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9-*O*-Acetylation of Exogenously Added Ganglioside GD3

*THE GD3 MOLECULE INDUCES ITS OWN O-ACETYLATION MACHINERY**

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Sialic acids are sometimes 9-*O*-acetylated in a developmentally regulated and cell-type-specific manner. Cells naturally expressing the disialoganglioside GD3 often *O*-acetylate the terminal sialic acid residue, giving 9-*O*-acetyl-GD3 (9AcGD3), a marker of neural differentiation and malignant transformation. We also reported that Chinese hamster ovary cells transfected with GD3 synthase can spontaneously *O*-acetylate some of the newly synthesized GD3. It is unclear whether such phenomena result from induction of the 9-*O*-acetylation machinery and whether induction is caused by the GD3 synthase protein or by the GD3 molecule itself. We now show that exogenously added GD3 rapidly incorporates into the plasma membrane of Chinese hamster ovary cells, and 9AcGD3 is detected after ~6 h. The incorporated GD3 and newly synthesized 9AcGD3 have a half-life of ~24 h. This phenomenon is also seen in other cell types, such as human diploid fibroblasts. Inhibitors of gene transcription, protein translation, or endoplasmic reticulum-to-Golgi transport each prevent induction of 9-*O*-acetylation, without affecting GD3 incorporation. Inhibition of the initial clathrin-independent internalization of incorporated GD3 also blocks induction of 9-*O*-acetylation. Thus, new synthesis of one or more components of the 9-*O*-acetylation machinery is induced by incorporation and internalization of GD3. Prepriming with structurally related gangliosides fails to accelerate the onset of 9-*O*-acetylation of subsequently added GD3, indicating a requirement for specific recognition of GD3. To our knowledge, this is the first example wherein a newly expressed or exogenously introduced ganglioside induces *de novo* synthesis of an enzymatic machinery to modify itself, and the first evidence for a mechanism of induction of sialic acid *O*-acetylation.

Gangliosides are glycosphingolipids containing one or more sialic acids and are commonly found on the outer leaflet of the plasma membrane (1, 2). With ceramide tails embedded in the membrane bilayer and glycans protruding from the surface, gangliosides are often clustered in microdomains and lipid rafts. Besides being major structural components in neural cell membranes, gangliosides have been found to play important roles in cell adhesion, cell recognition, signal transduction, and neural development (3–6). Early studies showed that when gangliosides in micellar form are exogenously added to cell culture media, they are capable of first becoming associated with cell surface proteins (a trypsin-sensitive component) and subsequently becoming inserted into the plasma membrane (becoming trypsin-resistant) (7). The latter molecules are then indistinguishable from their endogenous counterparts that are synthesized in the same cell (8, 9). This technique has since been widely employed to study the metabolic fate and functions of gangliosides. Radioactive and fluorescently labeled gangliosides have been used to elegantly trace the internalization route of exogenous gangliosides, demonstrating that such gangliosides are internalized from the plasma membrane by a clathrin-independent, caveolar-related mechanism, subsequently transported to endosomes, and eventually degraded in lysosomes, and their components are recycled (10–15). There is evidence that a small fraction of such internalized gangliosides can also be further glycosylated to make higher gangliosides (12, 16, 17).

9-*O*-Acetyl-GD3 (9AcGD3)[2] is a natural variant of the disialoganglioside GD3 that is cell-type and developmentally regulated.[3] Both GD3 and 9AcGD3 are prominently expressed during neuronal development, and their expression becomes very limited in adult tissues (18–20). In contrast, both are aberrantly expressed in basal cell carcinomas and in malignant melanomas of various species (21, 22). Several lines of evidence suggest that 9AcGD3 has its own biological significance, distinct from GD3. For example, unlike GD3, 9AcGD3 is a selective marker for germinal cells of the central nervous system (19) and shows a dorsoventral gradient across the developing retina (23). More recently 9AcGD3 has been shown to be associated with vinculin and β1-integrin at point contacts of neuronal growth cones (24). The CD60 subset of human T-lymphocyte markers includes GD3 and 9AcGD3 (25). Also, although certain cells are susceptible to GD3-mediated apoptosis, administration of 9AcGD3 can apparently prevent this process (26, 27).

It remains unclear why most cells that express GD3 also 9-*O*-acetylate GD3. Unfortunately, the enzymatic machinery involved has proven very labile and difficult to directly assay, purify, or clone. We have previously reported studies on the biosynthesis and turnover of 9AcGD3 in GD3-expressing melanoma cells and GD3-synthase-transfected Chinese hamster ovary cells (28–31). In the case of CHO cells which normally only express the precursor molecule GM3, new expression of GD3 caused by GD3-synthase transfection was accompanied by spontaneous appearance of 9AcGD3 (30). It remained unclear whether this involved a pre-existing *O*-acetyltransferase that was simply "waiting" for the GD3 substrate to appear, or whether the *O*-acetyltransferase is newly induced, and if the latter was the case, whether the GD3 synthase protein or the GD3 molecule itself was involved in the induction.

Here we describe the spontaneous 9-*O*-acetylation of exogenously added ganglioside GD3 that becomes incorporated into cells, presenting evidence for a new pathway in which GD3 induces 9-*O*-acetylation of itself. This 9-*O*-acetylation occurs in the absence of endogenous expression of GD3 synthase and requires new transcription and protein syn-

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[2] The abbreviations used are: 9AcGD3, 9-*O*-acetyl-GD3; CHO, Chinese hamster ovary; MFI, mean fluorescence intensity; ER, endoplasmic reticulum; GM3, Neuα2-3Galβ1-4Glcβ1–1′ceramide; GD3, Neu5A$c\alpha$2-8Neu5A$c\alpha$2-3Galβ1-4Glcβ1-1′ceramide.

[3] In 9AcGD3, the side chain of the terminal Neu5Ac molecule of GD3 carries an *O*-acetyl ester at hydroxyl group of the 9-carbon position.

thesis, presumably that of a specific 9-*O*-acetyltransferase, which may then work at the cell surface.

EXPERIMENTAL PROCEDURES

Materials—Ganglioside GD3 was purchased from Matreya. Tissue culture media were from Invitrogen Corp. Other reagents were from Sigma-Aldrich.

Cell Culture—CHO-K1, human skin fibroblast, and 293T cells were obtained from the American Type Culture Collection (ATCC CCL-61, CRL-1826, and CRL-11268, respectively). CHO-K1 cells were grown in α-minimum essential medium supplemented with 10% (v/v) fetal bovine serum. CHO-GD3, a cell line established previously by stable transfection of mouse GD3 synthase into parental CHO-K1 cells (31) were grown in the same medium containing 0.8 mg/ml G418, to assure continued expression of GD3 synthase. Fibroblast cells were grown in α-minimum essential medium supplemented with non-heat-inactivated 15% (v/v) fetal bovine serum, and 293T cells were grown in Dulbecco's modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum.

GD3 Feeding Study—Cells were grown in 6-well plates until ~60% confluent. Ganglioside GD3 (Matreya catalog 1504) in chloroform: methanol 1:1 was dried down under vacuum by using a Speedvac rotor, resuspended in serum-free medium, and briefly sonicated to ensure proper micellar suspension. GD3-containing medium was passed through a 0.2-μm sterilizing filter and diluted with fresh medium to a final 50 μM concentration. Cells were then incubated in GD3-containing medium in a 37 °C incubator for varying periods of time, as indicated in the text, figures, or figure legends.

Antibodies—The mouse IgG3 monoclonal antibodies R24 (32) against GD3 and 27A (33) against 9AcGD3 (kindly provided by Dr. M. Farquhar, UCSD) were purified from hybridoma culture supernatants by protein A-Sepharose (Amersham Biosciences) following the manufacturer's instructions.

Flow Cytometric Analysis —Cells (1×10^6) were trypsinized to eliminate nonspecifically adherent gangliosides and expose membrane-bound gangliosides. Briefly, standard EDTA/trypsin solutions normally used for passaging of tissue culture cells was added to minimally cover the cells. After incubation at 37 °C for 2 min, detached cells were collected, and the trypsin was quenched by washing twice with phosphate-buffered saline containing 1% bovine serum albumin. Each cell suspension was then incubated at 4 °C for 90 min with either 5 μg/ml R24 or 27A antibody precomplexed with phycoerythrin-conjugated goat anti-mouse secondary antibody. After the final washing, the fluorescent intensity on the cell surface was measured using a Becton Dickinson FACScan instrument. Mean fluorescence intensity (MFI) was calculated using FlowJo software.

As shown in the example in Fig. 1*A*, the forward and side scatter profiles of the CHO cells were very similar with or without GD3 addition. The gating shown in Fig. 1*A* excludes debris and obviously dead cells and is also typical of those used in all studies, capturing ~93% of the cells. Of the cells in the gated area, more than 98% were typically viable, as determined by propidium iodide incorporation, and there was no obvious toxicity of added exogenous GD3. Therefore, in subsequent experiments, we did not routinely use propidium iodide for such screening. However, we did routinely gauge the forward and side scatter and use similar gating in all studies.

Inhibitor Treatments—CHO-K1 cells were grown in 6-well plates until they were ~60% confluent. For inhibition of biosynthesis or trafficking, cells were incubated in serum-free media containing brefeldin A (10 μg/ml), actinomycin D (200 ng/ml), or cycloheximide (25 μM) for 18 h along with 50 μM GD3. Endocytosis inhibitors were added at appropriate concentrations according to Puri *et al.* (34). Briefly, cells were pretreated with chlorpromazine (6 μg/ml), nystatin (25 μg/ml), or genistein (200 μM) in serum-free medium for 2 h at 37 °C. Cells were then exposed to serum-free medium containing 50 μM GD3 in the presence of endocytosis inhibitors for 18 h. For delayed endocytosis inhibition cells were first incubated with 50 μM GD3 for 6 h, followed by addition of chlorpromazine (6 μg/ml), nystatin (25 μg/ml), or genistein (200 μM) and continued inhibition for another 13 h.

For each inhibitor treatment, no differences in cell viability were observed compared with sham treatment. To ensure that inhibitors did not nonspecifically induce degradation of 9AcGD3, they were also added to CHO-K1 cells that had been stably transfected with GD3 synthase (CHO-GD3). No difference in cell viability and no change in the expression of GD3 or 9AcGD3 were observed in CHO-GD3 cells over a period of 18 h.

RESULTS

Appearance of 9AcGD3 on the CHO-K1 Cell Surface upon Addition of Exogenous GD3 to the Media—Wild type CHO cells predominantly express the monosialoganglioside GM3 and no detectable GD3 (35–37). Previous studies have shown that when gangliosides are added to cell culture medium in the nanomolar to micromolar range, they are capable of inserting into the cell plasma membrane, becoming indistinguishable from endogenous gangliosides, and being subject to routine cellular trafficking and turnover (10). There is also some nonspecific adsorption to cell surface proteins, but these molecules can be removed by trypsin treatment (10) before detection of the membrane-inserted molecules by antibodies. This trypsin treatment (see "Experimental Procedures") also has the advantage that it better exposes the gangliosides for optimal detection by antibodies.

We first incubated CHO-K1 cells in medium containing 50 μM GD3 with reduced serum (5% v/v). Using monoclonal antibodies R24 (detects GD3) and 27A (detects 9AcGD3), we found that after a 3-day incubation, GD3 molecules were incorporated onto CHO-K1 cell surface in a trypsin-resistant fashion and that a small but noticeable amount was 9-*O*-acetylated (see Fig. 1*B*, *top panels*). After 7 days of incubation, a significant portion of the incorporated GD3 became 9-*O*-acetylated (see Fig. 1*B*, *bottom panels*). As indicated in Fig. 1*A* and "Experimental Procedures," the addition of GD3 caused no major change in forward and side scatter of the cells, and there was no change in cell viability (detailed data not shown).

A similar phenomenon was observed at a much faster rate, when GD3 was incubated in serum-free medium (serum is known to inhibit the incorporation of exogenously added gangliosides into the cell membrane) (10). As shown in Fig. 2, under serum-free conditions, GD3 molecules were inserted into the plasma membrane within the first 3 h of incubation. By the 6th hour, 9-*O*-acetylation was detected by antibody staining. At later time points, GD3 levels fluctuated as more molecules became incorporated and 9-*O*-acetylated. At the end of a 24-h incubation, a substantial amount of GD3 was 9-*O*-acetylated. It is not entirely clear why there is a broad peak of GD3 at 3 and 24 h but a tight and brighter peak at 6–9 h. We assume that only a finite amount of GD3 can become incorporated and that there is an equilibrium achieved with endocytic turnover from the cell surface, preventing unlimited GD3 accumulation. Also, as some of the GD3 becomes 9-*O*-acetylated at later time points, there would be correspondingly less GD3 detected on the cell surface, as the antibody R24 only recognizes the non-9-*O*-acetylated GD3. We did indirect fluorescence microscopy of these cells under

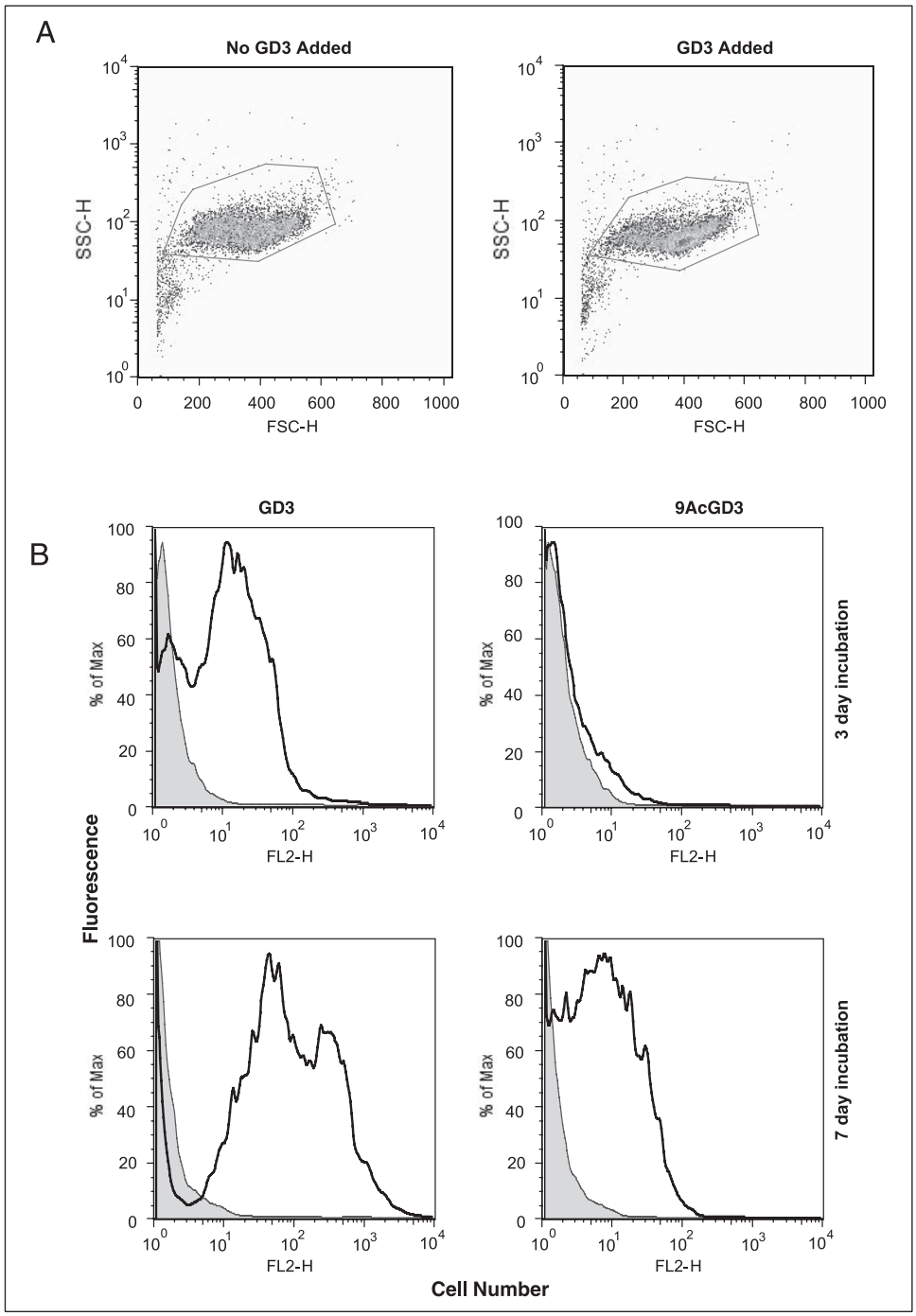

FIGURE 1. **Exogenous GD3 is incorporated into CHO-K1 cell membranes and becomes 9-*O*-acetylated.** CHO-K1 cells were incubated with 50 μM GD3 in α-minimum essential medium containing 5% fetal bovine serum for varying periods of time. *A*, there are no significant changes in the size or intracellular granularity of the cells upon addition of GD3, based on forward or side scatter profiles. Gating was used as indicated, to exclude dead cells and debris. Approximately 93–96% of cells were in the gated area and showed >98% viability on propidium iodide staining (not shown). *B*, after 3- or 7-day incubations, cells were trypsinized and stained with antibodies R24 (detects GD3) and 27A (detects 9AcGD3), and live cells were analyzed by flow cytometry. Cells that were treated with exogenous GD3 are shown in *black lines*, and sham-treated cells are shown as a *shaded histogram*. A minimal amount of 9AcGD3 observed after 3-day incubation. After 7 days, a larger amount of GD3 was incorporated, and 9-*O*-acetylation of GD3 became evident.

several of these conditions, and the findings were generally consistent with the flow cytometry data and the above interpretations.

We obtained similar results using another approach where cells were first serum-starved overnight to induce cellular quiescence, after which GD3 was added for 18 h in serum-free medium (data not shown). In follow-up experiments, it was observed that 9-*O*-acetylation only occurs if GD3 is maintained in the medium for more than 6 h. If GD3 is removed from the medium after 3 h of incubation, no 9-*O*-acetylation is observed at later time points, despite a substantial amount of GD3 molecules having already been incorporated (data not shown). This suggests that induction of the 9-*O*-acetylation machinery requires continued incorporation of new GD3 molecules, possibly to maintain a certain critical cell surface concentration for >3 h. All of these phenomena were best observed if cells were in a subconfluent stage prior to the start of the GD3 addition.

Turnover Rate of Incorporated GD3 and Newly Synthesized 9AcGD3—We determined the turnover time of exogenous GD3 and the resulting 9AcGD3 by first loading GD3 onto CHO-K1 cell surface for 24 h in serum-free medium. The GD3-containing medium was then removed and replaced with regular serum-free medium, and samples were taken at various time points to check for GD3 and 9AcGD3 expression. The MFI of GD3 and 9AcGD3 antibody staining was calculated using FlowJo software. The initial GD3 and 9AcGD3 MFIs at time 0 prior to chase were set at 100%. As shown in Fig. 3, the amounts of trypsin-resistant GD3 and 9AcGD3 increased during the first 3 h of the chase. This increase is likely because of the

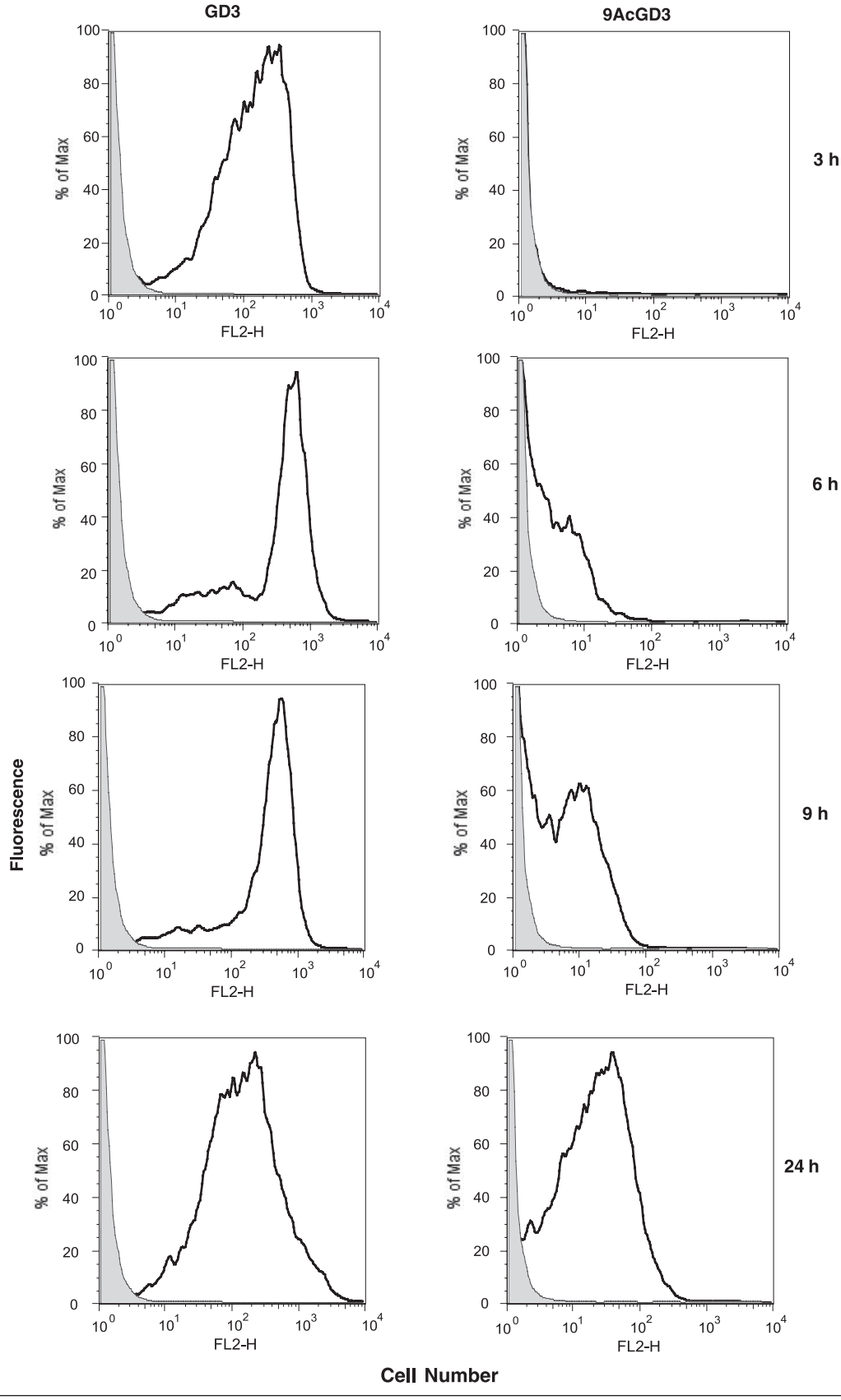

FIGURE 2. **Exogenous GD3 is rapidly incorporated into cell membranes in serum-free medium, and 9-*O*-acetylation appears within 6 h.** CHO-K1 cells were incubated with 50 μM GD3 in serum-free medium for 3, 6, 9, or 24 h. Both GD3-treated samples and corresponding controls were stained with R24 and 27A antibodies and analyzed by flow cytometry as described in the legend to Fig. 1. Negative controls are shown as *shaded histograms*, and GD3-treated cells are shown as *black lines*. A large amount of GD3 was incorporated into the plasma membrane within 3 h. However, 9-*O*-acetylation was not detectable until 6 h after GD3 was administered.

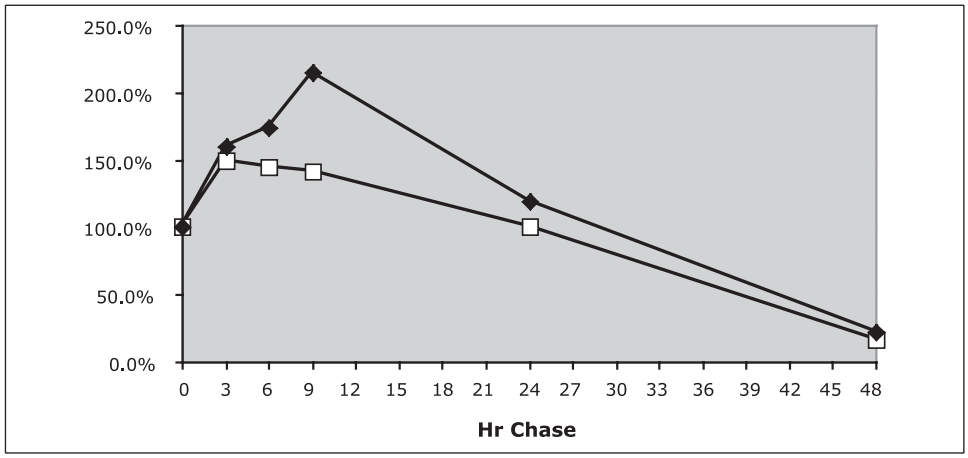

FIGURE 3. **Turnover rates of GD3 and 9AcGD3 from the cell surface.** CHO-K1 cells were incubated with 50 μM GD3 for 24 h. Medium was then removed, and fresh medium without GD3 was added. Samples were removed for R24 and 27A antibody staining at 0, 3, 6, 9, 24, and 48 h after GD3 removal and analyzed by flow cytometry, as described in the legend to Fig. 1. The MFI of GD3 and 9AcGD3 antibody staining was set as 100% at the time of GD3 removal. The relative change in MFI of GD3 over time is shown as *open squares*, and 9AcGD3 is as *solid diamonds*. GD3 incorporation continued for the first 3 h of chase, and then steadily declined over the next 48 h. 9-*O*-Acetylation continued to increase for the first 9 h but then declined similarly over the 48 h period. Note that the absolute MFI of GD3 was approximately three times greater than that of 9AcGD3, as seen in Fig. 1.

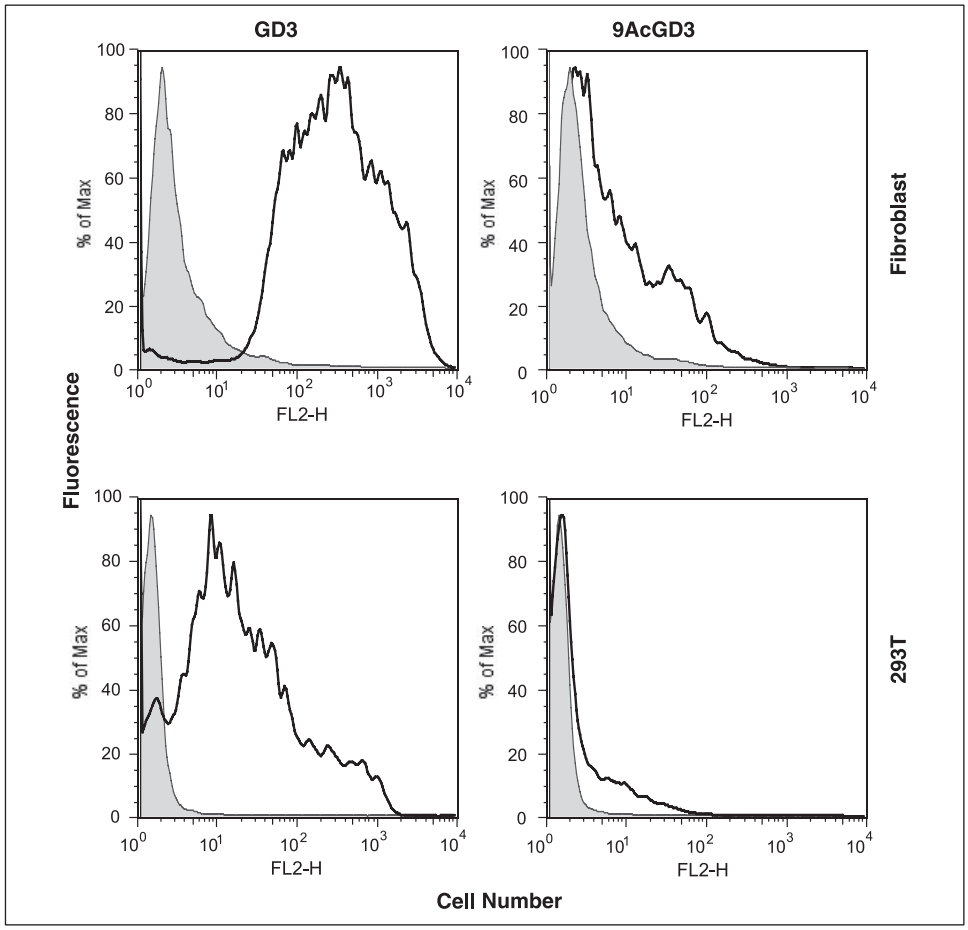

FIGURE 4. **Other cell lines are capable of 9-*O*-acetylating exogenously incorporated GD3.** Human skin fibroblasts (CCD919Sk) and 293T cells were analyzed by flow cytometry, as described in the legend to Fig. 1, and do not express detectable amount of GD3 and 9AcGD3 (shown as *shaded histograms*). After incubation with 50 μM GD3 for 18 h, cells were trypsinized and stained with R24 and 27A antibodies, and the results are shown as *black lines*. Similarly to CHO-K1 cells, both cell types can 9-*O*-acetylate exogenous GD3. However, the amount of 9-*O*-acetylation varies in different cell types.

slow incorporation of the remaining cell membrane-associated (trypsin-sensitive) GD3 into the membrane bilayer (trypsin-resistant). Following this initial increase, the amount of cell surface trypsin-resistant GD3 steadily declined. After a 30 h chase, the fluorescence intensity dropped by 50%, and very little GD3 remained on the cell surface after 48 h. On the other hand, total 9AcGD3 fluorescence intensity continued to increase for 12 h. However, after 12 h the level of 9AcGD3 decreased at a rate similar to GD3. This suggests that a continual presence of GD3 may be required to maintain 9-*O*-acetyltransferase activity. It is also possible that a negative feedback mechanism exists, to sustain an optimal ratio of GD3 to 9AcGD3. Otherwise, the remaining amount of GD3 should have continued to be 9-*O*-acetylated until it was all converted to 9AcGD3.

9-O-Acetylation of Exogenous GD3 in Other Cell Lines—In many instances where the GD3 ganglioside is found to be naturally expressed, there is already some level of expression of 9AcGD3. The current observation of 9-*O*-acetylation of exogenously added GD3 is thus likely to be mimicking this natural phenomenon. To rule out that this is strictly a CHO cell-specific phenomenon, we tested two other cell lines with very different properties that do not naturally express GD3 or 9AcGD3. Human skin fibroblast (CCD-919Sk) is a primary cell line and undergoes senescence with multiple cell culture passages, and 293T cells are

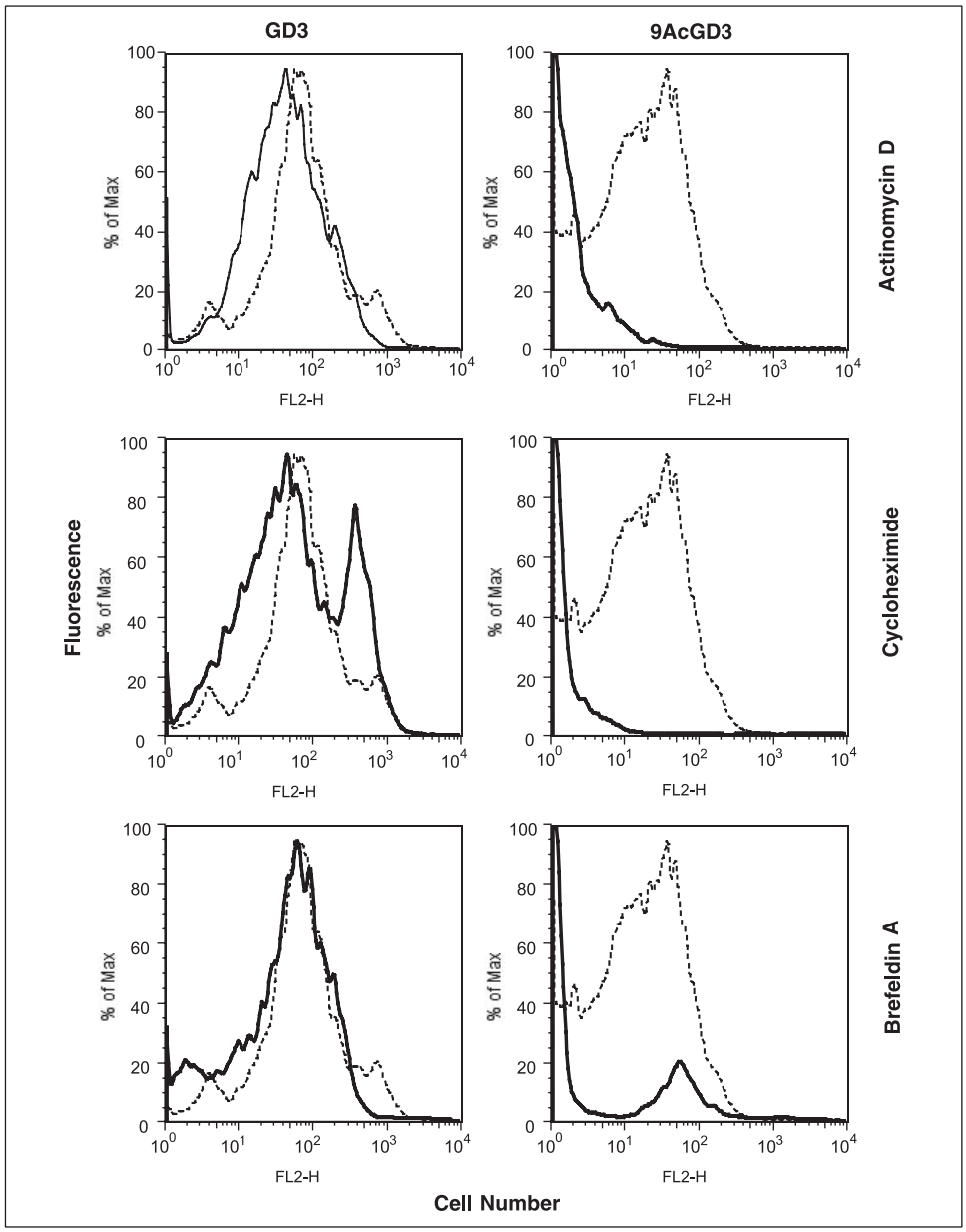

FIGURE 5. **9-*O*-acetylation of exogenous GD3 requires new synthesis of proteins in the ER, with delivery to the Golgi.** CHO-K1 cells were incubated with 50 μM GD3 along with actinomycin D, cycloheximide, or brefeldin A for 18 h and analyzed by flow cytometry, as described in the legend to Fig. 1. Inhibitor-treated cells (shown as *solid lines*) and sham-treated cells (shown as *dotted lines*) were stained with R24 and 27A antibody to detect cell surface GD3 and 9AcGD3. Actinomycin D and cycloheximide (inhibit transcription and translation, respectively) completely abolished 9-*O*-acetylation. Brefeldin A, which inhibits protein transport through the Golgi, also had a major impact on 9-*O*-acetylation. The inhibitors did not affect the amount of GD3 on the cell surface.

immortalized human kidney epithelial cells. These cells do not express detectable amounts of GD3 under typical cell culture conditions (see Fig. 4). Upon overnight incubation with GD3 in serum-free medium, both cell lines incorporated considerable levels of trypsin-resistant GD3 on the cell surface. Staining with 27A antibody revealed 9-*O*-acetylation of exogenous GD3, with CCD-919Sk cells expressing more than 293T cells. This indicates that 9-*O*-acetylation of exogenous GD3 occurs in more than one species and cell type and that the degree of 9-*O*-acetylation varies with the cell type.

9-O-Acetylation of GD3 Requires New Protein Synthesis—Based on our data up to this point, the GD3 9-*O*-acetyltransferase could either be constitutively expressed in CHO-K1 and other cell types (*i.e.* waiting for the appearance of the GD3 substrate) or be induced only when GD3 becomes available. There is unfortunately no reliable method to directly measure the 9-*O*-acetyltransferase enzyme activity using exogenous GD3 as a substrate. The enzyme activity is too labile after solubilization, and the efforts of many investigators have failed to isolate or clone sialic acid 9-*O*-acetyltransferases (30, 38, 39). Thus, we turned to other approaches to explore the phenomenon of *O*-acetylation of exogenously added GD3.

To determine whether new synthesis of proteins is required for the appearance of 9-*O*-acetylation, we inhibited protein production at the transcriptional, translational, or ER-Golgi transport levels. Actinomycin D binds to double stranded DNA and prevents RNA synthesis, cycloheximide inhibits protein synthesis by binding to the ribosome, and brefeldin A blocks membrane binding of ADP-ribosylation Factor 1, collapsing the *cis* and *medial* Golgi into the ER, and thereby blocking ER to Golgi transport. As shown in Fig. 5, both actinomycin D and cycloheximide completely abolished 9-*O*-acetylation induced by exogenous GD3 addition. Brefeldin A also exhibited a profound inhibitory effect. Because prior biochemical studies indicate that 9-*O*-acetyltransferases are localized in the late Golgi apparatus (40–42), we postulated that the addition of brefeldin A would prevent any newly synthesized 9-*O*-acetyltransferase molecules from reaching their natural localization. On the other hand, none of these inhibitors had any significant effect on the incorporation of GD3 into the plasma membrane. This data also eliminates the possibility that exogenous GD3

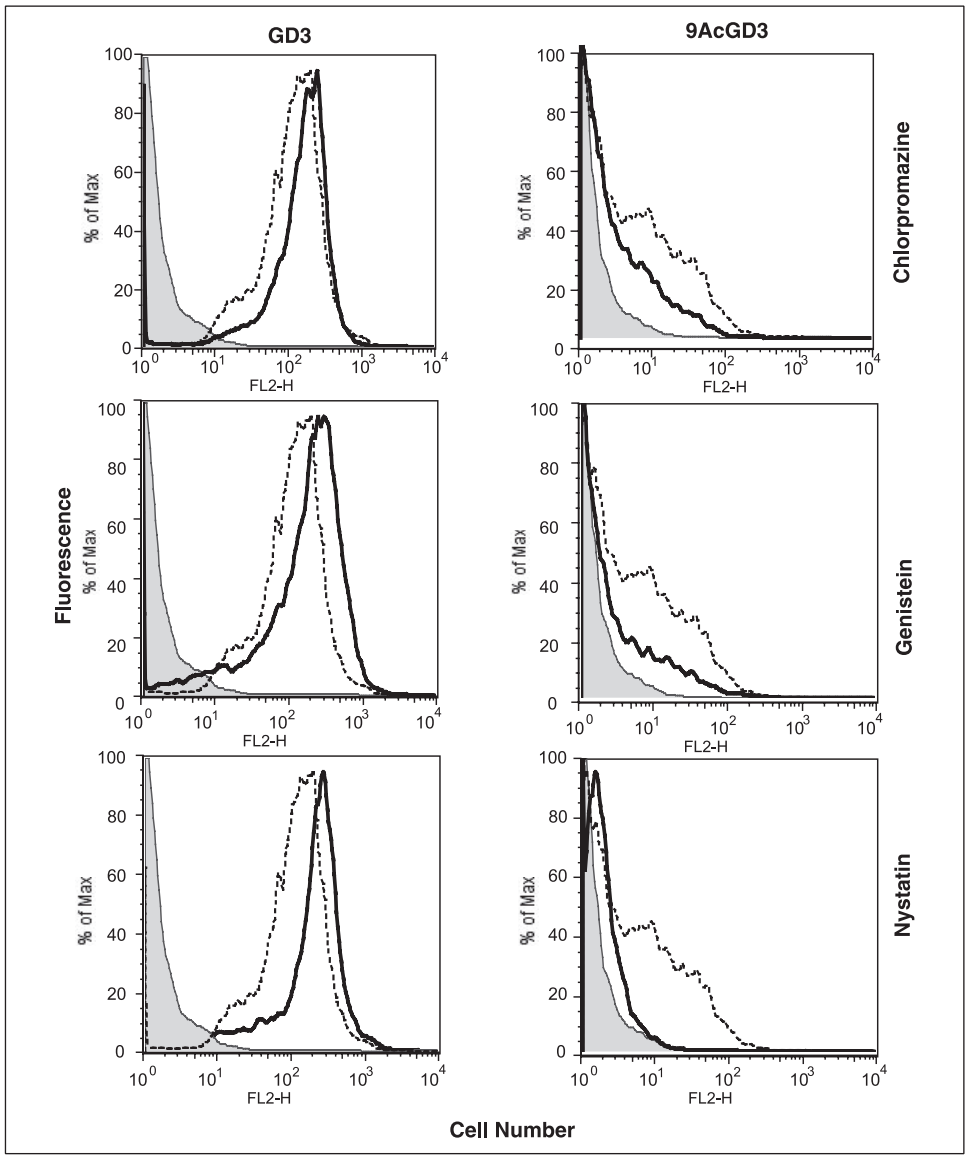

FIGURE 6. **Pretreatment of CHO cells with endocytosis inhibitors diminishes 9-*O*-acetylation of GD3.** CHO-K1 cells were pretreated with chlorpromazine, nystatin, and genistein for 2 h before 50 μM GD3 was added. After 18 h, cells were trypsinized, stained with R24 and 27A antibodies, and analyzed by flow cytometry, as described in the legend to Fig. 1. Sham-treated controls (no GD3 added) are shown as *shaded histograms*. *Dotted lines* depict cells treated with GD3 alone, and *solid lines* show cells treated with GD3 and various inhibitors. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, decreased 9-*O*-acetylation. Nystatin and genistein (inhibitors of clathrin-independent endocytosis), markedly reduced the capacity of the cells to 9-*O*-acetylate GD3. None of the inhibitors affected GD3 incorporation.

induces the expression of endogenous GD3 synthase molecule, which in turn synthesizes endogenous GD3 and 9AcGD3 from pre-existing GM3.

It is unclear why there is a doublet in the GD3 profile with cycloheximide treatment. This treatment can have additional effects by preventing the synthesis of various proteins, *e.g.* perhaps those involved in GD3 internalization. Regardless, the dramatic effects on blockade of *O*-acetylation are very clear, as is the interpretation of the overall result, especially when taken together with the actinomycin D and brefeldin A effects.

9-O-Acetylation of GD3 Is Decreased by Endocytosis Inhibitors—Cell surface gangliosides are typically found in microdomains and rafts (2, 43). No study has been done on the fate of plasma membrane-associated GD3. However, Sandhoff and colleagues reported that exogenously added radiolabeled GM2 (12) and biotinylated GM1 (13) are internalized and degraded in lysosomes. Using certain inhibitors, Pagano and colleagues (44) demonstrated that exogenously added BODIPY-labeled glucosylceramide is endocytosed primarily through clathrin-independent endocytosis. Although BODIPY-glycolipids are somewhat different from normal gangliosides (they can be removed from the plasma membrane by added albumin) we postulated that exogenous GD3 would also be endocytosed mainly through a clathrin-independent pathway. To determine whether 9-*O*-acetylation would be affected by endocytic processes, CHO cells were first pretreated with the same inhibitors, chlorpromazine, genistein, or nystatin. After pretreatment for 2 h, 50 μM GD3 was added into the medium and incubated overnight in the continued presence of inhibitors. Fig. 6 shows that the inhibitors had no adverse effect on the initial incorporation of GD3. In fact, GD3 levels were slightly higher, presumably because of inhibition of endocytosis and clearance from the surface. In contrast, 9AcGD3 expression decreased dramatically. Both chlorpromazine and genistein inhibited 9-*O*-acetylation by ~50%, and the addition of nystatin almost completely abolished it. These data suggest that induction of the 9-*O*-acetylation of GD3 relies on both clathrin-independent and -dependent pathways, with clathrin-independent endocytosis being dominant.

Of course, although these inhibitors are commonly used in standard endocytosis inhibition assays, they may have somehow affected exocytosis as well. If the 9-*O*-acetyltransferase were actually functioning on the cell surface, pretreating the cells with endocytosis inhibitors may have inadvertently hindered the transport of newly synthesized 9-*O*-acetyltransferase to the cell surface, resulting in the decreased 9-*O*-acetylation of GD3. The lack of a reliable method to detect the *O*-acetyltransferase makes it difficult to directly address this possibility.

Lack of Induction of the 9-O-Acetylation Machinery by Other Gangliosides—To ask whether the induction of the 9-*O*-acetylation machinery requires specific recognition of the GD3 molecule, we pre-incubated CHO-K1 cells with other gangliosides such as GM3 and GM1, following which GD3 was added. We reasoned that if other gangliosides could also turn on the 9-*O*-acetylation machinery in the same manner, the onset of 9-*O*-acetylation of subsequently added GD3 should be much more rapid. In fact we found that the kinetics of appearance remained unchanged, *i.e.* the 9-*O*-acetylation of added GD3 still took ~6 h (data not shown). These data indicate that the induction of the 9-*O*-acetylation machinery is not primarily based on addition of the ceramide tail of gangliosides, but rather, requires specific recognition of the glycan moiety of GD3.

DISCUSSION

The plasma membrane is a dynamic environment in which up to half of the membrane can be internalized via endocytosis each hour (45). Thus, membrane components such as gangliosides are constantly recycled, degraded, and replenished (and in some cases, recycled to the cell surface). The bulk of gangliosides that are internalized from the cell surface are delivered to endosomes and lysosomes, where they are degraded (12, 13). However, a small fraction of exogenously added gangliosides can be further glycosylated to make higher gangliosides (12, 16, 17), presumably in the endosomes or the *trans*-Golgi network. Studies using the fluorescent glycosphingolipid analog BODIPY-lactosylceramide demonstrated that these modified exogenous glycosphingolipids are rapidly internalized within minutes of incubation at 37 °C and quickly recycled back to the plasma membrane in ~20 min (15, 34). Golgi targeting of lactosylceramide can be seen following a 60-min incubation in the presence of bovine serum albumin (15).

Based on all of the above data, it is reasonable to suggest that if there is a pre-existing GD3 9-*O*-acetyltransferase located either on the cell surface, in the endosomal compartments or in the *trans*-Golgi network, 9-*O*-acetylation of GD3 should have been detected within the first few hours of GD3 incorporation. However, as shown in Fig. 2, significant amounts of 9AcGD3 were not detectable until 6 h of incubation with GD3. Furthermore 9-*O*-acetylation only occurs if GD3 is present for more than 6 h. If GD3 is removed after 3 h of incubation, no 9-*O*-acetylation is observed at later time points despite a significant amount of GD3 having already been incorporated (data not shown). This suggests that the constant presence of GD3 is needed to trigger 9-*O*-acetylation. Taken together, our data suggest that the accumulation of GD3 sends a cellular signal for new protein synthesis of a 9-*O*-acetyltransferase, or one of its critical components. Indeed, as shown in Fig. 5, inhibitors that blocked RNA synthesis, protein synthesis, or protein transport from the ER to the *trans*-Golgi network, all abolished 9-*O*-acetylation.

Studies with a preaddition of other gangliosides indicate that the induction of the 9-*O*-acetylation machinery requires specific recognition of the glycan moiety of GD3. It is likely that the GD3 9-*O*-acetyltransferase enzyme itself is being induced by GD3. Of course, we cannot exclude the possibility that an activator/inducer is synthesized to activate a pre-existing 9-*O*-acetyltranferase. An example of such post-transcriptional regulation of glycosyltransferases is the mammalian O-glycan core 1 β3-galactosyltransferase (C1β3Gal-T), which requires expression of a molecular chaperone Cosmc to assist the folding and stability of the C1β3Gal-T (46). Phosphorylation has also been postulated to be a post-transcriptional regulator of some sialyltransferase activities (47, 48).

Gangliosides are amphipathic molecules that cluster with cholesterol to form lipid rafts or microdomains (49). Unlike most cargos that utilize clathrin-pitted coats through the endocytic and late secretory pathways, the trafficking of membranes are thought to mostly utilize clathrin-independent endocytic pathways (50). We used inhibitors of both clathrin-dependent and -independent endocytosis to test their effects on 9-*O*-acetylation. All three inhibitors reduced 9-*O*-acetylation of GD3 (Fig. 5). A possible interpretation of this result is that the 9-*O*-acetyltransferase is in the Golgi and that blocking GD3 endocytosis resulted in the reduction of 9-*O*-acetylation. Another scenario could be that the 9-*O*-acetyltransferase is normally functional on the cell surface and was not transported to its destination because endocytosis inhibitors non-specifically affected exocytosis. Future studies are needed to address these possibilities, but they require reagents to detect the as yet undefined *O*-acetyltransferase.

Biochemical studies indicated that *O*-acetyltransferases responsible for 7- and 9-*O*-acetylation of α2–6-linked sialic acids on *N*-linked glycans are localized in the late Golgi (40, 41). Similarly, GD3 4-*O*-acetyltransferase activity has been found in isolated Golgi membranes of guinea pig liver (42). However, other studies have claimed to detect certain glycosylation activities on the plasma membrane. Preti *et al.* (51) have characterized a plasma membrane-anchored sialyltransferase that adds sialic acid to lactosylceramide and GM1 to make GM3 and GD1a. Sialidases have also been detected on the cell surface that remove sialic acids from gangliosides (11, 52, 53). Because sialic acids can be added and removed on the plasma membrane, it is conceivable that there exists a cell surface 9-*O*-acetyltransferase that modifies sialic acids. Our study does not rule out such a hypothesis, and the assay does not take into the possible interplay between 9-*O*-acetyltransferase and 9-*O*-acetylesterases, as well as possible intracellular pools of 9AcGD3 that were not detectable by cell surface antibody staining. Therefore, the ultimate proof would need first the isolation and cloning of the elusive 9-*O*-acetyltransferase.

The precise biological roles of GD3 9-*O*-acetylation also require further investigation. In the developing rat retina, GD3 is uniformly distributed, whereas 9AcGD3 appears in a gradient (54). In this regard, *in vivo* elimination of 9AcGD3 by transgenic expression of a viral 9-*O*-acetylesterase caused abnormalities in the retina and adrenal gland (55). Also, among human T cells, 9AcGD3 is specific for CD8+ cells (56). Thus 9AcGD3 may serve as a cellular marker or signal that is cell type-specific and developmentally regulated. The ratio of GD3 and 9AcGD3 also varies in different cell types, and usually only a fraction of GD3 is 9-*O*-acetylated.

The shedding of membrane gangliosides also occurs in normal and diseased states. Both human ovarian carcinoma cell line CABA1 and human malignant melanoma shed micelles containing GD3 (57, 58). Microglia can secrete GD3 upon exposure to inflammatory stimuli like lipopolysaccharide and inactivated bacteria, causing oligodendrocytes to undergo apoptosis (59). On the other hand, Malisan *et al.* (26) have shown that although GD3 induces apoptosis in HEK293 and U87 cells, 9-*O*-acetylation suppresses this proapoptotic activity of GD3. It is possible that cell types that are sensitive to exogenous GD3 are unable to induce the 9-*O*-acetyltransferase and are thus preprogrammed for GD3-mediated apoptosis.

In summary, we present evidence that exogenously added GD3 is capable of specifically inducing 9-*O*-acetylation of GD3 itself. This phenomenon can be seen in different cell types and is presumably closely mimicking the 9-*O*-acetylation of GD3 that occurs when cells newly express GD3 synthase, either under natural conditions, or following transfection. The mechanism involved requires the new transcription, translation, synthesis, and delivery of unknown proteins via the ER-

Golgi pathway. To our knowledge it is the first example of a ganglioside inducing new synthesis of an enzyme (or a regulator of an enzyme) that can modify the ganglioside itself.

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