrated antikeratin reactivity in the juxtanuclear region, predominantly located in the substrate focal plane in close contact with the nucleus, and in multiple fine filaments that streamed radially through the cytoplasm toward the cell borders. No v-*fms*-specific reactivity was observed in untransformed control cells (Fig. 5B). In contrast, the transformed cells demonstrated a disorganized antikeratin reactivity, again most intense in the perinuclear region (Fig. 5C). As compared with control cells, individual filaments were much more difficult to discern but could be visualized near the cell periphery. The v-*fms*-specific staining in transformed cells closely resembled that

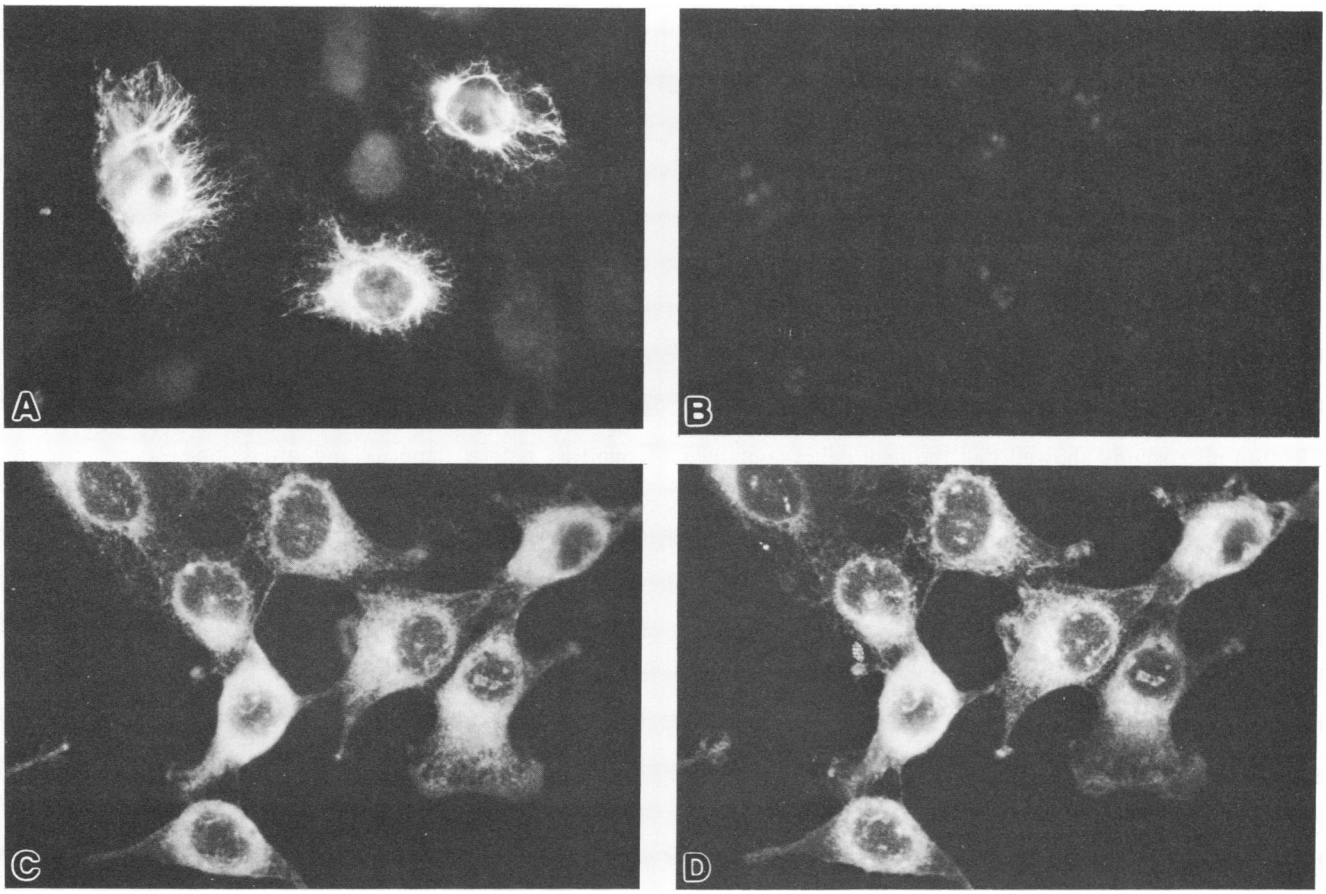

FIG. 5. Dual immunofluorescence of control NRK cells (A and B) and an SM-FeSV-transformed subclone (C and D) stained with rabbit antiserum to keratin (A and C) and rat antiserum to v-*fms*-coded glycoproteins (B and D). Staining was performed by using affinity-purified, fluorescein anti-rabbit IgG (A and C) and rhodamine anti-rat IgG (B and D). Matched fields and exposure times are shown for A and B and for C and D. The control NRK cells shown in A represent an uncloned mixed population of fibroblastic and epithelioid cells derived from an early passage seed stock. The transformed subclone (C) is apparently derived from the parental, keratin-positive population.

observed with antibodies against keratin, suggesting a strong site-specific correlation of the two classes of antigens (cf. the same field shown in Fig. 5C and D).

Two other fields of transformed cells stained with antikeratin (Fig. 6A and C) and anti-v-*fms* sera (Fig. 6B and D) are shown at higher magnification. Codistribution of the two classes of antigens was reproducibly observed. In cells that were well spread on the cover slips, the filaments radiated from the perinuclear region toward the cell border in organized arrays (arrows, Fig. 6A). The v-*fms*-specific fluorescence showed a more stippled appearance, although the longitudinal orientation was maintained (arrows, Fig. 6B). Matched overexposure of some fields of cells diminished the resolution of the intracellular filament arrays but enabled some surface fluorescence to be seen with the anti-*fms*-specific antiserum (arrows, Fig. 6D). This confirmed that even though most v-*fms*-coded molecules were sequestered internally, some were transported to the cell surface.

Considering the close association of the v-*fms* glycoproteins with rearranged IF antigens in transformed cells and previous reports that other virus-transformed cells displayed disrupted actin cables (11, 29, 46), actin-specific immunofluorescence was also studied (Fig. 7). Both control (Fig. 7A) and transformed (Fig. 7B) Mv 1 Lu cells contained well-organized actin cables above and below the nuclear focal plane. Except for numerous actin-rich ruffles at the borders of transformed cells, the normal and transformed cells appeared to be alike. Similar results were obtained with SM-FeSV-transformed NRK cell clones.

Cofractionation of IFs and v-*fms*-coded glycoproteins. To test for a possible association between v-*fms*-coded glycoproteins and IFs, a protocol for the purification of the muscle cell IF protein desmin (16) was adapted to isolate IFs from transformed mink cells. In brief, cells were disrupted by homogenization in hypotonic buffer, and the nuclei and attached juxtanuclear cytoskeletal mass were recovered by low-speed centrifugation. A series of extractions was then performed to remove the majority of non-IF proteins. Insoluble proteins were collected by low-speed centrifugation after each extraction step. DNase was introduced to degrade DNA before the final extractions. In agreement with previous studies (16), most of the microfilament protein (actin) could be removed by extraction with 600 mM potassium iodide, whereas most of the IF network remained insoluble. Electron microscopy showed that the final pellet contained primarily polymerized and aggregated filaments but few contaminating membranes or chromatin.

To assay the quantity of v-*fms* glycoproteins in various extracts and in the final pellet, equal amounts of protein from each fraction were denatured and subjected to electrophoresis. The separated proteins were transferred from the gel slab to nitrocellulose filters and reacted with ^{125}I-labeled monoclonal antibodies specific for v-*fms*-coded epitopes. The results of these immunoblotting experiments are shown in Fig. 8. Unfractionated cell lysates of transformed (lane 1) and control (lane 2) mink cells are shown for comparison. The proteins were derived from the postnuclear supernatant (lane 3) and from sequential extracts of the nuclear pellet (lanes 4 through 7). Proteins remaining insoluble after 600 mM potassium iodide extraction were denatured and blotted at three different concentrations (lanes 8 through 10). This fraction was found to be highly enriched for gp120^{fms} (Fig. 8A). After longer autoradiographic exposure (Fig. 8B), gP180$^{gag\text{-}fms}$ and gp140^{fms} were also detected. To control for the possibility that the v-*fms*-specific proteins copurified with contaminating membranes, the insoluble pellet was further extracted with 1% Triton X-100, 1% Triton X-100 and 0.5% deoxycholate, or 1% Triton X-100 followed by 50% (vol/vol) ether before immunoblotting. All of the v-*fms*-specific proteins remained physically associated with the insoluble, sedimentable filaments and were not detected in the detergent extracts.

Transformed mink cells infected with FeLV were subjected to fractionation and examined for the presence of the viral envelope glycoprotein gp70^{env} to determine whether another glycoprotein would aggregate with filaments during the procedure. Most gp70^{env} was liberated into the postnuclear supernatant under conditions in which v-*fms* antigens remained in the pellet (Fig. 8A, inset). The latter molecules could be sedimented with membranes by ultracentrifugation and were subsequently solubilized with detergent. Some gp70^{env} molecules also sedimented with the nuclei (Fig. 8, lanes 4 through 7) but were removed by the differential salt extraction. These results show that copurification with IFs is not a general property of glycoproteins from transformed cells.

In vitro assembly of v-*fms* antigens with IFs. As an alternative approach to testing for an in vivo association between v-*fms* glycoproteins and IFs, we took advantage of the fact that purified IF subunits from different cell types can reassemble into filaments in vitro (15, 16, 38, 51). Similar experiments were therefore performed with IFs purified from SM-FeSV-transformed cells to test whether the v-*fms*-coded proteins would be reincorporated during self-assembly. The purified IF preparation was disassembled by denaturation, renatured, and assayed for v-*fms*-specific antigens. Denaturation was performed with 1% SDS and 8 M urea, and the detergent was removed from the dissociated proteins by anion-exchange chromatography. The filaments were then renatured by dialysis against aqueous buffer and collected by low-speed centrifugation. Because filament assembly is concentration dependent, and because the filament aggregates are readily sedimented, most contaminating proteins remain in the supernatant fluid.

The sedimentable material obtained after self-assembly in vitro was composed of filaments (Fig. 9). When the polymerized IF proteins were denatured and examined for v-*fms* antigens by immunoblotting, v-*fms*-coded glycoproteins were detected. Figure 9B shows the intensity of the blot signal when equal quantities of protein were subjected to analysis before (lane 1) and after (lane 2) in vitro self-assembly. The intensity of the blot signal for the v-*fms* antigens was increased after IF assembly, and each of the three v-*fms*-coded glycoproteins (gP180$^{gag\text{-}fms}$, gp120^{fms}, and gp140^{fms}) were detected in proportions similar to those seen by immunoblotting in whole-cell lysates (Fig. 8, lane 1). These findings suggest that v-*fms* glycoproteins can associate with IFs in vitro.

DISCUSSION

The intracellular transport of v-*fms*-coded glycoproteins to particular sites in the cell and their subsequent interaction with as yet undefined target molecules must be critical in eliciting the transformed phenotype. Like other glycoproteins which are synthesized on membrane-bound polyribosomes and gain access to the cisternae of the ER, the v-*fms*-coded polypeptides cotranslationally acquire N-linked oligosaccharide chains that are remodeled during intracellular transport of the protein. The major form of the glycoprotein found in transformed cells, gp120^{fms}, is characteristic of molecules found within the cisternae of the ER in that it contains mannose-rich, endo H-sensitive oligosaccharide

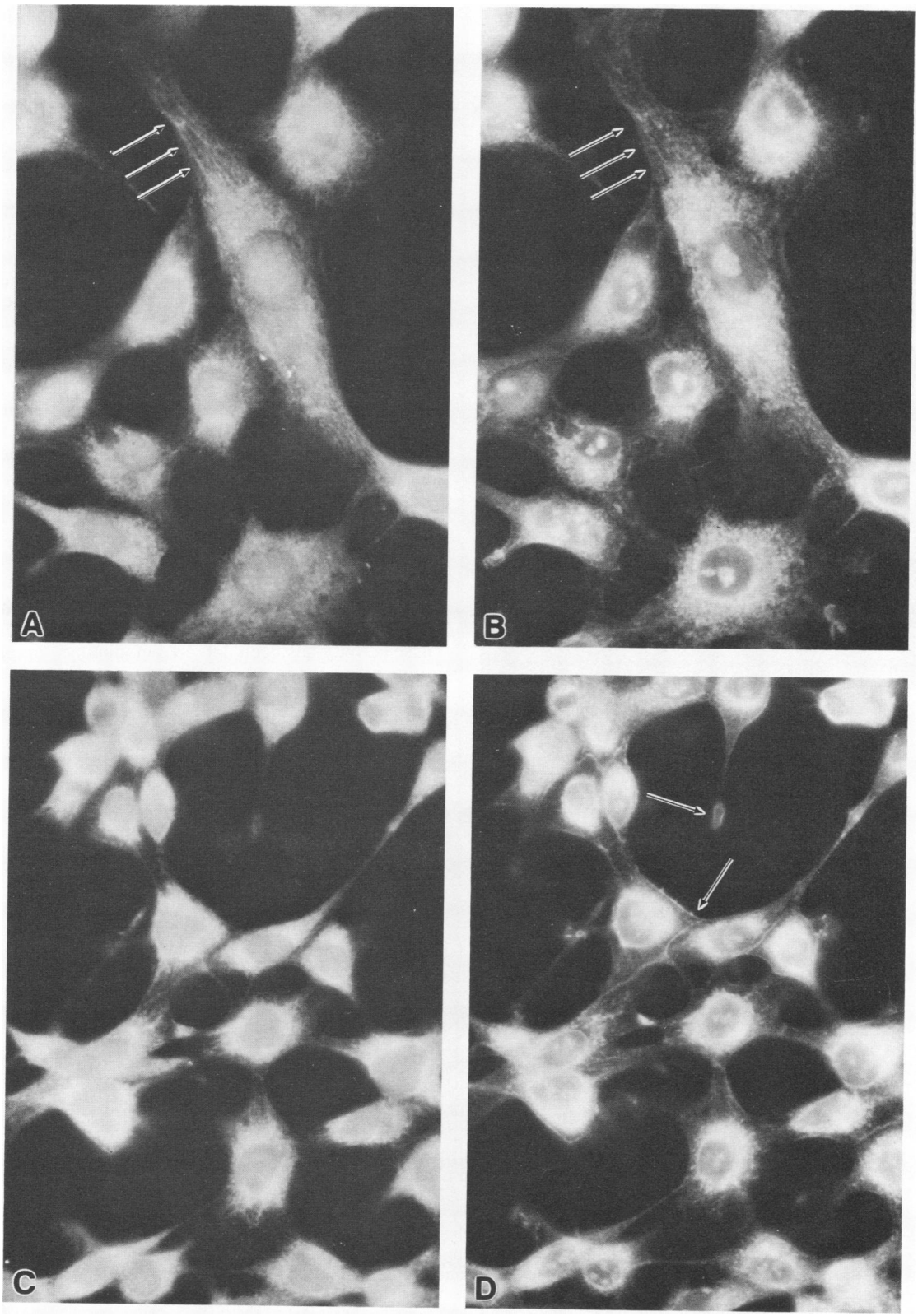

FIG. 6. Dual immunofluorescence staining of SM-FeSV-transformed NRK cells with rabbit antiserum to keratin (A and C) and rat antiserum to v-*fms*-coded glycoproteins (B and D). Staining was performed by using antibody conjugates as described in the legend to Fig. 4. Matched fields and exposure times are shown for panels A and B and for C and D. Arrows in A and B show colocalization of filament arrays (A) and v-*fms*-coded antigens (B). Arrows in D show staining of cell borders with the antiserum to v-*fms*-coded glycoproteins.

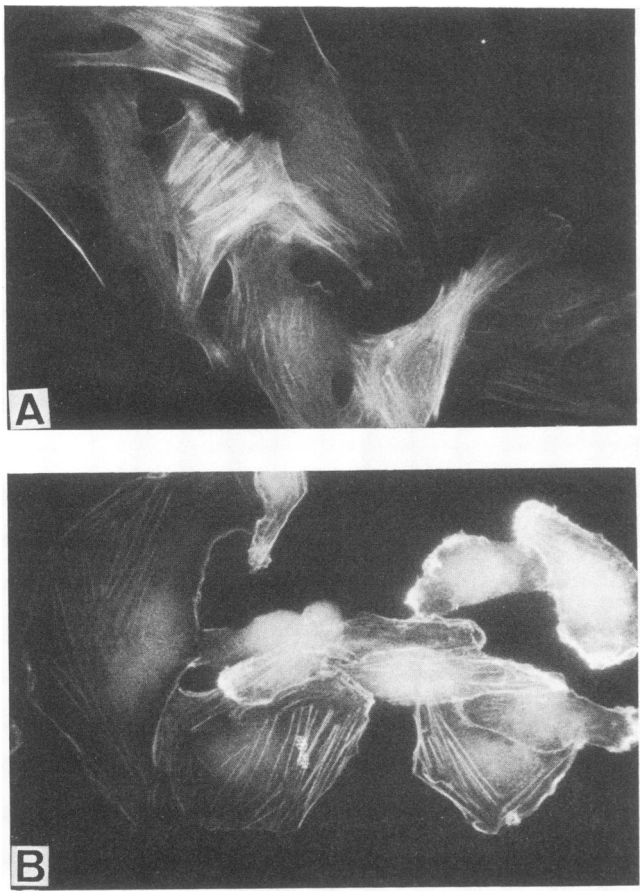

FIG. 7. Actin-specific immunofluorescence of mink Mv 1 Lu cells (A) and an SM-FeSV-transformed subclone (B). Fixation was with methanol.

chains lacking fucose and sialic acid residues. Only a relatively minor population of molecules, gp140^{fms}, undergoes remodeling of its carbohydrate chains, acquiring terminal sugars within the Golgi system. Kinetic experiments verified that most gp120^{fms} molecules remained in an unprocessed form for many hours, whereas few were converted to gp140 molecules within 30 to 120 min of synthesis. Hence, a substantial pool of molecules is not efficiently transported from the ER through the Golgi complex of transformed cells. Immunofluorescence staining with antisera to v-*fms*-coded antigens confirmed that most of the glycoprotein molecules remain internally sequestered. However, a small proportion of the v-*fms*-coded antigens localized to the cell surface as gp140^{fms} molecules.

The nucleotide sequence of SM-FeSV proviral DNA predicts that the v-*fms*-coded polypeptide is synthesized as an integral transmembrane protein (18). The basis for this conclusion is the presence of a strongly hydrophobic, 26-amino-acid stop transfer sequence near the middle of the v-*fms*-coded portion of the polyprotein. The location of the putative transmembrane region within the protein is unusual in that it occurs ca. 400 amino acids from the protein carboxyl terminus. We suggest, then, that the carboxyl-terminal half of the v-*fms* glycoprotein protrudes through the ER membrane into the cytoplasm and is maintained in that orientation throughout its intracellular transport. The terminal 200 amino acids at this end of the polypeptide are closely homologous to those oncogene products that exhibit tyrosine-specific protein kinase activities (18). However, whether the v-*fms* proteins themselves are active kinases in vivo remains controversial. In vitro tyrosine-specific protein kinase activity has been detected in immune complexes under some assay conditions (3; Rettenmier, unpublished data) but not others (33, 44), and no evidence for an in vivo enzymatic function has so far been obtained. The v-*fms*-coded molecules that reach the cell surface would be oriented with the

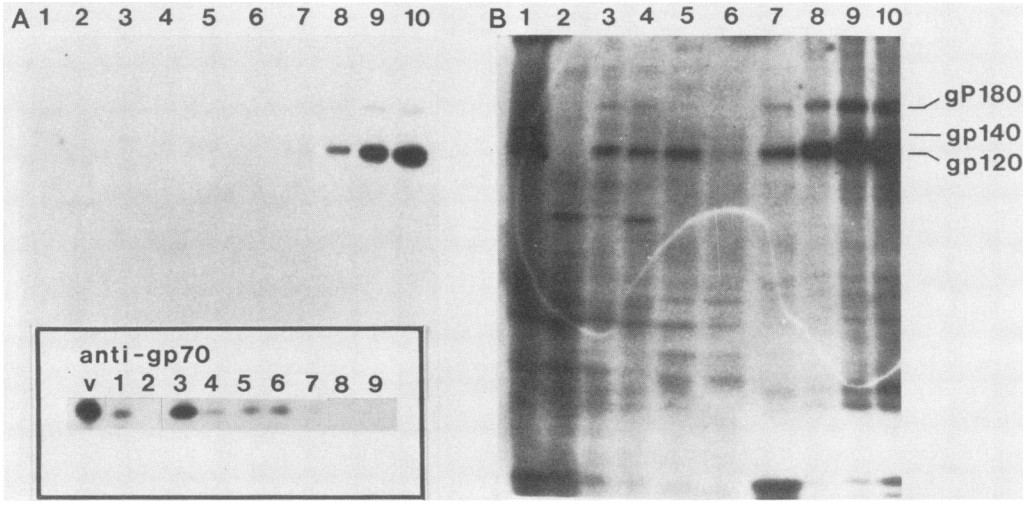

FIG. 8. Detection of v-*fms*- (A and B) and FeLV-coded (inset) glycoproteins in subcellular tractions from SM-FeSV-transformed mink cells. SM-FeSV-coded glycoproteins were detected by immunoblotting with radioiodinated rat monoclonal antibodies. The FeLV envelope glycoprotein gp70^{env} was detected by using an indirect method performed with goat antiserum to gp70^{env} and radioiodinated *Staphylococcus aureus* protein A. Lanes 1 and 2 represent total unfractionated lysates of transformed and control cells, respectively. Subcellular extracts of transformed cells include the postnuclear supernatant fluid (lane 3) and sequential extracts of the nuclear pellet with 150 mM KCl (lane 4), Tris-EGTA (lanes 5 and 6), 600 mM KI (lane 7), and KI-resistant filament proteins at threefold-increasing concentrations (lanes 8 to 10). Twenty micrograms of protein were separated in lanes 1 to 7 and 9. A and B represent 15- and 72-h exposures, respectively. The inset shows the distribution of gp70^{env} in fractions from transformed mink cells infected with FeLV. The lane labeled v contains protein from extracellular virus used as a positive control; all other lanes correspond to those described above. The exposure time for the gp70^{env} blot was 18 h.

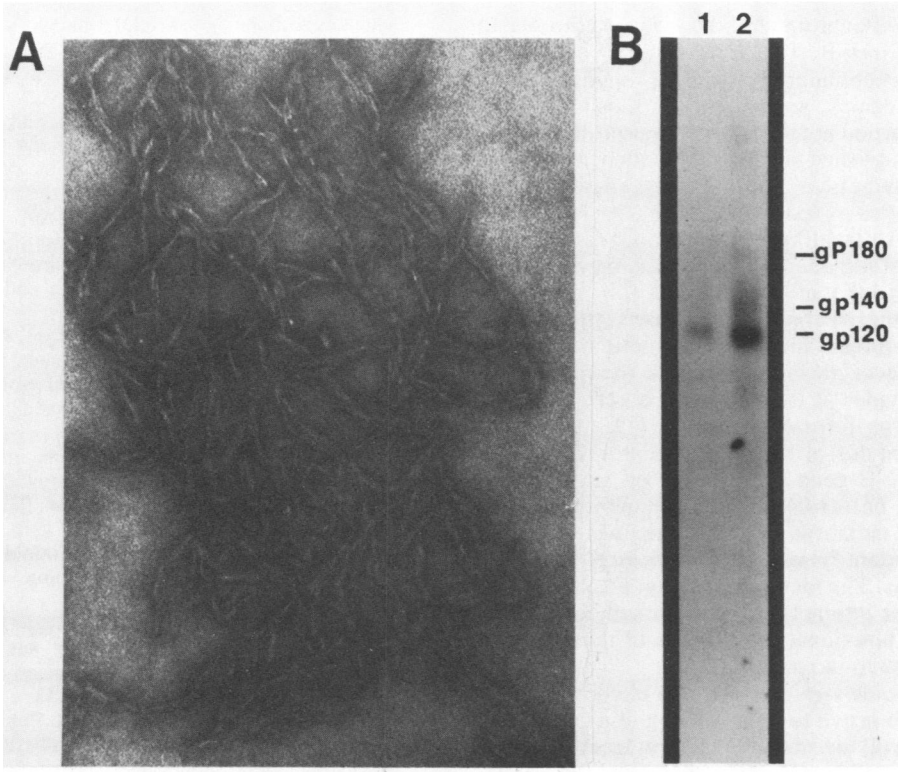

FIG. 9. Electromicrograph of intermediate filaments assembled in vitro from SM-FeSV-transformed cells (A) and an immunoblot of v-*fms*-coded antigens in the same preparation (B). The negatively stained filaments are shown at ×150,000 magnification. The immunoblotted proteins are derived from the filament fraction from SM-FeSV-transformed cells before (lane 1) and after (lane 2) self-assembly in vitro. Equal quantities of total protein (20 μg) were separated in each lane.

regions of kinase homology at the inner surface of the plasma membrane, similar to the localization of the Rous sarcoma virus transforming protein, pp60^{src} (7, 48). The latter protein is synthesized on free polyribosomes (27) and is posttranslationally modified by covalent attachment of fatty acids at the time of membrane insertion (14, 35). Hence, those v-*fms*-coded glycoproteins that reach the cell surface do so via a pathway different from that of pp60^{src}.

The v-*fms*-coded molecules that remain in the interior of the cell are associated with sedimentable organelles and are not detected in the cytosol (2, 3). In agreement with previous studies of some other transformed and rapidly proliferating cells (12, 37), IFs of v-*fms* transformants were rearranged and concentrated in the perinuclear region. By contrast, the organization of actin cables in transformed cells was not significantly altered. This differs from results obtained with several other oncogene-coded proteins that affect actin microfilaments. For example, microinjection of pp60^{src} into cells causes a rapid disassembly of actin cables (29). An unexpected finding was the topological association of IFs and v-*fms*-coded antigens.

Detergent-resistant, in situ cytoskeletal preparations retain lectin binding sites (5), implying that some glycoproteins can become physically associated with the cytoskeleton. However, it is difficult to exclude the possibility that glycoproteins artifactually bind to cytoskeletal elements after detergent treatment. The v-*fms* glycoproteins copurified with insoluble IFs and were resistant to subsequent extraction by nondenaturing detergents, high-ionic-strength buffers, ether, and DNase. By contrast, the FeLV envelope glycoprotein gp70^{env} did not remain associated with the filament pellet purified by the same fractionation procedure from productively infected FeSV-transformed cells. In addition, the v-*fms*-coded glycoproteins detected in the filament pellet by immunoblotting were shown to reassociate with IFs self-assembled in vitro. In the fractionation and self-assembly experiments, the strength of the immunoblot signal served as an estimate of v-*fms*-coded protein specific activity, since equal quantities of total protein were compared from each fraction. The intensity of the v-*fms* blot signal increased markedly during filament purification and in vitro self-assembly, pointing toward the possibility of a specific association of the two classes of molecules in transformed cells.

There are precedents for higher-molecular-weight, IF-associated proteins that form heteropolymers with IF subunits during in vitro assembly. For example, in avian erythrocytes the 230-kd protein synemin binds at regular intervals along the vimentin filament axis and is proposed to mediate interactions between individual filaments (17). In porcine spinal cord preparations, a protein of 210 kd can be incorporated at regular intervals into neurofilaments formed by self-assembly of 68-kd subunits (15); heteropolymers formed between 68-kd subunits and 210-kd proteins are shorter than homopolymers consisting only of the 68-kd protein. Despite its ability to become IF associated, the purified 210-kd protein itself has no capacity for self-assembly into polymers in the absence of the 68-kd neurofilament structural subunit. In both the erythrocyte and neural systems, the incorporation of the higher-molecular-weight, IF-associated proteins into IFs in vitro is an intrinsic property of the molecules, not requiring supplemental sources of energy, cofactors, or additional enzymatic activities (15, 16).

The v-*fms* gene codes for a protein that, in certain

respects, is similar to the product of the avian erythroblastosis virus oncogene v-*erb*-B. The latter gene encodes 62- to 68-kd glycoproteins containing N-linked oligosaccharide chains. These molecules accumulate intracellularly, although a small proportion are detected at the cell surface (19, 31). The recently published nucleotide sequence of the v-*erb*-B gene (49) predicts a product that shares several features with the v-*fms*-coded glycoprotein, such as (i) the protein contains an internal hydrophobic anchor sequence; (ii) sites for carbohydrate addition cluster amino-terminal to the membrane hydrophobic region; and (iii) the region carboxyl terminal to the anchor sequence shows strong homology to pp60^{src} even though intrinsic enzymatic activity has not been detected. In erythroid precursors, the presence of v-*erb*-B-coded molecules at the cell surface correlates with expression of the transformed phenotype (19). The recent observation that peptides of the EGF receptor show close homology to the *erb*-B gene product indeed suggests that transformation may be mediated by hormone-independent signals at the plasma membrane (9). Such findings emphasize the ambiguities in identifying the physiological targets of transforming proteins. The fact that many molecules localize to a particular site or interact with certain cellular proteins does not define the functional significance of these interactions in the transformation process.

Based on results with v-*erb*-B, it seems likely that the v-*fms* product will also prove to be an analog of a cell surface receptor. Studies involving site-directed mutagenesis of SM-FeSV proviral DNA will be critical in evaluating the correlations among retarded intracellular transport, IF rearrangement, cell surface localization, and transformation. The fact that each of these properties is of interest in and of itself suggests that manipulations of the cloned v-*fms* gene may provide unique opportunities for studies of protein traffic and targeting.

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