MOLECULAR AND CELLULAR BIOLOGY, Dec. 1985, p. 3467–3475
0270-7306/85/123467-09$02.00/0

Vol. 5, No. 12

Transformation by the v-*fms* Oncogene Product: Role of Glycosylational Processing and Cell Surface Expression

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The effect of glycosylational-processing inhibitors on the synthesis, cell surface expression, endocytosis, and transforming function of the v-*fms* oncogene protein (gp140^{fms}) was examined in McDonough feline sarcoma virus-transformed Fischer rat embryo (SM-FRE) cells. Swainsonine (SW), a mannosidase II inhibitor, blocked complete processing, but an abnormal v-*fms* protein containing hybrid carbohydrate structures was expressed on the cell surface. SW-treated SM-FRE cells retained the transformed phenotype. In contrast, two glucosidase I inhibitors (castanospermine [CA] and *N*-methyl-1-deoxynojirimycin [MdN]) blocked carbohydrate remodeling at an early stage within the endoplasmic reticulum and prevented cell surface expression of v-*fms* proteins. CA-treated SM-FRE cells reverted to the normal phenotype. Neither SW, CA, nor MdN affected either endocytosis or the tyrosine kinase activity associated with the v-*fms* gene product in vitro. These results demonstrate the necessity of carbohydrate processing for cell surface expression of the v-*fms* gene product and illustrate the unique ability to modulate the transformed state of SM-FRE cells with the glycosylational-processing inhibitors CA and MdN.

Oncogenes within the genomes of acute-transforming retroviruses have provided the most direct tools for analysis of molecular events leading to neoplastic transformation. In most of these retroviruses, a single transforming protein is synthesized from the viral oncogene, and this protein product is responsible for initiation and maintenance of the transformed state (4, 27). The retrovirus that we are interested in is the McDonough strain of feline sarcoma virus (SM-FeSV) (31, 42). This defective transforming virus was probably derived from a nondefective feline leukemia virus through in-frame insertion and replacement of part of the viral *gag* and all of the polymerase gene with an oncogene termed v-*fms* (10, 15). The primary translation product of the v-*fms* oncogene is therefore a fusion protein of 160 kilodaltons initiating in *gag* and terminating at the end of v-*fms* (P160$^{gag\text{-}fms}$) (1, 18). Signal sequences at the start of *gag* direct the protein to the endoplasmic reticulum (ER), where carbohydrate is added to asparagine residues to give gP180$^{gag\text{-}fms}$, and cleavage of the *gag* sequences yields gp120^{fms} plus p55^{gag}. A hydrophobic stretch of amino acids about midway through the sequence ensures a transmembrane orientation with the C-terminal end of the *fms* proteins in the cytoplasm (2, 18, 38). Further processing in the Golgi complex results in a gp140^{fms} species that ultimately is expressed on the plasma membrane (2, 30, 38). The gp140^{fms} is associated with coated pits on the cell surface, is processed through endocytosis (30), and may function as a modified growth factor receptor on the surface of SM-FeSV-transformed cells (50).

Since the transforming protein encoded by the oncogene v-*fms* is known to undergo glycosylational processing, we decided to determine whether this process was necessary for its cell surface expression. In view of the putative role of this protein as a growth factor receptor, the effects of glycosylation on cell surface expression might have important consequences in growth regulation.

Our approach was to use inhibitors which prevent glycosylation or interfere with the glycosylational processing reactions occurring on the oligosaccharides after their attachment to the protein (11, 34, 47–49). These inhibitors include swainsonine (SW), castanospermine (CA), *N*-methyl-1-deoxynojirimycin (MdN), and tunicamycin (TU). Both CA and MdN inhibit the same processing enzyme, glucosidase I, resulting in glycoproteins with oligosaccharides containing 1 to 3 glucose residues and 7 to 9 mannose residues (21, 39, 39a, 44, 49). SW, on the other hand, inhibits Golgi mannosidase II, resulting in glycoproteins accumulating hybrid-type oligosaccharides with one branch containing a high mannose structure and the other containing a complex-type structure (17, 24, 56, 57). The action of the nucleoside antibiotic TU, which inhibits the formation of dolichol-*P*-*P*-*N*-acetylglucosamine, results in the complete blockage of oligosaccharide chain addition to potential N-linked sites (51, 54, 55).

MATERIALS AND METHODS

Cells and growth conditions. Fischer rat embryo (FRE) cells nonproductively transformed with the McDonough strain of feline sarcoma virus (SM-FRE cells) were obtained from Fred Reynolds, Oncogene Sciences, Inc., Mineola, N.Y., and Gardner-Rasheed (GR) FeSV-transformed FRE cells (GR-FRE cells) were from S. Rasheed, University of Southern California. All cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Soft agar colony assays were performed in 24-well plates (Costar) as described by Crowe et al. (9). SM-FRE cells were suspended in 1 ml of 0.36% agar above a 0.6% agar bottom layer. When present, inhibitors were added to both agar layers. Colonies were recorded after 8 days of growth and counted with the aid of a grid.

The glycosylational-processing inhibitors were included in the growth medium at the concentrations indicated in the figure legends. CA and TU were purchased from Calbiochem-Behring, SW was generously provided by Harry Broquist, Vanderbilt University, and MdN was obtained from Y. Aoyagi, Nippon Shinyaku Co. Ltd., Kyoto.

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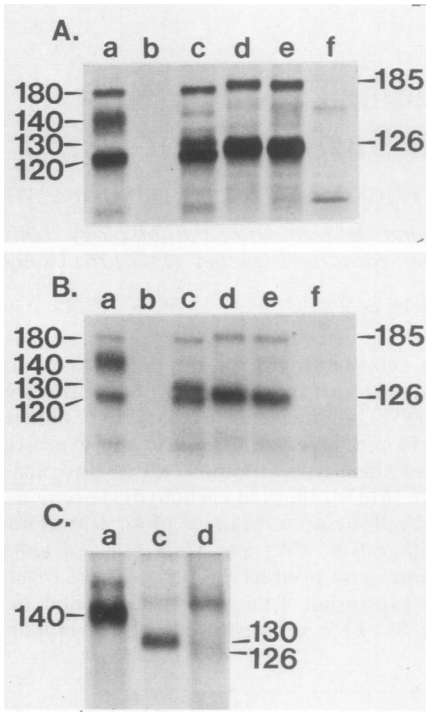

FIG. 1. Influence of carbohydrate-processing inhibitors on metabolism, kinase activity, and cell surface expression of the v-*fms* proteins. SM-FeSV-transformed FRE fibroblasts were grown in the absence (lanes a and b) or presence (lane c) of either 58 μM SW, 0.13 mM CA (lane d), 1 mM MdN (lane e), or 0.12 mM TU (f). Lane b represents immune precipitates from normal serum controls. (A) Metabolism. Cells were metabolically labeled with L-[^{35}S]methionine and extracted with detergent, and v-*fms* proteins were immunoprecipitated and analyzed on SDS-polyacrylamide gels. (B) Kinase activity. Phosphorylation of the v-*fms* proteins was measured in immunoprecipitates as described in Materials and Methods. Products from control or inhibitor-treated cells were analyzed by SDS-polyacrylamide gel electrophoresis. (C) Cell surface labeling. Cells were incubated with the appropriate inhibitor for 48 h and surface labeled with ^{125}I by using chloramine T as previously described (30). Iodinated *fms*-proteins were analyzed on an SDS-polyacrylamide gel. The iodinated protein bands in the 150- to 160-kilodalton range in panel C are nonspecific background.

Radiolabeling and immunoprecipitation. The synthesis and processing of the v-*fms* proteins were measured after metabolic labeling with L-[^{35}S]methionine. Cells were incubated overnight in Dulbecco modified Eagle medium containing 5% fetal bovine serum and the respective inhibitor. The media were then replaced with methionine-free Dulbecco modified Eagle medium containing 5% dialyzed fetal bovine serum, and the cells were labeled for 4 h with L-[^{35}S]methionine (100 μCi/ml) in the absence or presence of the respective inhibitor. Cells were finally washed three times in ice-cold phosphate-buffered saline, RIPA (150 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 300 Kallikrein inhibitor units of Trasylol per ml) detergent extracts were prepared, and v-*fms* proteins were detected by immune precipitations with 5 μl of rat polyclonal anti-*fms* serum, as we have previously described (30). Immune complexes were washed four times in RIPA buffer and once in lysis buffer (100 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 0.5% Nonidet P-40) and analyzed on SDS-polyacrylamide gels (30).

Kinase assays were performed with immunoprecipitates prepared from unlabeled cells as described above. The precipitates were suspended in 25 μl of kinase buffer (20 mM Tris hydrochloride [pH 7.5], 10 mM $MgCl_2$), [γ-^{32}P]ATP was added, and the mixture was incubated for 10 min at room temperature. The reaction was stopped by addition of 10 mM EDTA, washed three times in lysis buffer, and analyzed by SDS-polyacrylamide gel electrophoresis.

Cell surface labeling with ^{125}I was performed exactly as previously described (30). Cell extracts were assayed for protein by the fluorescamine assay (58) and normalized before immune precipitation. Iodinated *fms*-proteins were analyzed on an SDS-polyacrylamide gel. Molecular weight markers were coelectrophoresed.

Immunofluorescence and immunoperoxidase staining. Details of immunofluorescence and immunoperoxidase staining have been described in a previous publication (30).

RESULTS

Metabolic processing and kinase activity. The effect of the various glycosylational-processing inhibitors on the synthesis and expression of the v-*fms* proteins was examined in SM-FRE cells. Immunoprecipitation of extracts of [^{35}S]methionine-labeled fibroblasts with antisera to v-*fms*-encoded determinants demonstrates the normal complement of v-*fms* gene proteins (gP180$^{gag\text{-}fms}$, gp140^{fms}, and gp120^{fms}) in untreated control cells (Fig. 1A, lane a). When the SM-FRE cells were grown in the presence of the mannosidase II inhibitor SW, synthesis of the gp140^{fms} protein was prevented and a gp130^{fms} species was found instead (Fig. 1A, lane c). Inhibition of glucosidase I in SM-FRE cells with either CA or MdN resulted in an accumulation of v-*fms* proteins gP185$^{gag\text{-}fms}$ and gp126^{fms} (Fig. 1A, lane d and e). Pulse-chase analysis after removal of CA and MdN showed that the gP180$^{gag\text{-}fms}$ and gp120^{fms} were derived from the gP185$^{gag\text{-}fms}$ and gp126^{fms} species (data not shown). The 5-kilodalton decrease in the mass of these proteins was probably due to removal of terminal glucose residues and perhaps some subsequent trimming of exposed mannose residues, as has been reported for other glycoproteins synthesized in the presence of these inhibitors (5, 16, 28, 36). TU treatment of SM-FRE cells resulted in the synthesis of unglycosylated proteins P160$^{gag\text{-}fms}$ and 95^{fms} (Fig. 1A, lane f), as reported previously (1).

The v-*fms* proteins that were produced in the presence of the various inhibitors contained altered carbohydrate moieties, yet the associated in vitro tyrosine kinase activity of these proteins was largely unaltered. The results in Fig. 1B show that all processing intermediates produced in the presence of either SW, CA, of MdN contained a tyrosine kinase activity that phosphorylated the respective v-*fms* proteins in vitro. An exception was the unglycosylated proteins made in the presence of TU (lane f). The P160$^{gag\text{-}fms}$ and p95^{fms} incorporated very little radioactive phosphorus in these experiments.

Cell surface expression. The expression of the v-*fms* proteins on the surface of SM-FRE cells grown in the presence of the glycosylational-processing inhibitors was assessed by both cell surface iodination and viable cell fluorescence studies. The results in Fig. 1C demonstrate the cell surface iodination experiments. Normally, only the gp140^{fms} species is detectable on the surface of SM-FeSV-transformed cells (2, 30) (Fig. 1C, lane a). When SW is included in the growth medium, gp140^{fms} is not detectable on these cells (in agreement with the results shown in Fig. 1A), but the new species, gp130^{fms}, is expressed on the cell surface (Fig. 1C, lane c).

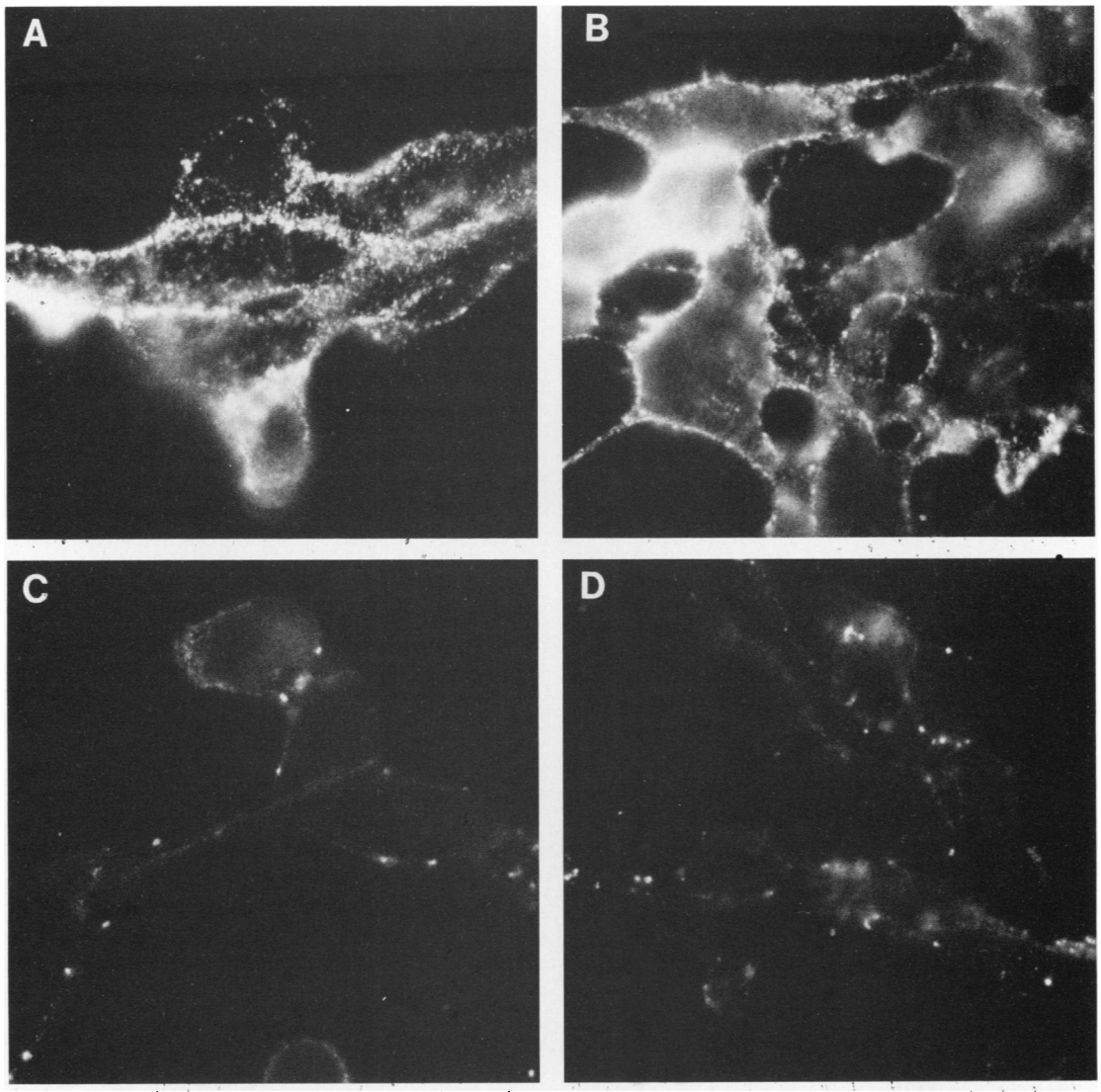

FIG. 2. Cell surface expression determined by viable cell immunofluorescence. SM-FRE cells were plated onto glass cover slips in the bottom of 24-well plates and growth for 24 h in either normal growth medium (A) or growth medium containing 58 μM SW (B), 0.13 mM CA (C), or 2 mM MdN (D). The expression of *fms*-specific determinants on the surface of the live SM-FRE cells were analyzed by indirect immunofluorescence on viable cells. Cells treated with 0.12 μM TU also were tested for cell surface expression of *fms* proteins, but were completely negative (not shown). The punctate fluorescence in panels C and D is nonspecific background that also was seen on uninfected cells (30).

Very little if any of the v-*fms* proteins were found on the cell surface of CA- (lane d) or MdN- (not shown) treated cells.

The expression and distribution of v-*fms* proteins on the surface of SM-FRE cells as examined by viable cell immunofluorescence with anti-*fms* sera is shown in Fig. 2. As we have shown previously (30), untreated SM-FRE cells exhibited a punctate distribution of gp140^{fms} on the cell surface (Fig. 2A). This pattern is due in part to the association of gp140^{fms} with coated pits (30). SM-FRE cells grown in the presence of SW exhibited a similar distribution and intensity of fluorescence as that seen in the untreated control cells (Fig. 2B). Both the metabolic labeling and the cell surface iodination experiments indicated that in the presence of SW, a gp130^{fms} species was formed instead of the gp140^{fms}, and the gp130^{fms} is expressed on the cell surface. This expression is qualitatively and quantitatively similar to that seen in untreated control SM-FRE cells.

In contrast to cells grown in the presence of SW, both CA- and MdN-treated SM-FRE cells exhibited a greatly reduced level of cell surface fluorescence (Fig. 2C and D). This weak cell surface fluorescence was diffuse and not punctate as on the control or SW-treated SM-FRE cells. In the presence of CA or MdN, only gP185$^{gag\text{-}fms}$ and gp126^{fms} species were detectable. The results of both the cell surface iodination and immunofluorescence experiments indicated that neither of these *fms* proteins was expressed on the cell surface.

TU treatment of SM-FRE cells completely prevented the expression of any *fms*-related proteins at the cell surface (data not shown).

Endocytosis. Our previous studies demonstrated that the cell surface gp140^{fms} was to a large extent associated with clathrin-coated pits and that the cell-surface-expressed *fms* protein was rapidly taken into the cell through endocytosis (30). In the present studies we examined the effects of the

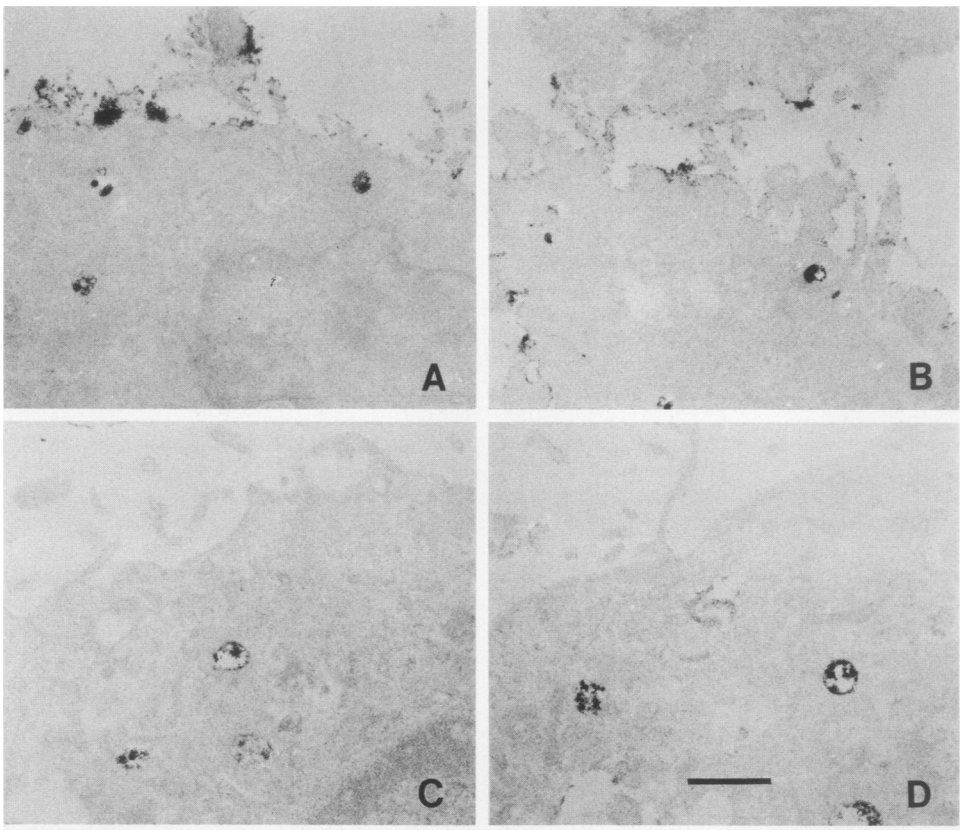

FIG. 3. Endocytosis of v-*fms* proteins in the presence of inhibitors. SM-FRE cells, either untreated (A) or treated with 58 μM SW (B), 0.13 mM CA (C), or 2 mM MdN (D), were reacted with anti-*fms* serum on ice. After application of immune reagents for peroxidase staining, the cells were warmed to 37°C for 10 min, fixed, permeabilized, and incubated with diaminobenzidine. Thin sections, not counterstained with either uranyl acetate or lead citrate, were viewed and photographed by the electron microscope. In all cases peroxidase-positive cytoplasmic vesicles were observed; however, there were fewer in both CA- and MdN-treated cells (see text). Control and SW-treated SM-FRE cells exhibited conspicuous cell surface peroxidase labeling (A, B), which was not readily apparent on CA- or MdN-treated cells (C, D). Bar, 1 μm.

glycosylational-processing inhibitors on endocytosis of cell-surface-expressed *fms* proteins. SM-FRE cells were incubated with SW, CA, or MdN at the same concentrations used for immunofluorescence microscopy, and the cellular uptake of *fms* proteins into endocytotic vesicles was determined by immunoperoxidase electron microscopy as previously described (30).

Sections of peroxidase-stained SM-FRE cells viewed in the electron microscope displayed stainable peroxidase reaction product within cytoplasmic vesicles regardless of the inhibitor treatment used (Fig. 3). However, the number of these positive vesicles per cell was dependent on the inhibitor treatment. To quantitate this effect for each treatment, cross-sections of 50 cells were viewed at random, and the number of stained vesicles per cell was determined. The glucosidase I inhibitors (CA and MdN) had the greatest effect, reducing the number of positive vesicles to 2.8 ± 2.6 (arithmetic mean ± standard deviation) in MdN-treated cells and 4.9 ± 3.5 in CA-treated cells. This was in comparison with the number of positive vesicles in untreated control SM-FRE cells at 10.3 ± 4.9. SW-treated cells exhibited nearly the same number of positive vesicles as the controls, at 8.9 ± 4.1. Analysis of variance revealed that the reduction in the number of positive vesicles with either CA or MdN inhibitor was highly significant.

The decreased number of peroxidase-positive endocytotic vesicles in the CA- and MdN-treated cells was probably due to the small amount of v-*fms* protein reaching the cell surface, even in the presence of the inhibitors. This small amount was cleared completely from the cell surface within 10 min (Fig. 3C and D), whereas cell surface v-*fms* protein was still evident on the surface of both control and SW-treated cells after the same incubation time (Fig. 3A and B).

Growth in soft agar. If the expression of the v-*fms* protein product gp140^{fms} at the cell surface is important for oncogenic transformation, two of the carbohydrate-processing inhibitors (CA and MdN) may also modulate the transformed state of SM-FeSV transformed cells. We tested this possibility by determining the anchorage dependence of growth (soft agar colony assay) with SM-FRE cells grown in the presence and absence of the inhibitors (Fig. 4). SM-FRE cells in the absence of any inhibitor formed macroscopic colonies with about 60% efficiency. In the presence of SW, no decrease in colony size or number was noted; in fact, in two separate experiments SW treatment resulted in a slightly higher efficiency of colony formation. In contrast to growth in the presence of SW, growth of SM-FRE cells in the presence of either CA or MdN caused a 10-fold decrease in the ability of these cells to form colonies in soft agar. Even the colonies that did form in the presence of CA or MdN were much smaller than those that formed in the untreated control wells. The ability of these cells to form colonies in soft agar in the presence of CA or MdN may be due to impaired permeability of these drugs, or these cells may

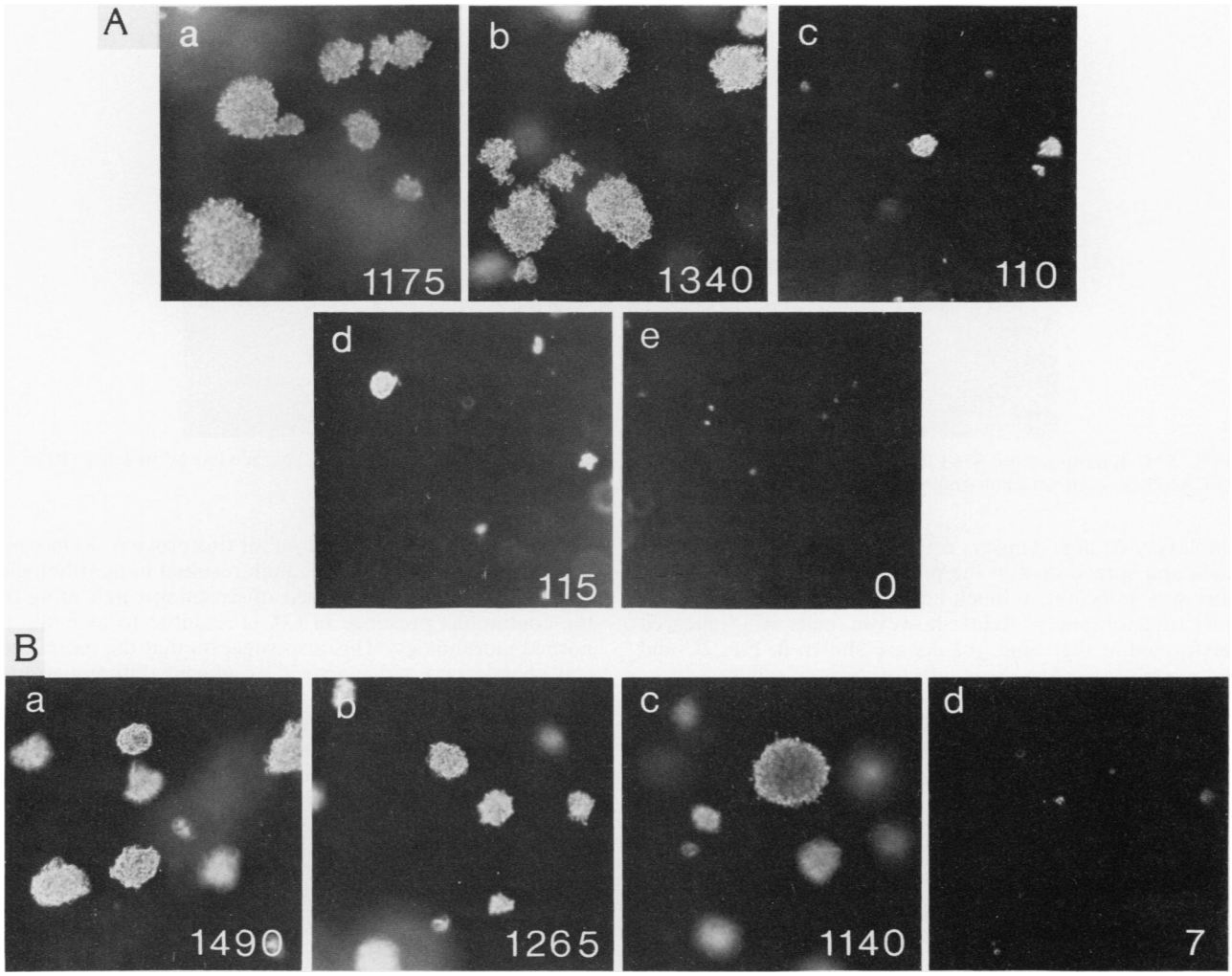

FIG. 4. Anchorage-independent growth measured in the presence and absence of carbohydrate-processing inhibitors. (A) SM-FRE cells (2×10^3) were grown in agar suspension containing either no additions (a), or 58 μM SW (b), 0.13 mM CA (c), 2 mM MdN (d), or 0.12 μM TU (e). At 8 days after seeding, the total number of colonies (>10 cells) in duplicate wells were counted with the aid of a grid; the average values are shown in each panel. The range between duplicates never exceeded 20% of the average value. (B) GR-FRE cells (2×10^3) were grown in agar suspension as described above in the absence (a) or presence (b) of 0.13 mM CA. In the same experiment, 2×10^3 SM-FRE cells were grown in the absence (c) or presence (d) of 0.13 mM CA. The total number of colonies (>10 cells) were counted in duplicate wells; the average values are presented in each panel.

express conformationally altered glycosylational-processing enzymes (owing to mutation) which are insensitive to inhibition. Alternatively, these cells may be utilizing a pathway independent of glycosylational processing in directing the transforming protein to the cell surface. These possibilities are currently under investigation. It also was obvious that neither CA nor MdN was toxic, since the treated cells appeared viable and could be picked from the agar and regrown in culture (personal observation). TU treatment, however, was toxic, and no colonies formed in the presence of this inhibitor owing to cell death.

We considered the possibility that the reversal of anchorage independence of growth caused by CA and MdN in SM-FRE cells could have been due to the indirect action of these inhibitors on a cellular glycoprotein rather than directly on the v-*fms* glycoproteins. This notion was tested by comparing the effect of CA on the ability of GR-FRE cells and the SM-FRE cells to form colonies in soft agar. The GR strain of FeSV carries the v-*fgr* oncogene (33), whose protein product is associated with a tyrosine kinase activity (32), but is not expressed on the cell surface and is not glycosylated (R. Manger, S. Rasheed, and L. Rohrschneider, manuscript in preparation). CA had no significant effect on the formation of GR-FRE cell colonies on soft agar, whereas in this experiment CA caused a 100-fold reduction in the number of SM-FRE cell colonies. These results indicate that the actions of the drugs are specific for SM-FeSV-transformed cells. Both CA and MdN returned SM-FRE cells to more anchorage-dependent growth. This effect is most probably due to the prevention of cell surface expression of the v-*fms* gene product, although it is still possible that CA exerts its effects on a cellular glycoprotein.

Cell morphology. SM-FRE cells grown in the presence of either CA or MdN for at least 48 h exhibited a flattened morphology, in contrast to the more rounded morphology of untreated control cells (Fig. 5). Treatment of SM-FRE cells with SW had no effect (data not shown). Cells that were plated at low density in the presence of CA grew only to a

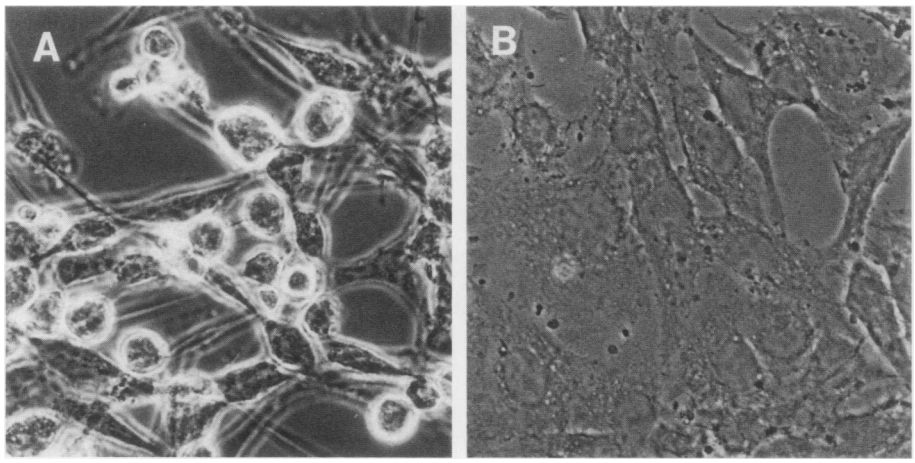

FIG. 5. Cell morphology. SM-FRE cells were plated on glass cover slips and grown for 3 days in the absence (A) or presence (B) of 0.13 mM CA. Phase contrast photographs were taken of the cells after fixation in 4% formaldehyde.

monolayer. At high density, cells that did not have room to attach and spread died in the presence of CA. Twenty-four hours was sufficient to block cell surface expression of the v-*fms* transforming proteins; however, cells still appeared transformed at that time (results are shown in Fig. 2C and D).

Morphological reversion to the normal phenotype occurred only after at least 2 days of exposure to the drug. Because gp140^{fms} disappears from the cell surface at least 24 h before reversion to the normal morphology, some slower cellular events may be required for this process. Removal of the drug from the culture medium resulted in morphological retransformation (unpublished observation), indicating that the continuous presence of CA is required to maintain the normal morphology. This also suggests that the morphological change is not due to a CA-induced differentiation of SM-FRE cells.

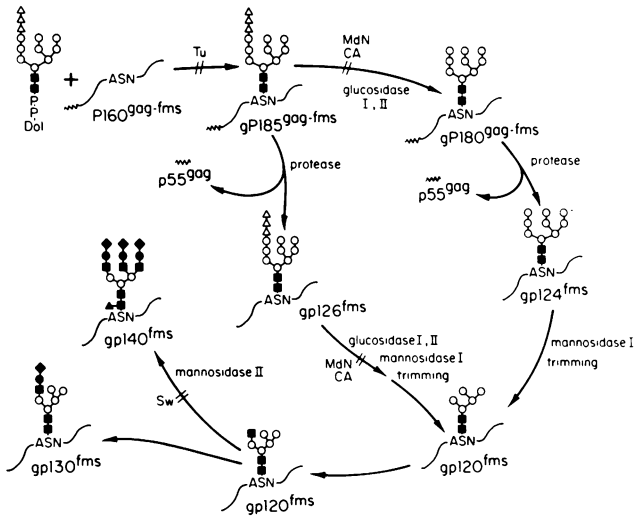

FIG. 6. Processing scheme for the v-*fms* gene products. The above glycosylational-processing pathway is based on actual intermediates that we have detected and on the known mechanism of action of these inhibitors. Only a short segment of the amino acid sequence surrounding a single asparagine (ASN) carbohydrate addition site is shown for the *fms* proteins. Eleven such sites exist in the extracellular domain of the *fms* protein (18). The name and molecular weight of each *fms* species is given according to standard nomenclature (7), and the specific points at which the inhibitors act are indicated. The normal processing pathway follows the circumference of the scheme and leads to gp140^{fms}. Symbols: ■, *N*-acetylglucosamine; ○, mannose; ●, galactose; ◆, sialic acid; ▲, fucose; △, glucose.

Besides morphology, other features of the CA-treated SM-FRE cells indicated they had reverted to the normal phenotype. The medium from SM-FRE cells grown in either CA or MdN was consistently less acidic than that from controls, indicating decreased overall metabolism. Also, fibronectin was again expressed in the extracellular matrix of the treated cells, and bundles of microfilaments could be seen (manuscript in preparation). These properties are consistent with the reexpression of the normal phenotype in CA-treated SM-FRE cells.

Processing of v-*fms* proteins. The schematic depicted in Fig. 6 summarizes the glycosylational-processing sequence for the v-*fms* gene product and indicates the point at which each inhibitor acts. The molecular weights of the protein products in the scheme are based on actual species detected either in the absence or presence of inhibitors. The initial glycosylated product, gP185$^{gag\text{-}fms}$, is generated in the lumen of the ER by en bloc transfer of a $Glc_3Man_9GlcNAc_2$ oligosaccharide from a dolichol phosphate lipid carrier to asparagine residues of P160$^{gag\text{-}fms}$ undergoing synthesis on membrane-bound ribosomes. The nucleoside antibiotic TU, which inhibits the formation of dolichol-*P*-*P*-N-acetylglucosamine, prevents glycosylation of P160$^{gag\text{-}fms}$ (54, 55). The newly transferred $Glc_3Man_9GlcNAc_2$ oligosaccharide then undergoes a series of processing reactions during its transport through the ER and Golgi complex (22, 23). Cleavage of the outer glucose residues by glucosidases I and II (6, 12, 23, 26) gives rise to gP180$^{gag\text{-}fms}$. Inhibition of glucosidase I by either CA or MdN prevents the formation of gP180$^{gag\text{-}fms}$, resulting in an accumulation of gP185$^{gag\text{-}fms}$, which is proteolytically cleaved to yield a nonglycosylated p55^{gag} protein and a glycosylated gp126^{fms} that is not expressed on the cell surface. Removal of the glucose residues by both glucosidases I and II and the α1-2-linked mannose residues by mannosidase I generates gp120^{fms} containing the $Man_5GlcNAc_2$ oligosaccharide. Addition of GlcNAc to the α1-3-branch mannose (of the trimannosyl core) present on gp120^{fms} allows the removal of both the α1-6 and α1-3

branching mannose residues by Golgi-mannosidase II (20, 52, 53). Further addition of *N*-acetylglucosamine, galactose, fucose, and sialic acid results in the complex-type carbohydrate structure(s) appearing on the mature glycoprotein ($gp140^{fms}$). If SW is present, mannosidase II is inhibited and the $gp120^{fms}$ is shunted along a pathway leading to the synthesis of hybrid-type oligosaccharides (17, 24, 56, 57). Of all the processing intermediates, only the $gp140^{fms}$ and $gp130^{fms}$ are expressed on the cell surface.

DISCUSSION

The results presented in this paper indicate that both proper carbohydrate processing and cell surface expression of the v-*fms* gene product are necessary for maintaining the transformed phenotype of SM-FeSV-infected cells. Glycosylational-processing inhibitors that block carbohydrate remodeling at an early stage within the rough ER (i.e., CA and MdN) prevent further processing, and the v-*fms* proteins do not reach the cell surface. These proteins presumably accumulate in the ER and still retain an associated tyrosine kinase activity that protrudes into the cytoplasm from the ER membrane. The cells, however, appear morphologically normal and do not grow well in soft agar. CA-treated cells also reexpress fibronectin in the extracellular matrix and reform stress fibers (data not shown). In contrast, even when an abnormal processing intermediate of v-*fms* (i.e., the $gp130^{fms}$ species produced in the presence of SW) is expressed at the cell surface, the transformed state is maintained. Although it is still possible that CA acts via a normal cellular glycoprotein, we believe this is less likely. Rather, by analogy with other *onc* proteins (8, 46), the results support the notion that not only a functional transforming protein but also its transport to the appropriate subcellular target are necessary for transformation. Presumably, the targets for the v-*fms* protein reside in the plasma membrane, but could be situated at some point in the endocytotic pathway as well.

Two other reports also suggested that the cell surface expression of the v-*fms* proteins is necessary for transformation (38, 40). In these cases, deletions in the primary amino acid sequence were generated, and these affected both the intracellular precursors and the final $gp140^{fms}$ product. One mutant lacked an associated kinase activity altogether. It was therefore not possible to distinguish between transforming functions of the precursors and those of the cell-surface-expressed $gp140^{fms}$. The glycosylational-processing inhibitors, on the other hand, do not affect the primary amino acid sequence or the associated tyrosine kinase activity of the v-*fms* gene products. The mechanism of action of these inhibitors indicates that only carbohydrate processing is blocked within the transformed cells. This blockage at a step prior to surface expression was able to prevent anchorage-independent growth and revert cells to the normal phenotype.

The drugs that were effective at preventing transformation by SM-FeSV (CA and MdN) are not toxic to normal cells and did not reverse transformation by a retrovirus carrying a different oncogene (Fig. 4). No overt toxicity was observed either in vitro (unpublished observations) or in short-term tests (3 to 7 days) in vivo (43). High dosage levels, however, were toxic to mice (A. D. Elbein, personal communication). Also, similar drugs were without effect on normal erythroid differentiation (46).

It is surprising that cells can still survive and grow in the presence of these glucosidase I inhibitors. Fibronectin, however, can still function in CA-treated cells. N-linked carbohydrate on fibronectin does not appear to play a role in either fibronectin secretion or its ability to promote cell attachment and spreading (3, 35). Therefore, fibronectin may still function in the observed reversion and spreading of the SM-FRE cells grown in the presence of CA. Perhaps most cellular glycoproteins can still reach the cell surface and function even when grown in the presence of CA or MdN. Also, there may be something unique about the carbohydrate structure of $gp140^{fms}$ that makes it so sensitive to inhibition by the glucosidase I inhibitors. We do not yet know which, if any, of these alternatives is correct.

Both drugs that were effective at preventing cell surface expression of $gp140^{fms}$ and reversing the transformed state blocked carbohydrate processing at the same early step. CA and MdN inhibited α-glucosidase I and prevented trimming of the outermost glucose on the initial carbohydrate structure added to asparagine residues on the polypeptide backbone of the v-*fms* protein. $gP185^{gag\text{-}fms}$ and the proteolytically processed products $gp126^{fms}$ and $p55^{gag}$ accumulated in the ER compartment. Presumably, a certain carbohydrate structure past this step is required for "ticketing" to the next compartment (the Golgi complex). Endocytotic uptake and intracellular targeting of lysosomal enzymes has been shown to depend on recognition of phosphomannosyl residues present on these glycoproteins (13, 25). It has been proposed that some ER lectins may recognize specific carbohydrate structures and direct attached glycoproteins along proper pathways (14, 29). Such a model would be consistent with the lack of further transport and accumulation of $gP185^{gag\text{-}fms}$; however, it also is possible that the carbohydrate plays a more indirect role. Perhaps carbohydrate moieties determine a specific protein conformation required for transport.

These are both attractive models, and as far as we could determine, the carbohydrate did not have any appreciable effect on any function or activity of the v-*fms* proteins other than limiting the cell surface expression. All the precursors, whether abnormally processed or not, still became phosphorylated in the in vitro kinase assay. Surprisingly, endocytosis of the v-*fms* proteins also was not affected by either SW, CA, or MdN. SW-treated SM-FRE cells were near normal in their endocytosis of v-*fms* proteins, and in the CA- or MdN-treated cells endocytosis still occurred, even though very little v-*fms* protein found its way to the cell surface. These data suggest that carbohydrate structures affected by SW, CA, or MdN are probably not involved as determinants that specify accumulation in coated pits and subsequent endocytosis.

Glycosylational processing inhibitors similar (but not identical) to those used in the present studies were without dramatic effect on *erbB*-transformed cells (46). Carbohydrate processing was abnormal, yet the *erbB* glycoprotein product was still expressed on the cell surface, and the cells remained transformed. This lack of effect may be due either to qualitative or quantitative differences in carbohydrate on *erbB* versus *fms* proteins, but also could result from the use of less potent inhibitors (39a). We are presently examining the carbohydrate structure on the v-*fms* protein as well as the sensitivity of *erbB* transformation to CA or MdN.

Recently, the protein product of the c-*fms* proto-oncogene has been identified in both cats (37) and humans (59). This product is a glycoprotein expressed on the surface of cells in the monocyte-macrophage lineage, and may function as the CSF-1 receptor (50). It is not known yet whether activation or abnormal expression, or both, of this particular proto-oncogene is involved in any types of human tumors; how-

ever, there are indications that this may be the case (41). If so, the glycosylational-processing inhibitors (CA and MdN) should be tested as anticancer drugs for these tumors. In addition, other *onc* proteins, whose transformation is mediated through glycosylated intermediates (19, 45), may prove susceptible to chemotherapy with these inhibitors. Prevention of cell surface expression of oncogene products may be an effective means of regulating the transformed state.

ACKNOWLEDGMENTS

We thank Jon Cooper for critical review of the manuscript, Harry Broquist for the swainsonine, and Lyle Najita for expert technical assistance.

This work was supported by Public Health Service grants CA20551 and CA28151 from the National Cancer Institute. R.M. was supported by a National Institutes of Health fellowship.

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