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Regulation of Sialic Acid 9-*O*-Acetylation during the Growth and Differentiation of Murine Erythroleukemia Cells*

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Sialic acids are typically found at the terminal position on vertebrate oligosaccharides. They are sometimes modified by an *O*-acetyl ester at the 9-position, potentially altering recognition of sialic acid by antibodies, lectins, and viruses. 9-*O*-Acetylation is known to be selectively expressed on gangliosides in melanoma cells and on *N*-linked chains in hepatocytes. Using a recently developed probe, we show here that in murine erythroleukemia cells, this modification is selectively expressed on another class of oligosaccharides, *O*-linked chains carried on cell surface sialomucins. These cells also express 9-*O*-acetylation on the ganglioside G_{D3}, but this modification appears to be undetectable on the cell surface. Increasing cell density in culture is associated with a decrease in cell surface 9-*O*-acetylation of sialomucins. This change correlates with the spontaneous differentiation toward a mature erythroid phenotype. This down-regulation upon differentiation and entry into the G_0/G_1 stage of the cell cycle is confirmed by differentiation-inducing agents. In contrast, cells arrested in G_2/M by the microtubule depolymerizing agent nocodazole show increased expression of cell surface 9-*O*-acetylated sialomucins (but not the 9-*O*-acetylated ganglioside). However, the microtubule stabilizer taxol does not induce this increase, showing that the nocodazole effect is independent of cell cycle stage. Indeed, direct analysis showed no correlation of 9-*O*-acetylation with cell cycle stage in rapidly growing cells, and shorter treatments with nocodazole also increased expression. Western blots of cell extracts confirmed that changes caused by differentiation and nocodazole are not due to redistribution of molecules from the cell surface. Indeed, following selective removal of 9-*O*-acetyl groups from the cell surface by a specific esterase, the recovery of expression is mediated by new synthesis rather than by redistribution from an internal pool. Thus, 9-*O*-acetylation on these sialomucins appears to be primarily regulated by the rate of synthesis, and the increase with nocodazole treatment is likely due to the inhibition of turnover of cell surface molecules. These data show that 9-*O*-acetylation of sialic acids in murine erythroleukemia cells is a highly regulated modification, being selectively expressed in a cell type-specific manner on certain classes of oligosaccharides and differentially regulated with regard to subcellular localization and to the state of cellular differentiation.

The sialic acids (Sias)[1] are a family of 9-carbon monosaccharides usually found as the terminal units of animal oligosaccharides (1). The most common modifications of Sias are *O*-acetyl substitutions at the C-7 or C-9 positions (2, 3). Since *O*-acetyl esters at C-7 position spontaneously migrate to the C-9 position at physiological extracellular pH (4, 5), 9-*O*-acetylated Sias predominate on cell surface glycoconjugates. Several observations show that these modifications can affect the physicochemical and biological properties of the parent molecule (2, 3), affecting the activity of microbial sialidases (6), the specificity of sialyloligosaccharide recognition by viruses (7, 8), recognition by mammalian lectins and monoclonal antibodies (9, 10), and potentially altering activation of the alternative complement pathway (11, 12). These modifications also show remarkable tissue-specific, molecule-specific, and developmentally regulated expression in a variety of systems. For example, structural studies of different *O*-acetylated gangliosides from melanoma cells have shown that the 9-*O*-acetyl group is invariably located on a particular terminal α2,8-linked Sia originating from the β1,4-linked galactose of lactosylceramide (13, 14); in the same cell type, Sias on glycoproteins do not appear to be *O*-acetylated (15). In contrast, rat hepatocytes express 9-*O*-acetylation selectively on the Sia residues of *N*-linked sugar chains (16). Since the *O*-acetylation of Sias takes place after transfer of Sias to glycoconjugates (2, 3), these findings suggest that this reaction may be regulated in a molecule- and tissue-specific fashion.

Other studies have shown *O*-acetylated Sias as distinct markers for human lymphocyte subsets (10, 17–21), and variable expression has been reported in human leukemic cells (22). Murine erythroleukemia (MEL) cells are virus-transformed erythroid precursors that rapidly proliferate in culture (23). These cell lines have served as classic models for *in vitro* differentiation along the erythrocyte pathway (23), which can be initiated by a number of agents and results in decreased cell size, restricted proliferative capacity, expression of mature erythrocyte antigens, and terminal differentiation (24–26). Because of these characteristics, MEL cells are useful to investigate the mechanisms by which proliferating transformed precursor cells in a differentiation lineage withdraw from the cell division cycle and express the genes characteristic of the nor-

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[1] The abbreviations used are: Sia, sialic acid, type unspecified; mAb, monoclonal antibody; HPTLC, high performance thin layer chromatography; PBS, phosphate-buffered saline; CHE-Fc, chimeric protein made of InfCHE (influenza C hemagglutinin-esterase with the fusion peptide eliminated by mutation) and the Fc portion of human IgG_1; CHE-FcD, DFP-treated CHE-Fc (esterase activity irreversibly inactivated); HMBA, hexamethylene-bisacetamide; Me_2SO, dimethyl sulfoxide; MEL, murine erythroleukemia cells; DFP, diisopropyl fluorophosphate; BSA, bovine serum albumin; OSGPase, *O*-sialoglycoprotease; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; PNGase, peptide-*N*-glycosidase F; BKG, background.

mal differentiated phenotype. Sia *O*-acetylation has also been reported on murine erythrocytes (11, 27, 28) and on MEL cells (29). However, the class of sugar chains (gangliosides, *N*-linked or *O*-linked) to which these *O*-acetyl groups were attached was not known. Recently, murine erythrocytes have been shown to bear ligands for sialoadhesin, a macrophage-specific I-type lectin known to selectively recognize the Siaα2–3Galβ1–3GalNAc terminal structure that can occur on either *O*-linked chains or gangliosides (30). In this case, de-*O*-acetylation increased binding (31), suggesting that at least a portion of the target structures might be masked by *O*-acetyl groups.

With the exception of certain ganglioside antigens recognized by specific monoclonal antibodies (32–35), it was previously not possible to detect 9-*O*-acetyl sialic acids on cell surfaces without resorting to biochemical analyses. We and others (20, 36, 37) have suggested the use of the whole influenza C virion as a probe for this purpose. This virus expresses a membrane-bound hemagglutinin-esterase (CHE), which can both detect and cleave 9-*O*-acetyl residues on sialic acids, regardless of the underlying oligosaccharide structure to which they are attached. However, the intact influenza C virions are unstable, are subject to steric hindrance in binding, and are impractical for many applications. To address this deficiency, we created a recombinant soluble chimera of the influenza C hemagglutinin-esterase fused to the hinge and Fc regions of human IgG_1 (38). The purified molecule (CHE-Fc) retains the specific 9-*O*-acetyl esterase activity of the parent viral glycoprotein (38). Irreversible inactivation of the enzyme activity with DFP gives the derivative CHE-FcD, which serves as a specific probe for detection of 9-*O*-acetylated Sias. Using these probes, we demonstrated the widespread but selective expression of 9-*O*-acetylation in various rat tissues (39) and occurrence of 9-*O*-acetyl groups masking sialylated ligands for the B-cell-specific lectin CD22 (10). We have now used these reagents to explore the expression, regulation, and turnover of 9-*O*-acetyl groups during growth and differentiation in MEL cells.

EXPERIMENTAL PROCEDURES

Materials—Most of the materials used were obtained from Sigma. The following materials were obtained from other sources as indicated. Biotin-conjugated Lc-hydrazide and fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab was from Pierce; proteinase K was from Life Technologies, Inc.; alkaline phosphatase-conjugated streptavidin and alkaline phosphatase substrate package were from Life Technologies, Inc.; peroxidase-conjugated goat anti-mouse IgG Ab and alkaline phosphatase-conjugated goat anti-human IgG Ab were from Bio-Rad; fluorescein isothiocyanate-conjugated goat anti-human IgG was from CalTag Laboratories; propidium iodide was from Calbiochem; diisopropyl fluorophosphate (DFP) was from Aldrich; fetal calf serum was from Hyclone; peptide:*N*-glycosidase F was from Genzyme; high performance thin layer chromatography (HPTLC) plates (silica Gel-60 10 × 10 cm) was from Merck. Protein assays were determined with the bicinchoninic acid protein assay reagent kit (Pierce) using BSA as a standard. The *O*-sialoglycoprotease enzyme was a kind gift from Dr. Alan Mellors, University of Guelph, Canada. All other chemicals were of reagent grade or better and were obtained from commercial sources.

Cell Lines and Monoclonal Antibodies—Murine erythroleukemia (MEL) cells were obtained from Dr. George Palade, UCSD (24, 40), and cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum. Human melanoma cells (Melur) were from David Cheresh, Scripps Research Institute, and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The mouse IgG_3 anti-G_{D3} monoclonal antibody R-24 (American Type Culture Collection), and the IgG_3 anti-9-*O*-acetyl-G_{D3} monoclonal antibody 27A (M. Farquhar, UCSD) were used as the hybridoma supernatants.

Chimeric CHE-Fc and CHE-FcD—The soluble chimeric protein CHE-Fc, consisting of the extracellular domain of influenza C hemagglutinin-esterase fused to Fc portion of human IgG_1, was generated and characterized as described elsewhere (38). The modified form CHE-FcD was generated by treating CHE-Fc with 1 mM diisopropyl fluorophosphate to irreversibly inactivate the esterase as described (38). CHE-Fc specifically releases 9-*O*-acetyl esters from Sias, whereas CHE-FcD specifically recognizes and binds to 9-*O*-acetylated Sias (38).

Extraction of Gangliosides from Cultured Cells—Washed cell pellets were resuspended in 3 volumes of ice-cold deionized water, homogenized at 4 °C, and the homogenate was added dropwise to 10.6 volumes of methanol at room temperature under constant stirring. Chloroform (5.3 volumes) was then added to the suspension. After centrifugation, the supernatant was collected and adjusted to a final chloroform/methanol/water ratio of 4:8:5.6 (v/v/v). After phase separation, gangliosides were enriched in the hydrophilic upper phase, which was dried down, resuspended in methanol, and kept at −20 °C until use.

Extraction of Proteins from Cultured Cells—Washed cell pellets were resuspended into a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitors (1 mM EDTA, 50 μg/ml leupeptin, and 4 μg/ml pepstatin) and incubated 10 min at room temperature with occasional mixing. After centrifugation, the supernatant was saved for analysis.

O-Sialoglycoprotease Treatments—Proteins (40-μg aliquots) from MEL cells were incubated with 2.5 milliunits of enzyme in 100 mM HEPES (pH 7.4), in the presence of BSA (stabilizer) for 2 h at 37 °C. For treatment of cell surface molecules, MEL cells (100 μl, 10^6 cells/ml) in Hanks' balanced saline solution, 1% fetal calf serum, 0.1% NaN_3, and 10 mM Neu2en5Ac (sialidase inhibitor) were incubated for 2 h at 37 °C in the presence or absence of 5 milliunits of *O*-sialoglycoprotease. The cells were then washed with Hanks' balanced saline solution,, 1% fetal calf serum, 0.1% NaN_3 prior to immunostaining.

Trypsin Treatment—Cells were washed three times with ice-cold PBS and finally resuspended in 0.5 ml of PBS containing 0.1% NaN_3 and 1% trypsin and incubated at 37 °C for 1 h. The trypsin was inactivated by adding an excess of medium containing fetal calf serum. The cells were washed with staining buffer prior to immunostaining.

PNGase F Treatment—Protein samples were denatured as described (42) and treated with 1 milliunit of PNGase F and incubated overnight at 37 °C. The enzyme was thereafter heat-inactivated for 3 min at 100 °C.

Removal of 9-O-Acetyl Esters from Cell Surface Sias—2×10^6 MEL cells were resuspended in 100 μl of PBS, containing 0.02% NaN_3 (control cells) or 100 μl of PBS, containing 0.02% NaN_3 with 10 μg of CHE-Fc. After incubation at 37 °C for 60 min, the cells were washed 3 × in ice-cold PBS and then stained for flow cytometry analysis as described below. For studies requiring pulse-chase analysis, the incubations were done in Hanks' balanced saline solution, and the cells were placed back in full culture medium for varying chase periods.

SDS-PAGE and Western Blot Analysis—Proteins were separated by SDS-PAGE in 7.5% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), and the blots incubated overnight at 4 °C with CHE FcD (10 μg/ml) in PBS, 1% BSA. Glycoproteins reacting with the chimera were colorimetrically detected with a goat anti-human IgG antibody conjugated with alkaline phosphatase.

ELISA Plate Assays for 9-O-Acetyl Gangliosides—Total ganglioside extracts prepared as above were studied by lipid ELISA as described previously (15, 41). Lipids were applied to the plate in 45% methanol, allowed to dry, and then blocked with 5% BSA in PBS overnight. The effects of base on reactivity were assessed by treatment with 0.1 M NaOH at 4 °C for 30 min. After treatment, the plates were extensively washed with PBS, blocked with 2% BSA in PBS for 1 h, and then incubated with the mAb mixture for 2 h. After washing three times with 1% BSA, a mixture of horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-human IgG antibodies was added (each at 1:1000 dilutions) for 1 h. After washing, the reaction was developed as described. Background levels determined with the secondary antibody alone were subtracted in all cases.

HPTLC Immuno-overlay of Gangliosides—Gangliosides were separated on aluminum-backed Silica Gel-60 HPTLC plates; the plates were plasticized and overlaid with antibodies as described previously (15, 41). Horseradish peroxidase-conjugated goat anti-mouse IgG recognizing mouse IgG 1:2000 (27A) was reacted with the plate for 1 h at 4 °C. After washing, the reaction was developed with diaminobenzidine.

Treatment of MEL Cells with Biological Modulators—MEL cells were routinely grown to ~90% confluency before splitting for passage. Cells were used during asynchronous near-confluent growth or were arrested at the G_2/M stages of the cell cycle using nocodazole (1 μg/ml) or Taxol (1 μg/ml) for varying periods of time. Optimal enrichment of G_2/M-arrested cells was obtained when the anti-microtubule agents were added to cells at 50% confluency. The percentage of G_2/M cells was determined by flow cytometric cell cycle analysis as described below. Synchronization of cells at the G_1/S boundary was carried out by incubating near-confluent MEL cells with thymidine (5 μg/ml, 24 h). The

cells were then chased with normal medium for 8 h and then incubated with nocodazole for 10 h.

Growth and Differentiation of MEL Cells—A culture of cells at various densities (2.5×10^5/ml to 4×10^6/ml) in complete medium was studied by measuring the extent of spontaneous differentiation, using the benzidine assay to measure hemoglobin accumulation, and DNA content to determine cell cycle phase status. Differentiation was induced by growing MEL cells (starting inoculum of 5×10^5 cells/ml) in the presence of 2% Me_2SO in complete medium for 3 days.

Staining of MEL cells for Flow Cytometry Analysis—MEL cells were washed three times with 1% BSA in PBS, and incubated with a mixture of fluorescein isothiocyanate-conjugated goat anti-human IgG and CHE-FcD for 2 h at 4 °C. Control cells were incubated with similar amounts of human IgG_1 in 1% BSA in PBS. Cells were washed once in PBS, fixed in 2% formaldehyde in PBS, and analyzed by flow cytometry using a Becton Dickinson FACScan instrument. Intact cells were gated based on their forward and side scattering characteristics.

Propidium Iodide Staining of MEL Cells and Flow Cytometry Analysis of Cell Cycle Status—Cells were washed and stained with CHE-FcD as described above. After fixing with 2% formaldehyde in PBS, the cells were treated with 0.03% saponin at 37 °C for 15 min and stained with propidium iodide (20 μg/ml) in PBS containing RNase A (40 μg/ml, Boehringer Mannheim) at 37 °C for 30 min. The DNA content was measured using FACScan (Lysis program, Becton Dickinson).

Sia Side Chain Oxidation and Labeling with Biotin Hydrazide—Control and base-treated glycoproteins were incubated with freshly prepared 2 mM sodium periodate in phosphate-buffered saline (pH 6.5) at 4 °C for 30 min in the dark. The resulting aldehydes were reacted with an excess of biotin-labeled hydrazide at room temperature for 1 h. The labeled sialoglycoproteins were dialyzed against PBS (pH 7.0) overnight at 4 °C, separated by SDS-PAGE, transferred onto nitrocellulose, and analyzed using streptavidin-alkaline phosphatase developing system.

RESULTS

9-O-Acetylation of Sias on MEL Cells Is Predominantly Expressed on Cell Surface Mucin-type Glycoproteins—In earlier studies, we reported detection of 9-*O*-acetylated Sias in murine erythroleukemia (MEL) cells but did not identify the type of oligosaccharides carrying these modifications (29). To pursue this question, membrane proteins from cultured MEL cells were studied by Western blotting using two different approaches. In the first, membrane Sias were labeled via selective oxidation of their side chains with mild periodate oxidation, followed by covalent coupling with biotin hydrazide. The biotin residues could then be detected on the blot using a streptavidin/horseradish peroxidase probe (see Fig. 1*A*.). Under the conditions used, mild periodate will not react with 9-*O*-acetylated Sias (29), which would hence be protected from biotinylation. De-*O*-acetylation of these molecules with base treatment exposes them to subsequent periodate oxidation and biotinylation. As shown in Fig. 1*A*, base treatment increased overall staining of many proteins, especially two bands with approximate apparent molecular mass of 110 and 87 kDa (some of the increased diffuseness of staining could be due to occasional cleavage of peptide bonds by the base treatment). These data suggested that several cell surface glycoproteins might carry the *O*-acetylated Sias. To confirm this, we probed other untreated blots with the CHE-FcD probe which is specific for binding to 9-*O*-acetylated Sias. As shown in Fig. 1*B*, major bands from MEL cells with apparent masses of 140, 110, and 90 kDa carry 9-*O*-acetylated Sias. The staining of these bands by the CHE-FcD probe is unaffected by prior PNGase F treatment (see Fig. 1*B*), indicating that the 9-*O*-acetyl groups are not carried on the Sias of *N*-linked oligosaccharides. On the other hand, their staining is markedly reduced by prior treatment of the membrane extract with *O*-sialoglycoprotease (OSGPase), an enzyme known to selectively proteolyze only mucin-type glycoproteins that carry clustered *O*-linked oligosaccharides (42, 43). To confirm that the protein bands found by SDS-PAGE represent cell surface 9-*O*-acetylation, intact MEL cells were studied by flow cytometry for staining with the CHE-FcD probe, with and without prior treatment with OSGPase. As shown in Fig. 2, this probe detects 9-*O*-acetylation on the cell surface, and OSGPase treatment markedly reduces staining. These data indicate that in contrast to melanoma cells and hepatocytes, most of the cell surface Sia 9-*O*-acetylation in murine erythroleukemia cells is selectively expressed on *O*-linked oligosaccharides of mucin-type glycoproteins.

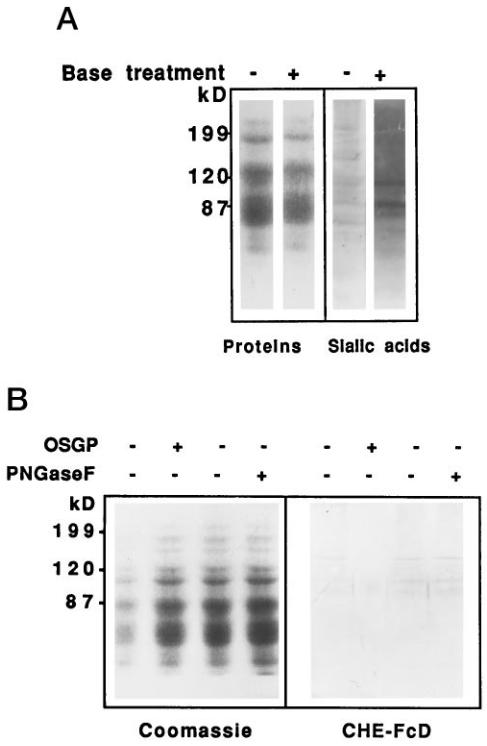

FIG. 1. *O*-Acetylation is expressed on sialomucins in MEL cells. *A*, staining for total Sias before and after de-*O*-acetylation. Total proteins from MEL cells (20 μg/lane) were treated with combinations of alkali (de-*O*-acetylation) and/or mild periodate oxidation/biotin hydrazide (see "Experimental Procedures" for details) and separated by SDS-PAGE under reducing conditions. Total proteins were detected by Coomassie Blue staining (*left panel*). Total non-*O*-acetylated Sias labeled by mild periodate oxidation/biotin hydrazide were detected by a horseradish peroxidase-conjugated streptavidin probe after transferring of proteins to nitrocellulose (*right panel*). *B*, direct detection of *O*-acetylation on sialomucins. Samples were digested with PNGase F or *O*-sialoglycoprotease and subjected along with sham-treated controls to SDS-PAGE under reducing conditions. Total proteins were detected by Coomassie staining (*left panel*) and 9-*O*-acetylated Sias by a Western blot probed with CHE-FcD (*right panel*). Bound probe was detected using goat anti-human IgG-conjugated alkaline phosphatase as described under "Experimental Procedures."

9-O-Acetylation of Sias on MEL Cells Is Also Found on Gangliosides, but These Are Not Detectable at the Cell Surface—Our prior studies showed that in human melanoma cells, 9-*O*-acetylation of Sias is selectively expressed on gangliosides and not on glycoproteins (15). The most common of these gangliosides 9-*O*-acetyl-G_{D3} can be selectively detected in melanoma cells by cell surface reaction with the specific mAbs Jones or 27A. However, the CHE-FcD probe (which should recognize all 9-*O*-acetylated Sias regardless of the underlying glycoconjugate) does not as easily detect this type of 9-*O*-acetylation at the intact cell surface. When Melur melanoma cells that express 9-*O*-acetyl-G_{D3} are studied with the CHE-FcD probe, staining was not fully revealed unless the cells were pretreated with trypsin, to remove cell surface glycoproteins that mask the epitope (Fig. 3*A*). This is interpreted to mean that this probe (unlike the mAbs) is unable to approach close enough to the ganglioside, which is a small molecule embedded in the outer membrane bilayer. Thus, 9-*O*-acetylated gangliosides could

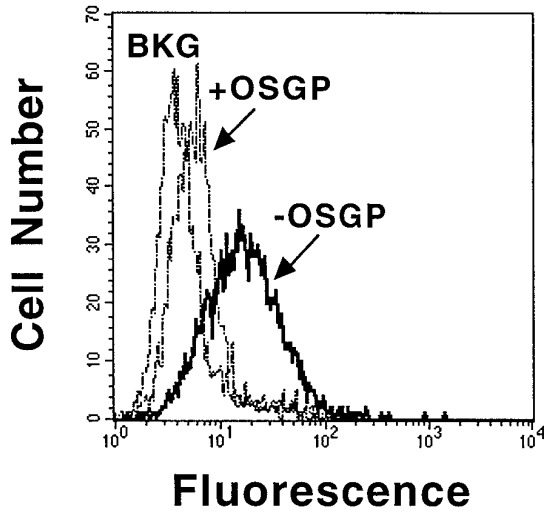

FIG. 2. **Cell surface expression of *O*-acetylated sialomucins in MEL cells.** MEL cells were either sham-treated or treated with *O*-sialoglycoprotease (*OSGP*ase) as described under "Experimental Procedures." The washed cells (10^6) were stained with CHE-FcD followed by FITC-conjugated goat anti-human IgG (*BKG* = secondary antibody alone) and analyzed by flow cytometry.

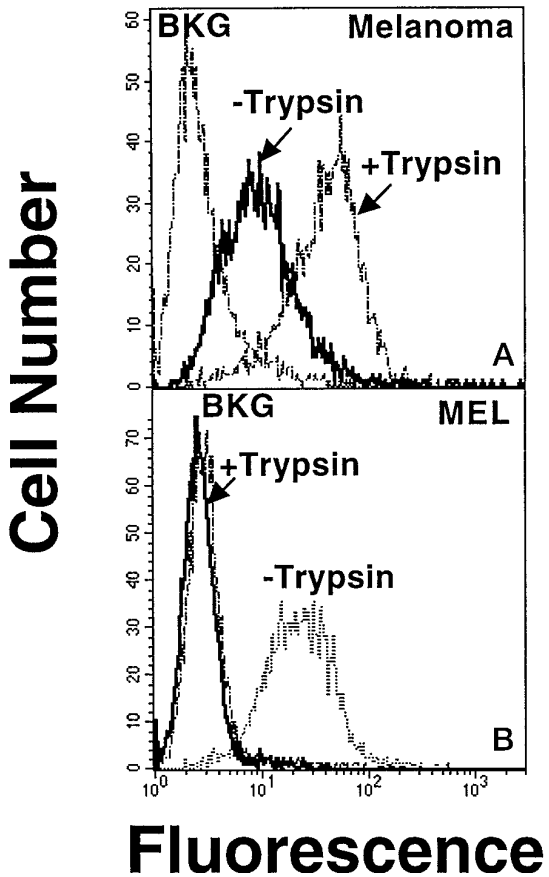

FIG. 3. **Differential effects of trypsin treatment on the detection of *O*-acetylated cell surface molecules in MEL and melanoma cells.** MEL or Melur melanoma cells (10^6) were either sham-treated or treated with trypsin at 37 °C for 1 h as described under "Experimental Procedures." The washed cells were stained with CHE-FcD followed by FITC-conjugated goat anti-human IgG (BKG = secondary antibody alone) and analyzed by flow cytometry.

also be present in the MEL cells and be masked from detection by the CHE-FcD by endogenous glycoproteins. To explore this, we re-examined staining following trypsin treatment. In contrast to the melanoma cells, FcD staining was completely lost

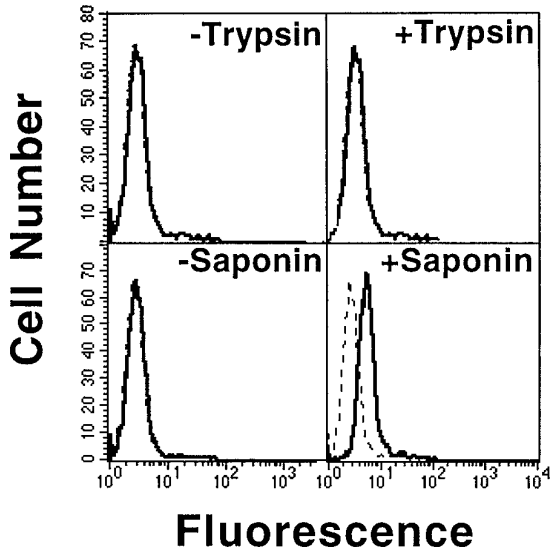

FIG. 4. **Detection of a cryptic pool of 9-*O*-acetylated gangliosides in MEL cells after permeabilization.** MEL cells were treated with trypsin or saponin, respectively, as described under "Experimental Procedures." The washed cells were stained with mAb 27A against 9-*O*-acetyl G_{D3}, followed by FITC-conjugated goat anti-mouse IgG. *Dotted line*, secondary antibody alone.

from the MEL cells after this treatment (Fig. 3*B*), indicating the absence of accessible 9-*O*-acetylated gangliosides on the cell surface. In keeping with this, there was no staining with mAb 27A on flow cytometry analysis, even after trypsin treatment (Fig. 4, *upper panels*). However, upon permeabilization with saponin, some reactivity with 27A was detected (Fig. 4, *lower panels*). In keeping with this, MEL cells (like melanoma cells) were found to contain the precursor G_{D3} as one of the major gangliosides (detected by TLC with resorcinol spray and by reactivity with mAb R24, data not shown). The presence of 9-*O*-acetyl-G_{D3} was confirmed in ganglioside extracts by lipid ELISA (Fig. 5), as well by overlay with CHE-FcD or 27A on HPTLC plates (data not shown). Thus, unlike the case with melanoma cells, 9-*O*-acetylation of Sias in murine erythroleukemia cells is found both on the glycolipid and the *O*-linked glycoprotein fraction. However, in contrast to the *O*-acetylated sialomucin glycoproteins, 9-*O*-acetyl-G_{D3} is present in low amounts and is either confined to an internal compartment or is inaccessible to the antibodies and CHE-FcD, even after trypsinization.

The Expression of Cell Surface 9-O-Acetylated Sialomucins Varies with Cell Density—In the course of these studies, we noticed some variations in the extent of MEL cell surface *O*-acetylation (detected by CHE-FcD) in batches of cells collected on different days. The explanation was found in the fact that cells grown at higher cell densities have marked reduction in 9-*O*-acetylation (see Table I). When MEL cells are grown at higher densities, they spontaneously differentiate toward a mature erythroid phenotype. Indeed, as shown in Table I, the loss of *O*-acetylation with increasing density seems to parallel the appearance of hemoglobin expression, a known marker of MEL cell differentiation. This change is also paralleled by an increase in the proportion of cells in the G_0/G_1 phases of the cell cycle (see Table I).

Induced Differentiation of MEL Cells Results in Loss of Surface 9-O-Acetylation—The above data suggested that differentiation and/or cell cycle status may be associated with a decrease in 9-*O*-acetylation of MEL cells. To check this, cultures were treated with 2% Me_2SO, a well-known differentiating agent for these cells (23). Indeed, as shown in Fig. 6*A*, fully differentiated cells undergo a marked reduction in cell surface

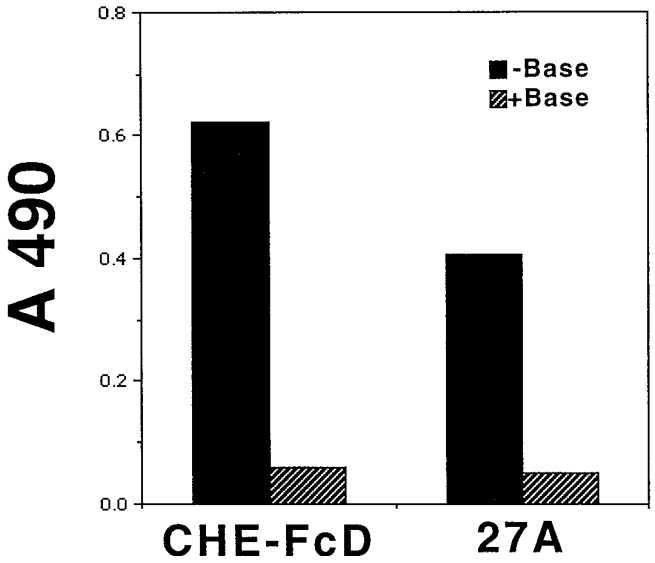

FIG. 5. **The presence of 9-*O*-acetyl-G_{D3} is confirmed in ganglioside extracts from MEL cells.** Gangliosides were extracted from MEL cells as described under "Experimental Procedures." Base-treated or non-base-treated gangliosides were coated on ELISA plates and incubated with the mAb 27A or the CHE-FcD probe. Binding was detected using goat anti-mouse IgG or goat anti-human IgG-conjugated horseradish peroxidase, respectively, as described under "Experimental Procedures." The data are the mean of triplicates.

O-acetylation, to a nearly undetectable level. To rule out an effect of Me_2SO unrelated to the differentiation process, cultures were subjected to serum starvation, which also causes cells to differentiate (as shown by hemoglobin accumulation). This was also accompanied by a significant loss of *O*-acetylation, although not the extent seen with Me_2SO (Fig. 6*B*). Similar effects were seen with hexamethylene-bisacetamide (HMBA), another inducer of differentiation (data not shown). Notably, none of these treatments was associated with a major change in total cell surface sialylation (detected by flow cytometry using wheat germ agglutinin-FITC, data not shown).

Effects of Cell Cycle Stage upon the Expression of 9-O-Acetylation—Since all treatments that induce differentiation (high cell density, Me_2SO, serum starvation, or HMBA) also arrest cells in G_0/G_1 of the cell cycle, the relationship of 9-*O*-acetylation to cell cycle stage was analyzed in rapidly growing cells that are not committed to the differentiated phenotype. No loss of *O*-acetylation is seen in rapidly growing cells that are in the G_1 phase (Fig. 7). Thus, the loss of *O*-acetylation seen upon differentiation is not simply a consequence of entry into G_0/G_1. However, a small increase in cell surface 9-*O*-acetylation was observed when rapidly growing cells are in the G_2/M phase (Fig. 7). To study this further, cells were also treated with nocodazole (or colcemid), to arrest them in the G_2/M phase. A more substantial increase of cell surface *O*-acetylation (>5 times control) was observed after this treatment. However, nocodazole could also be causing this change by virtue of its ability to alter microtubule dynamics and membrane recycling. The MEL cells were also therefore treated with taxol, another G_2/M phase blocker which has (in contrast to nocodazole) a microtubule stabilizing effect. While taxol-treated cells were also found to be blocked in G_2/M to the same extent as those treated with nocodazole (data not shown), this was not accompanied by such a marked increase in cell surface *O*-acetylation (Fig. 8*B*). Indeed, the very small increase seen could be explained by the increase in cell size that occurs during the G_2/M phase of the cell cycle. In keeping with this, shorter treatments with nocodazole (3–6 h, too short to cause extensive accumulation in the M phase) still caused an increase in cell surface 9-*O*-acetylation (analyzed by flow cytometry, data not shown).

The effects of cell cycle, nocodazole, and taxol on the cellular content of 9-*O*-acetylated G_{D3} was also investigated. None of these appeared to have any effect on the content or the internal localization of this form of 9-*O*-acetylated molecules (staining with mAb 27A, with and without saponin pretreatment and analyzed by flow cytometry, data not shown). Thus, depolymerizing microtubules affect the expression and localization of 9-*O*-acetylated sialomucins but not of 9-*O*-acetylated G_{D3}.

Changes in Cell Surface 9-O-Acetylated Sialomucins after Induced Differentiation or Nocodazole Treatment Are Not Due to Redistribution—As indicated above, the changes in *O*-acetylation seen with induced differentiation (decrease) or nocodazole treatment (increase) are not due to arrest in the G_2/M phase. Alternatively, that could be due to a redistribution of the *O*-acetylated sialomucins rather than changes in actual cellular content. To rule out this possibility, a Western blot of total cell glycoproteins from each situation was probed with CHE-FcD. As shown in Fig. 9, this approach confirmed a substantial decrease of protein-associated 9-*O*-acetylation upon Me_2SO-induced differentiation and a significant increase with nocodazole treatment, particularly in some high molecular weight sialoglycoproteins.

Increase in 9-O-Acetylation Following Nocodazole Treatment Requires New Synthesis and Is Due to Accumulation on the Cell Surface—In the past, it was not possible to selectively remove 9-*O*-acetyl groups from cell surface molecules and to follow the subsequent fate of the Sias. Using the recombinant soluble influenza C 9-*O*-acetyl esterase (CHE-Fc), we have explored conditions for complete removal of cell surface 9-*O*-acetyl groups from MEL cells. As shown in Fig. 10, complete removal is possible, and the cells can be returned to culture for a chase period. As shown in the examples in Fig. 10*A*, 9-*O*-acetylation gradually returned to the cell surface over a period of 2–3 h. However, simultaneous treatment with nocodazole had no effect upon the rate of recovery (Fig. 10*B*), indicating that this compound may not affect the rate of new synthesis of 9-*O*-acetylated Sias.

To confirm that the restoration of cell surface 9-*O*-acetylation after selective removal was due to new protein synthesis, we studied the effects of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 11, this treatment markedly blunted the restoration of expression of 9-*O*-acetylation. Taken together, the data indicate that the mucin molecules that were de-*O*-acetylated at the cell surface did not undergo re-*O*-acetylation and re-expression. Thus, the effects of nocodazole are most likely explained by the accumulation of newly synthesized molecules on the cell surface, *i.e.* a decreased rate of internalization and turnover.

DISCUSSION

Friend murine erythroleukemia cells (MEL cells) were one of the first cellular models where "normal" differentiation could be induced and recapitulated *in vitro* (23), as determined by the expression of molecules such as the globins. Erythroid differentiation normally takes place in a defined microenvironment in the bone marrow, where erythroblasts interact via cell surface glycoproteins with stromal cells (fibroblasts and macrophages) and with extracellular matrix components (44). Thus, besides the coordinated expression of globin genes, controlled expression of cell surface sialoglycoconjugates may characterize the differentiated phenotype of erythrocytes. Murine erythrocytes also present *O*-acetylated sialoglycoconjugates on their cell surface (11, 27, 28). Although the precise function of this modification is still an open question, *O*-acetylation of Sias may alter the binding of some viruses and is predicted to affect the activation of the alternative complement pathway.

TABLE I
Effect of cell culture density on cell cycle, differentiation, and cell surface 9-O-acetylation
MEL cells were seeded and grown to different densities in culture as indicated, and aliquots were studied for the various parameters indicated.

Cell density	G_0/G_1[a]	G_2/M	Hemoglobin-positive[b]	Relative cell surface *O*-acetylation[c]
cell/ml	%	%	%	%
2.5×10^5	62	38	0	93
5×10^5	61	39	0	94
1×10^6	63	38	0	93
2×10^6	64	37	0	92
4×10^6	93	7	50	17

[a] Cells were stained with propidium iodide, and DNA content was analyzed by flow cytometry as described under "Experimental Procedures."
[b] Cells were stained for hemoglobin expression with benzidine as described under "Experimental Procedures."
[c] Cells were stained with CHE-FcD and studied by flow cytometry as described under "Experimental Procedures." The mean fluorescence intensity is presented, relative to control cells stained with secondary antibody alone.

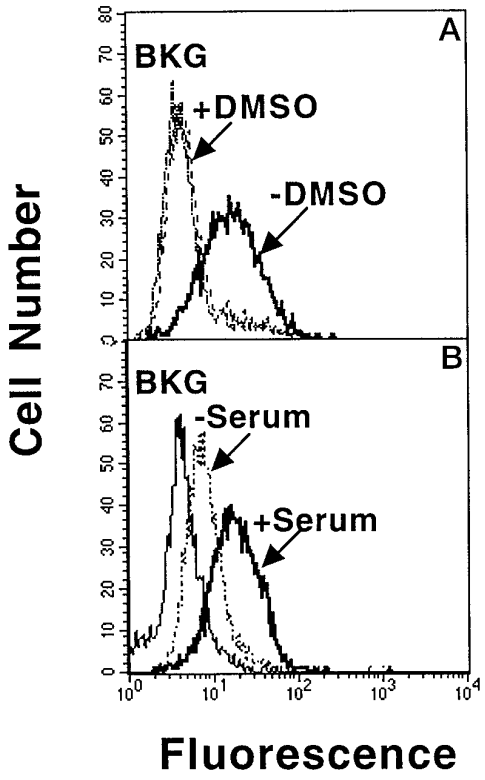

FIG. 6. **Effect of differentiation state on the expression of cell surface 9-*O*-acetylated sialomucins in MEL cells.** MEL cells were grown in the presence of 2% Me_2SO (*DMSO*) or serum-free media for 3 days, and washed cells were stained with CHE-FcD and analyzed by flow cytometry as described under "Experimental Procedures" (*BKG*, secondary antibody alone).

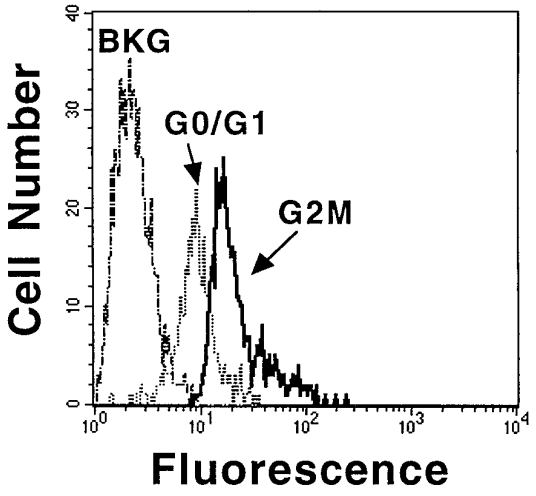

FIG. 7. **Relationship of cell cycle to expression of 9-*O*-acetylated sialomucins in rapidly growing MEL cells.** MEL cells were grown in logarithmic growth phase and double-stained with CHE-FcD and propidium iodide as described under "Experimental Procedures." Flow cytometry analysis was set up with FITC/propidium iodide fluorescence compensation. The G_0/G_1 and G_2/M cells in the histogram were gated from analysis of a dot plot display of the double-stained MEL cells (not shown), (*BKG*, secondary antibody alone).

Here we have identified the nature of *O*-acetylated sialoglycoconjugates in MEL cells and studied the control of their expression upon differentiation. Both glycoproteins and glycolipids of MEL cells were found *O*-acetylated. Among glycoproteins, several high molecular weight polypeptides were the major *O*-acetylated species. These glycoconjugates were sensitive to *O*-sialoglycoprotease but not to PNGase F, thus identifying them as mucin-like molecules carrying clusters of *O*-linked chains. The identity of the polypeptides that carry this modification needs to be investigated. Among glycolipids, MEL cells contain both G_{D3} and its 9-*O*-acetylated form, but the latter is a minor species that is present either in an internal compartment or it is not readily accessible on the cell surface, as discussed for other cellular models (45). This is in contrast to human melanoma cell lines, which express both G_{D3} and 9-*O*-acetylated G_{D3} on the cell surface, but not 9-*O*-acetylated sialoglycoproteins. Also, unlike the MEL cells, 9-*O*-acetylated G_{D3} was readily identified on the cell surface of Melur cells using specific mAbs and becomes unmasked for CHE-FcD detection after cell surface trypsinization. On the other hand, MEL cells do not express any trypsin-resistant sialoglycoconjugate, and 9-*O*-acetyl-G_{D3} could be observed only after treatment of cells with saponin (which permeabilizes cells by extracting cholesterol from the plasma membrane). In separate work, we have shown that 9-*O*-acetylation is found primarily on *N*-linked glycoproteins of hepatocytes (16). Thus, 9-*O*-acetylation appears to be differentially regulated in different cell types in a molecule-specific and location-specific manner.

We next analyzed the expression of 9-*O*-acetylated sialomucins on the cell surface of MEL cells during their growth and differentiation. At cell densities of $>4 \times 10^6$/ml, we observed an arrest of cells in G_0/G_1, accompanied by spontaneous production of hemoglobin, and a marked reduction of 9-*O*-acetylation. Induced differentiation with serum starvation, Me_2SO, or HMBA caused a more marked increase in hemoglobin accumulation and arrest in G_0/G_1 phases and was accompanied by a more marked loss of cell surface expression of 9-*O*-acetylation, without any accompanying major changes in the level of cell surface sialylation. Since all of these treatments arrested cells in the G_0/G_1 phase, we evaluated a possible cell cycle dependency of this Sia modification. Cells in G_2/M displayed a small (~2-fold) increase of CHE-FcD reactivity over those in G_0/G_1. However, if one assumes that the cells in G_2 phase would double their volume before division, an actual increase of about 60% in cell surface area is expected. To explore this, we induced

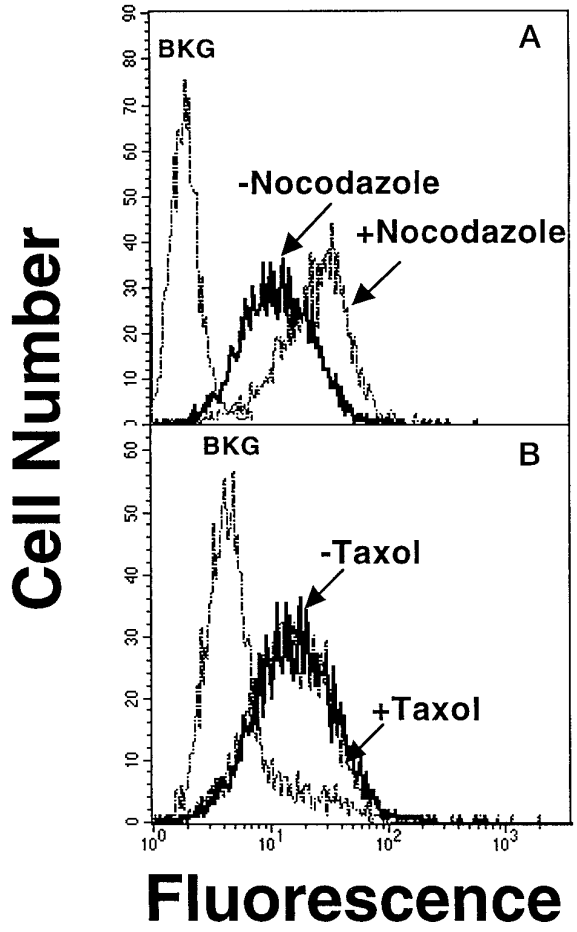

FIG. 8. **Effects of drugs altering microtubule dynamics upon the expression of 9-*O*-acetylated sialomucins in MEL cells.** MEL cells grown to confluence were incubated with indicated drugs. After 15 h, the cells were harvested and stained with CHE-FcD as described under "Experimental Procedures" (*BKG*, secondary antibody alone).

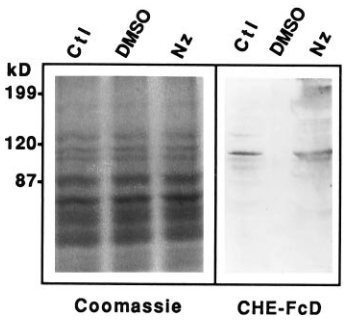

FIG. 9. **Changes in cell surface *O*-acetylation upon differentiation and nocodazole treatment are not due to redistribution.** MEL cells grown to confluence were incubated with no additions, with 2% Me_2SO (*DMSO*), or with nocodazole (*Nz*). The cells were harvested, total proteins extracted, 20 μg/lane separated by 7.5% SDS-PAGE, and Western blotted with CHE-FcD and goat anti-human IgG-conjugated alkaline phosphatase (developed with nitro blue tetrazolium and bromochloroindolyl phosphate substrate). *1st to 3rd lanes*, Coomassie staining; *4th to 6th lanes,* Western blot.

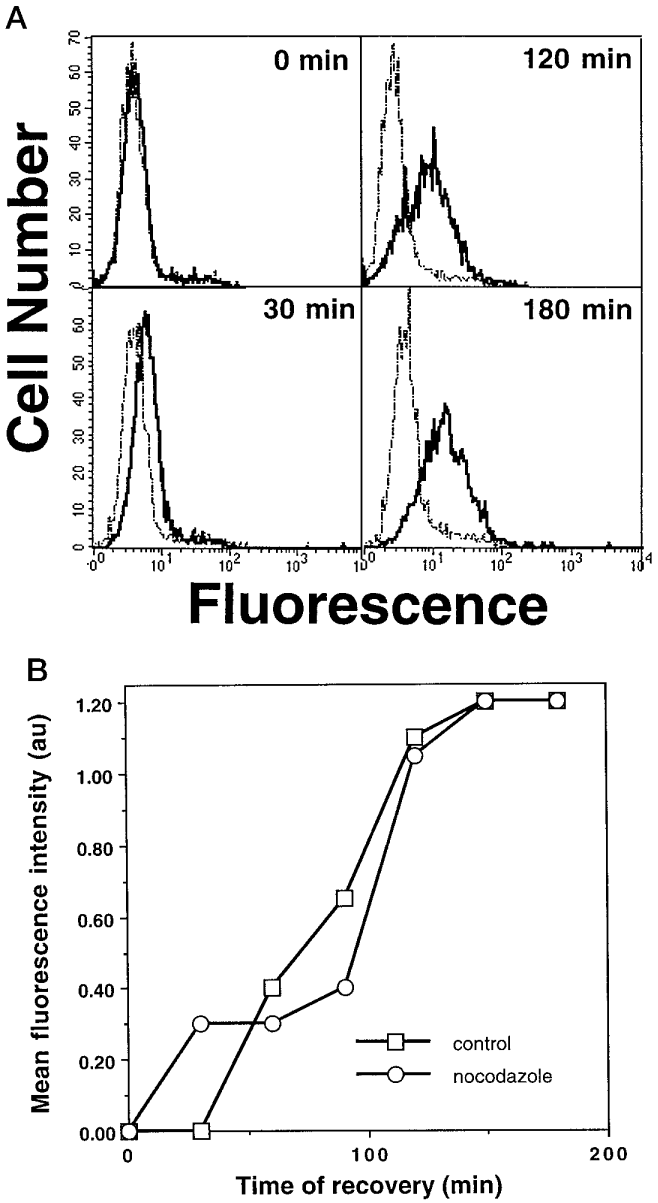

FIG. 10. **Recovery of cell surface 9-*O*-acetylation following selective de-*O*-acetylation, lack of effect of nocodazole.** MEL cells (4×10^6) grown to confluence were incubated with CHE-Fc at 37 °C for 1 h to remove 9-*O*-acetyl groups, followed by washing and incubation in culture medium for the indicated time points. The cells were harvested and incubated with CHE-FcD as described under "Experimental Procedures." *A*, flow cytometry to assay *O*-acetylation recovery. *Solid line*, CHE-FcD-stained cells; *dashed line*, secondary antibody alone. *B*, effect of nocodazole on *O*-acetylation recovery, assessed by flow cytometry.

the arrest of MEL cells in G_2/M phase, using drugs that altered microtubule dynamics such as nocodazole, colchicine, and taxol (46). While nocodazole and colchicine block microtubule polymerization, taxol acts as a stabilizer of microtubules. We found that nocodazole and colchicine, but not taxol, induced accumulation of 9-*O*-acetylated sialomucins on the surface of MEL cells. Taken together, these results indicate that (i) there is no significant cell cycle-dependent change on cell surface *O*-acetylation of MEL cells; (ii) the loss of 9-*O*-acetylation upon MEL cell differentiation is part of the differentiation program of this particular cell line; and (iii) nocodazole and colchicine induce accumulation of cell surface *O*-acetylated sialomucins via a mechanism independent of their effects on cell cycle.

To explore the last matter further, we needed to selectively remove 9-*O*-acetylation from the cell surface and to then monitor its recovery. This has not been possible in the past. However, we show here for the first time such a removal and recovery experiment, using the recombinant soluble form of the influenza C HE protein as a soluble 9-*O*-acetyl esterase. The results show that there is no recycling with re-*O*-acetylation in this system and that new protein synthesis is required for recovery. We could not address the same question regarding the 9-*O*-acetylation of G_{D3}, since this molecule was not acces-

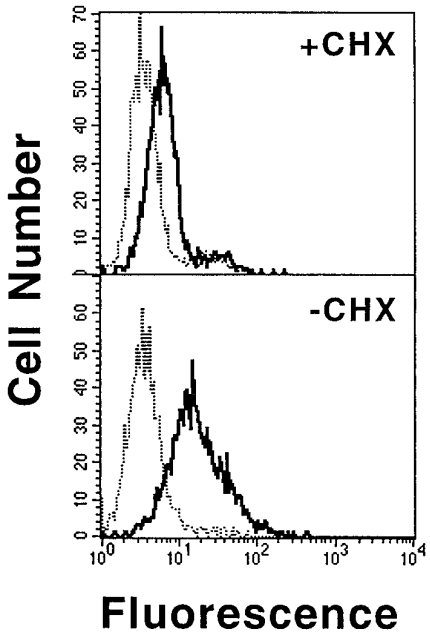

FIG. 11. **Effects of cycloheximide on the recovery of cell surface 9-*O*-acetylation following selective de-*O*-acetylation.** MEL cells grown to confluence were treated with CHE-Fc at 37 °C for 1 h to remove 9-*O*-acetyl groups, followed by washing and incubation in culture medium in the presence or absence of cycloheximide (*CHX*) at 37 °C for 3 h. The cells were harvested and stained with CHE-FcD as described under "Experimental Procedures." *Solid line*, CHE-FcD-stained cells; *dashed line*, secondary antibody alone.

sible for de-*O*-acetylation at the cell surface. Thus, the effects of nocodazole and colcemid must be due to delay of normal cell surface turnover via endocytosis. This interpretation is given credence by earlier studies of the transferrin receptor, which accumulates on the cell surface after endocytosis blockage by agents like nocodazole (47–51).

As mentioned above, murine erythrocytes present some 9-*O*-acetylated sialoglycoconjugates on their surface (11, 27, 28). Thus, despite being a suitable model for differentiation regarding globin gene expression, MEL cells do not constitute a representative model for "normal" differentiation regarding cell surface Sia *O*-acetylation. On the other hand, it represents a valuable system for general studies on the control of *O*-acetylation of Sias, an issue that is still very poorly understood. *O*-Acetylation of sialoglycoconjugates depends on the activity of two opposing enzymatic systems, *O*-acetyltransferase(s) and Sia-specific esterases (2, 3). Although the activity of *O*-acetyltransferases could be detected in a series of different cellular systems, the enzyme(s) have resisted all attempts to date for purification or molecular cloning. At least two Sia-specific esterases were identified (2, 3), one a cytosolic protein and the other one a lysosomal enzyme, which has recently been cloned (52). In preliminary studies, we have noted that during Me_2SO-induced differentiation there is only a marginal increase on total esterase specific activity.[2] Thus, loss of *O*-acetylation on differentiated MEL cells is more likely associated with a decreased activity of *O*-acetyltransferase(s). Unfortunately, the activity of the latter enzyme cannot be accurately measured in cell extracts. An alternative, but less likely, explanation for loss of 9-*O*-acetylation upon differentiation would be down-regulation of a unique subset of sialomucins that are specific substrates for the *O*-acetyltransferase(s) expressed on growing MEL cells.

[2] R. Chammas and A. Varki, unpublished data.

Analysis of the 9-*O*-acetylated Sias described so far in nature indicates that there may be separate *O*-acetyltransferase activities directed toward α2,6-linked Sias (*e.g.* for *N*-linked sialoglycoproteins from hepatocytes) and toward α2,8-linked Sias (*e.g.* for G_{D3} in human melanoma cells). It is still not known if α2,3-linked Sias can be 9-*O*-acetylated. Since MEL cells clearly expressed 9-*O*-acetyl-G_{D3}, they must be expressing an *O*-acetyltransferase activity toward α2,8-linked Sias. The nature of the Sia linkage on the 9-*O*-acetylated sialomucins remains to be determined. Regardless, it is evident that the *O*-acetyltransferase(s) present in these cells show some specificity, since they do not modify the Sias on the *N*-linked oligosaccharides of cell surface sialoglycoproteins.

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