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Kinetic and Spatial Interrelationships between Ganglioside Glycosyltransferases and *O*-Acetyltransferase(s) in Human Melanoma Cells*

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The melanoma-associated disialogangliosides 9(7)-*O*-acetyl-G_{D3} and 9(7)-*O*-acetyl-G_{D2} have been structurally well characterized. However, the compartmentalization and sequence of action of the biosynthetic activities responsible for synthesizing these molecules remain obscure. Here, we have studied the spatial and temporal interrelationships among the activities responsible for the later stages of ganglioside biosynthesis and those for *O*-acetylation in cultured human melanoma cells. First, brefeldin A treatment was used to separate biosynthetic steps into compartments distal or proximal to the transport block imposed by the drug. In keeping with prior reports, G_{M2}/G_{D2} synthase was consistently rendered inaccessible to its acceptors G_{M3} and G_{D3}. In contrast, the effect on G_{D3} biosynthesis was cell line-specific. Synthesis of G_{D3} was nearly abrogated in two lines, while it accumulated in a third line. This indicates that the spatial organization of ganglioside processing activities can vary even between similar cell lines. However, in all cell lines studied, the ratio of 9(7)-*O*-acetyl-G_{D3} to G_{D3} was not changed by brefeldin A, indicating that the majority of ganglioside *O*-acetyltransferase activity is co-localized with G_{D3} biosynthetic activity in the same Golgi subcompartment(s). As an alternative approach, Golgi-enriched fractions from melanoma cells were incubated with radiolabeled and nonlabeled nucleotide sugars or acetyl-CoA. In these preparations, biosynthesis is dependent upon the co-localization of appropriate sugar nucleotide transporters, glycosyltransferases, and acceptors that are endogenously present within intact topologically correct compartments. Incubations with CMP-Neu5Ac and acetyl-CoA corroborated the results with brefeldin A, co-localizing ganglioside *O*-acetyltransferase activity in compartments where G_{D3} biosynthesis takes place. Analyses with CMP-Neu5Ac and UDP-GalNAc showed that G_{D2} and G_{D3} synthesis occur in partially overlapping compartments. Labeling with acetyl-CoA and UDP-GalNAc indicated that although labeled acetate can be transferred from acetyl-CoA directly to G_{D2}, ganglioside *O*-acetyltransferase activity does not substantially overlap with the biosynthetic compartment(s) for G_{D2}. Instead, *O*-acetyl-G_{D3} appears to be co-localized with the compartment of G_{D2} biosynthesis and serves as an acceptor for G_{D2} synthase. Thus, both 9-*O*-acetyl-G_{D3} and G_{D2} can be precursors of 9-*O*-acetyl-G_{D2}, but apparently in distinct compartments.

Gangliosides are a structurally diverse sialylated subset of glycosphingolipids that are expressed predominantly on the outer leaflet of animal cell surface membranes (1–3). The biological roles of gangliosides have become increasingly appreciated, particularly in regard to intercellular adhesion (4–10), immune modulation (11–13), growth control and receptor function (14–20). The later stages of ganglioside biosynthesis occur by the action of highly specific sequentially interdependent glycosyltransferases (21–25). Akin to many other glycosylation pathways, the active sites of ganglioside glycosyltransferases are oriented toward the lumen of the Golgi apparatus, enabling them to transfer monosaccharides from activated sugar nucleotide donors to growing oligosaccharide acceptor chains (25–29). Cytosolic sugar nucleotide donors are transported into and concentrated within the Golgi lumen by specific sugar nucleotide transporters (29–31). The enzymes involved in the initial steps of ganglioside biosynthesis may be exceptions, with their active sites being oriented toward the cytosolic surface of the Golgi apparatus (32, 33). The later steps in ganglioside biosynthesis therefore require the co-localization of appropriate glycosyltransferases, sugar nucleotide transporters, and ganglioside acceptors within the same Golgi apparatus subcompartment. Throughout this paper we refer to ganglioside "biosynthetic activities" or "biosynthetic machinery," rather than to the enzymes themselves. This is purposely done to emphasize that the functional requirements for any given biosynthetic step include not only the relevant enzyme, but also the appropriate acceptor and the correct sugar nucleotide.

The sialic acids are a family of 9-carbon anionic monosaccharides found on various glycoconjugates. While the most common form of sialic acid is *N*-acetylneuraminic acid (Neu5Ac),[1] several different modifications give rise to over 30 sialic acid derivatives, adding considerable structural complexity to sialoglycoconjugates (34, 35). *O*-Acetylation of sialic acids on gangliosides has been the subject of particular inter-

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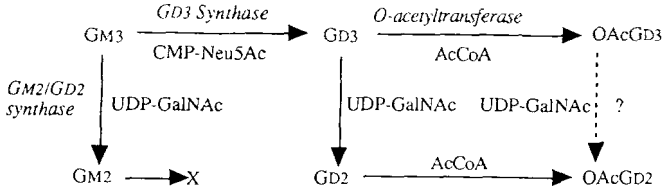
[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; Neu5,(7)9-Ac_2, 9(7)-*O*-acetyl-*N*-acetylneuraminic acid; mAb, monoclonal antibody; HPTLC, high performance thin layer chromatography; OAc, 7- or 9-*O*-acetyl group; Ac-CoA, acetyl-coenzyme A; NEM, *N*-ethylmaleimide; ELISA, enzyme-linked immunosorbent assay; BFA, brefeldin A. The various ganglio series gangliosides discussed in this study are designated according to Svennerholm (105). G_{T_x} refers to unidentified trisialoganglioside(s).



SCHEME I

est. Evidence to date indicates *O*-acetylation of melanoma-associated gangliosides occurs by the action of ganglioside *O*-acetyltransferase(s) using acetyl-coenzyme A (Ac-CoA) as a donor (36, 37). *O*-Acetyl esters are added to C7 and/or C9 hydroxyl groups on the glycerol-like side chain of a specific terminal α2–8-linked sialic acid residue (36, 37). A C7 *O*-acetyl ester can then migrate spontaneously to the C9 position under physiological conditions (38, 39). *O*-Acetylated gangliosides show remarkable tissue-specific and developmentally regulated expression patterns in tissues of neuroectodermal origin and are re-expressed as oncofetal antigens in certain malignancies (36, 40–48). Studies with transgenic mice have suggested that ganglioside *O*-acetylation may play a role in organizing developing neuroectodermal tissues (49). The structures of several *O*-acetylated gangliosides have been well characterized (36, 41, 43, 50–54), including 9(7)-$OAcG_{D3}$ (OAc-Siaα2–8Siaα2–3Galβ1–4Glcβ1–1′-ceramide) and 9(7)-$OAcG_{D2}$ (OAc-Siaα2–8Siaα2–3(GalNAcβ1–4)Galβ1–4Glcβ1–1′-ceramide) in melanoma cells. However, many details about their biosynthesis remain obscure, particularly regarding the compartmentalization and sequential action of the related biosynthetic activities. Elucidation of these issues is important because one potential control point for the regulated expression of ganglioside *O*-acetylation could be the organization of ganglioside biosynthesis within Golgi apparatus subcompartments. Here, we use brefeldin A (BFA) treatment and a biochemical "freeze frame" Golgi labeling reaction to explore the compartmentalization and sequential action of the G_{D3} and G_{M2}/G_{D2} ganglioside biosynthetic activities relative to ganglioside *O*-acetyltransferase(s) in human melanoma cells. Previously described pathways (23, 37, 55) of relevance to the current article (*solid arrows*) and that explored for the first time in this paper (*dotted arrows*) are illustrated above (Scheme I), along with the relevant nucleotide donors for each step.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from sources indicated: Silica Gel 60 high performance thin layer (HPTLC) plates, EM Science; *N*-ethylmaleimide (NEM), pepstatin, and leupetin, Sigma; and EN^{3}HANCE fluorography spray was from Du Pont-New England Nuclear. Diisopropyl fluorophosphate from Aldrich was stored as a 1 M stock solution in isopropyl alcohol and was used with the appropriate precautions. BFA was obtained from Epicenter Technologies and stored as 2 mg/ml stock solution in ethanol. HPLC solvents were of HPLC grade and all other chemicals were of reagent grade or better.

Monoclonal Antibodies—Monoclonal antibodies (mAbs) recognizing 9-*O*-acetyl-G_{D3} were kindly provided by Dr. Colin Barnstable, Yale University, School of Medicine (JONES), and Marilyn Farquhar, University of California, San Diego (27A). MAbs 14.G2A (recognizing G_{D2} and $OAcG_{D2}$) and MB3.6 (recognizing G_{D3}) were generous gifts from Dr. Ralph Reisfeld, Scripps Clinic and Research, La Jolla, CA.

Radioisotopes—[*acetyl*-^{3}H]Ac-CoA (2.3 Ci/mmol), CMP-[4-^{3}H]Neu5Ac (10 Ci/mmol), CMP-[9-^{3}H]Neu5Ac (20 Ci/mmol), and UDP-[6-^{3}H]GalNAc (6 Ci/mmol) were synthesized as described (56, 57). [4,5-^{3}H]Gal (15 Ci/mmol) was from American Radiolabeled Chemicals.

Cell Culture—The sources and propagation of the human melanoma cell lines Melur, M14, and M21, have been previously described (36, 37).

Analysis of Lipids—Total lipids from lyophilized cell pellets or vesicles from Golgi labeling reactions were extracted as described (37). Glass or aluminum backed Silica Gel 60 HPTLC plates were activated at 110 °C for 20 min. Desalted radiolabeled gangliosides were spotted at the origin and developed in chloroform, methanol, 0.02% $CaCl_2$ (60:40:9, v/v/v), and detected by spraying the HPTLC plates with EN^{3}HANCE (Du Pont-New England Nuclear), and fluorography using Kodak X-Omat AR film. Gangliosides were quantitated by cutting out bands of interest from aluminum-backed plates for scintillation counting or by densitometry of the fluorograms using an LKB Ultrascan XL laser densitometer. To examine *O*-acetylation, samples were prespotted onto HPTLC plates and exposed to ammonia vapors overnight in a sealed chamber as described (54). After sufficient aeration to remove ammonia vapors, an equivalent amount of fresh sample was spotted alongside the saponified one and the plate was immediately developed as described above.

DEAE-HPLC and Lipid ELISA—Total lipid extracts from metabolically labeled melanoma cells or Golgi-enriched fractions labeled with sugar nucleotides were fractionated over a TSK-DEAE-HPLC column as described (37). Fractions were monitored for reactivity to G_{D2}, $OAcG_{D3}$, and G_{D3} with mAbs 14.G2A, JONES, and MB3.6, respectively, by lipid ELISA as described (37).

Brefeldin A Treatment of Melanoma Cells—To 60% confluent cultures of M14, M21, or Melur cells, BFA (2 mg/ml ethanol) was added to a final concentration of 1 μg/ml. Preliminary dose-response experiments indicated this concentration to be optimal. An equivalent amount of the carrier ethanol (0.05% final) was added to control cultures. After 3 h, [4,5-^{3}H]Gal was added to 2 μCi/ml, and metabolic labeling continued for 21 h. At 24 h, viability was >98% in both control and BFA-treated cells as assessed by trypan blue dye exclusion. Cells were harvested by scraping into ice-cold phosphate-buffered saline and pelleted. Cell pellets were washed three times with ice-cold phosphate-buffered saline and lyophilized prior to lipid extraction. In experiments where recovery of ganglioside biosynthesis from BFA was being studied, four plates were studied in parallel: two of the plates were treated as described above, while a third was treated with BFA for 24 h followed by 3 washes with phosphate-buffered saline to remove BFA. After a 6-h incubation in the absence of BFA, 2 μCi/ml [4,5-^{3}H]Gal was added to the culture supernatant for 21 h, after which the cells were harvested. As a control for potential confluency induced alterations in ganglioside biosynthetic profiles, a fourth plate was treated similarly to plate three, but without BFA.

Isolation of Golgi-enriched Fractions from M21 Cells—Golgi-enriched fractions from M21 cells were isolated as described (36). Briefly, cells were trypsinized from 20 p150 plates, washed in phosphate-buffered saline, suspended in 0.25 M sucrose in 25 mM potassium phosphate buffer, pH 7.0 (SK buffer), and disrupted by nitrogen cavitation at an equilibration pressure of 1,000 p.s.i. (5 min/ml suspension). The homogenization buffer contained 1 μg/ml each of NEM, pepstatin, leupeptin, and 1 mM diisopropyl fluorophosphate to prevent proteolysis. The addition of NEM also prevents any potential vesicular transport by the inhibition of the NEM-sensitive factor (58), and diisopropyl fluorophosphate inhibits *O*-acetylesterases (59). After a low speed (500 rpm, 10 min) spin to remove unbroken cells and nuclei, the homogenate was adjusted to 1.2 M sucrose, placed in the bottom of a 35-ml ultracentrifuge tube, and overlaid with 10 ml of 1.1 M sucrose followed by 7 ml of 0.25 M sucrose. After centrifugation at 85,000 × *g* (Beckman SW-28 rotor) for 1.5 h, a "light" membrane fraction floated up and was removed from the 1.1–0.25 M interface. This was adjusted to 0.25 M sucrose as determined by refractometry, and pelleted at 100,000 × *g* for 30 min. The isolated light membranes were gently resuspended in an appropriate volume of SK buffer without protease inhibitors, and 400-μl aliquots were used immediately in labeling reactions. The light membranes (100–150 μg of protein per plate of starting cells) were judged to be enriched in Golgi fractions based on a 12–15-fold enrichment in GlcNAc:β1–4 galactosyltransferase activity.

Labeling of Golgi-enriched Fractions with Sugar Nucleotides and Acetyl-CoA—15 μCi of CMP-[4-^{3}H]Neu5Ac, UDP-[6-^{3}H]GalNAc, or [*acetyl*-^{3}H]acetyl-CoA were dried along with varying concentrations of any nonlabeled sugar nucleotides or Ac-CoA in 1.5-ml microcentrifuge tubes. 400-μl aliquots of Golgi-enriched fractions from M21 cells were added to these tubes and incubated at 24 °C for 20 min. Reactions were then transferred to ultracentrifuge tubes, diluted with 4 ml of ice-cold SK buffer, and pelleted at 100,000 × *g* for 30 min. The pellets were surface washed 3 times with ice-cold SK buffer, and

transferred to glass conical vials for lyophilization and lipid extraction. No difference was observed when samples were quenched with a 100-fold molar excess of unlabeled sugar nucleotide. When labeling with UDP-GalNAc, the addition of 1 mM $MnCl_2$ was necessary, because it was shown to be saturating for transfer of [^{3}H]GalNAc to endogenous ganglioside acceptors (data not shown).

Protection of Radiolabeled Melanoma Glycoproteins from Proteases—To determine if incorporated label is protected from proteolysis, 1.5-ml aliquots of Golgi-enriched fractions were labeled with CMP-[4-^{3}H]Neu5Ac or UDP-[6-^{3}H]GlcNAc plus 1 mM $MnCl_2$, then aliquoted into 3 × 400-μl aliquots for treatment with proteinase K, proteinase K plus 0.15% Triton X-100, or buffer alone for 60 min at 24 °C. The concentration of proteinase K used was 1:50 that of the Golgi-enriched protein concentration. Ice-cold perchloric acid was added to 8%, and the samples centrifuged at 15,000 rpm for 10 min in a microcentrifuge to pellet precipitable radioactivity. These conditions are specific for precipitation of proteins but not gangliosides. The pellets were solubilized in 0.2 M NaOH overnight, neutralized with 0.2 M HCl, removed into scintillation vials, and quantitated by scintillation counting.

Identification of O-Acetyl Esters in Radiolabeled Gangliosides—*O*-Acetylated forms of G_{D2} and G_{D3} were identified by comigration with authentic standards, susceptibility to base, susceptibility to influenza C esterase, and detection by specific mAbs. Samples were developed on HPTLC plates alongside authentic $OAcG_{D3}$ or $OAcG_{D2}$ isolated from melanoma cells, with and without prior treatment with ammonium hydroxide vapors as described above. One-half of the plate was impregnated with polyisobutylmethacrylate followed by immunooverlay as described (36, 60) with mAbs directed against 9-$OAcG_{D3}$ (JONES) or G_{D2} and $OAcG_{D2}$ (14.G2A) (36, 46). The other half was subjected to fluorography. Note that doublets shifting to the position of parent gangliosides after ammonia treatment cannot be lactone forms, since ammonia vapors convert lactones into amides, which will migrate between the intact lactone and the parent ganglioside (61). Furthermore, lactones themselves migrate more rapidly than their *O*-acetylated counterparts in this HPTLC solvent system.

RESULTS

Effects of Brefeldin A on Melanoma Ganglioside Biosynthesis Is Cell Line-specific—BFA inhibits anterograde but not retrograde vesicular transport between the Golgi apparatus and endoplasmic reticulum, at least partly by interferring with reversible binding of coat proteins to the cytoplasmic surface of the Golgi apparatus (62–66). In most cell types, this causes partial or complete fusion of the two compartments, allowing mixing of the normally segregated endoplasmic reticulum and Golgi macromolecules and enzymes access to one another (67, 68) and/or the production of Golgi remnants (69). In contrast to the Golgi apparatus itself, the *trans*-Golgi network is said to remain physically distinct during this treatment (66, 70). Others have previously demonstrated dramatic effects of BFA on ganglioside biosynthesis in cultured cell lines, noting a buildup of G_{M3} and G_{D3} with an abrogation of G_{M2}/G_{D2} biosynthesis, presumably by rendering the G_{M2}/G_{D2} synthase inaccessible to its precursors (71, 72). Since G_{D3} is an immediate precursor of $OAcG_{D3}$ (37), we reasoned that BFA should be useful in exploring the compartmentalization of the *O*-acetylation reaction relative to the other late acting ganglioside biosynthetic activities. We therefore examined the effect of BFA on ganglioside biosynthesis in three human melanoma cell lines, Melur, M14, and M21. The Melur cell line expresses G_{M3}, G_{D3}, and $OAcG_{D3}$ (37), while the M14 and M21 cell lines express in addition, G_{M2}, G_{D2}, and $OAcG_{D2}$ (36). Cells were pretreated with BFA for 3 h, followed by a 21-h pulse of [^{3}H]Gal in the continued presence of the drug. These conditions were chosen since shorter pulses resulted in an artificial skewing towards less complex glycolipids such as glucosylceramide and G_{M3} (data not shown). After the 24 h of BFA treatment, the cells appeared morphologically normal as judged by phase-contrast microscopy, and >98% were viable as judged by trypan blue dye exclusion. As illustrated by Fig. 1 and Table I, biosynthesis of G_{M2} and G_{D2} appeared to be virtually eliminated by BFA treatment in the two cell lines normally expressing these gangliosides. Further proof of the ganglioside products was obtained by DEAE-HPLC of total lipid extracts, monitoring the elution profile with specific mAbs directed against G_{D2}, G_{D3}, and $OAcG_{D3}$ (Fig. 2). These results confirm previous results from the groups of Young (71) and Sandhoff (72), and indicate that G_{M2}/G_{D2} biosynthesis occurs in a compartment distal to the BFA transport block. Surprisingly, BFA effects on G_{D3} biosynthesis were cell line-specific, with markedly decreased synthesis in the Melur and M14 cell lines *versus* a slight buildup in the M21 cell line. Since G_{D3} biosynthesis in the latter line was not inhibited, nonspecific effects of BFA on the G_{D3} biosynthetic machinery can be ruled out. Furthermore, removal of BFA from the melanoma cells resulted in a slow qualitative, if not quantitative recovery of ganglioside biosynthesis (data not shown), similar to that observed in previous BFA studies on ganglioside biosynthesis (71). Thus, even in very similar cell lines, spatial organization of the G_{D3} biosynthetic machinery along the Golgi apparatus may vary.

Ganglioside O-Acetyltransferase Cannot be Separated from the G_{D3} Biosynthetic Compartment by BFA Treatment—To examine if addition of *O*-acetyl groups to G_{D3} occurs proximal or distal to the transport block imposed by BFA, total lipid extracts from the experiments described above were fractionated by DEAE-HPLC, and the fractions containing $OAcG_{D3}$ and G_{D3} were pooled (Fig. 2). This HPLC fractionation is required prior to TLC analysis because $OAcG_{D2}$ co-migrates with G_{D3} on HPTLC plates (36). While BFA inhibition of G_{D3} biosynthesis in the Melur line was too extensive to allow analysis of the $OAcG_{D3}/G_{D3}$ ratio, this matter could be examined in the M14 and M21 cell lines. As shown in the example in Table II, the ratio of $OAcG_{D3}/G_{D3}$ was unaffected by BFA. This demonstrates that the addition of *O*-acetyl esters to G_{D3}, *i.e.* the functional compartment of ganglioside *O*-acetylation, occurs proximal to the transport block imposed by BFA, and may be present in the same subcompartment(s) as G_{D3} biosynthesis. Further evidence for the co-localization of ganglioside *O*-acetyltransferase and G_{D3} synthase is provided below.

Golgi-enriched Fractions Isolated from M21 Melanoma Cells Are Intact and of the Correct Topological Orientation—It is well accepted that oligosaccharide chain elongation on both glycoproteins and gangliosides occurs within the lumen of the Golgi apparatus through the action of specific glycosyltransferases (73). However, the appropriate acceptor and sugar nucleotide transporter must also be co-localized in the same subcompartment with the glycosyltransferase for a given biosynthetic step to occur. The passage of gangliosides through normal Golgi compartments is rapid and dynamic, making it difficult to dissect individual steps in the intact cell. We reasoned that intact Golgi-enriched fractions should contain endogenous ganglioside acceptors co-localized with the glycosyltransferases and sugar nucleotide transporters required for the subsequent step of biosynthesis. Furthermore, such intact fractions should facilitate normal transferase reactions by concentrating the sugar nucleotides into the compartments (74). In the absence of vesicular transport between compartments, incubation of such Golgi-enriched fractions with radiolabeled sugar nucleotides or Ac-CoA should therefore detect co-localization of the biosynthetic reactions of interest. While vesicular transport between Golgi compartments should not occur in the absence of added cytosolic components (75), NEM was also added to rule out this possibility (58). This type of "Golgi labeling reaction" (as it is termed here-

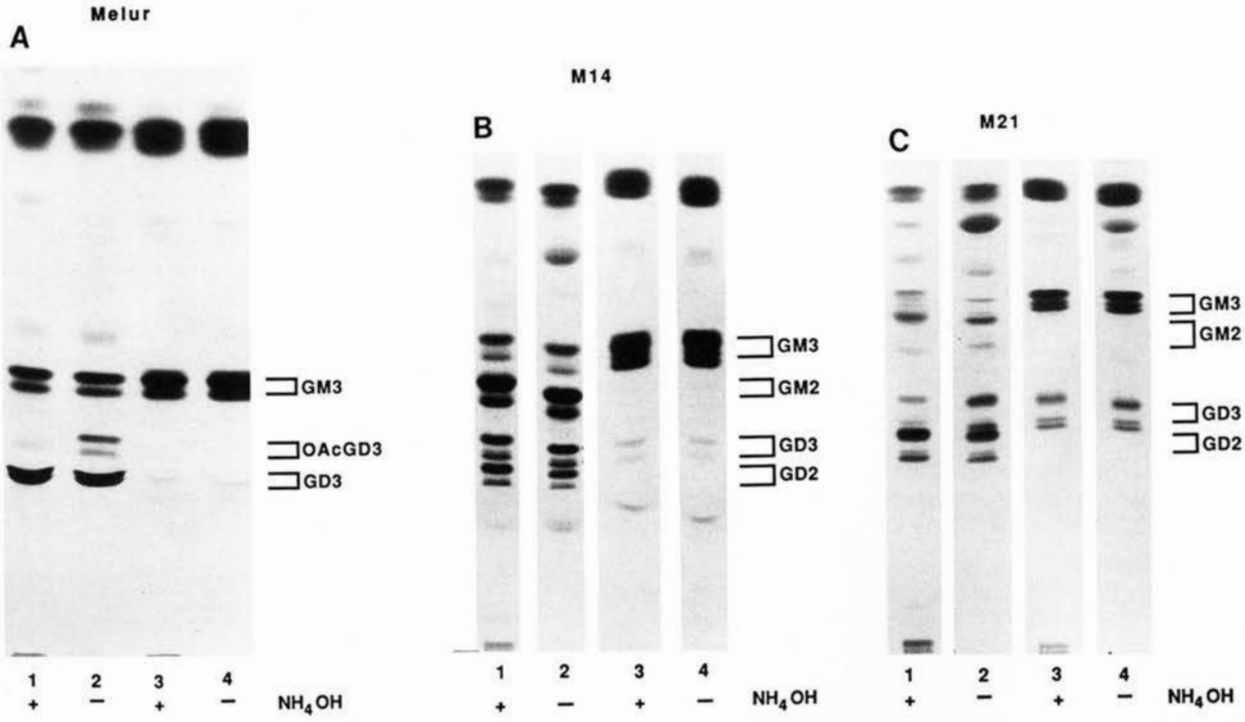


FIG. 1. **BFA-induced inhibition of ganglioside biosynthesis in melanoma cell lines.** Three human melanoma cell lines (Melur, M14, and M21) were treated with BFA (1 μg/ml) and labeled with [^{3}H]Gal as described under "Experimental Procedures." Total lipids were extracted and aliquots (~20,000 cpm/lane) analyzed by HPTLC. *Lanes 1* and *2*, control labeling; *lanes 3* and *4*, BFA-treated samples. In each case, *lanes 2* and *4* were treated with ammonium hydroxide vapors prior to development.

TABLE I

Effects of brefeldin A on the distribution of [^{3}H]Gal label in melanoma gangliosides

The relative amounts of gangliosides synthesized in the presence or absence of brefeldin A was quantitated from metabolically labeled total lipid extracts by TLC, fluorography, and densitometry (M21 and M14 cell lines) or by DEAE-HPLC (Melur cell line). Saponification is required prior to quantitation since $OAcG_{D2}$ migrates with G_{D3} on HPTLC plates.

Ganglioside	Cell line					
	M21		M14		Melur	
	Control	BFA	Control	BFA	Control	BFA
	%		%		%	
G_{M3}	12	70	20	95	36.5	97
G_{M2}	24		44			
G_{D3}	11	30	19	5	63.5	3
G_{D2}	53		17			

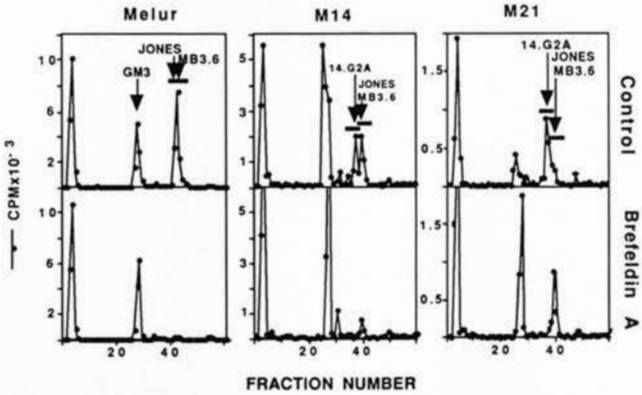


FIG. 2. **DEAE-HPLC fractionation of total lipid extracts from melanoma cells treated in the presence or absence of BFA.** Three human melanoma cell lines (Melur, M14, and M21) were treated with BFA and labeled as described in the legend to Fig. 1. Total lipid extracts were fractionated by DEAE-HPLC as described under "Experimental Procedures." The elution profile was followed by lipid ELISA with mAbs to G_{D3} (MB3.6), G_{D2} (14.G2A), and $OAcG_{D3}$ (JONES).

after) should represent a biochemical freeze frame picture of ganglioside biosynthesis at the time of cellular disruption. This type of analysis is particularly well suited for the co-localization of distinct glycosyltransferase activities within a particular Golgi subcompartment, as shown in the example in Fig. 3.

In order for the results obtained with this Golgi labeling reaction to be valid, it is critical that the compartments in question retain membrane integrity and correct topological orientation. The topology of ganglioside biosynthesis in the Golgi labeling reaction was examined by determining if the radiolabeled ganglioside products were protected from sialidase treatment after labeling with CMP-[4-^{3}H]Neu5Ac. Golgi fractions enriched 12–15-fold in the Golgi-specific marker β1–4 galactosyltransferase were isolated from M21 cells and incubated with 2 μM CMP-[4-^{3}H]Neu5Ac (note that the K_m of the CMP-Neu5Ac sugar nucleotide transporter is ~2–4 μM, which is 1–2 orders of magnitude below the K_m of many glycosyltransferases) (23, 74, 76–78). The radiolabeled gangliosides synthesized in these fractions were >99% protected from sialidases (data not shown). Although this result suggests intactness and correct topology, the conclusion could be misleading, since sialidases are known to require detergent for optimal activity towards ganglioside substrates (79, 80). Since glycolipid and glycoprotein biosynthesis occur in the same Golgi apparatus compartments, the topology of newly synthesized glycoproteins was therefore examined. The radioactivity incorporated into glycoproteins was subjected to proteinase K treatment with and without addition of detergent to disrupt membrane integrity. As illustrated in Fig. 4, *top*, >98% of the

TABLE II

Effect of brefeldin A on distribution of [^{3}H]Gal in $OAcG_{D3}$ and G_{D3} in melanoma cells

DEAE-HPLC fractions corresponding to G_{D3} and $OAcG_{D3}$ (but not G_{D2}) were pooled as indicated in Fig. 2, desalted, and analyzed by HPTLC with and without exposure to ammonia vapors prior to development of the chromatogram. Bands corresponding to $OAcG_{D3}$ and G_{D3} were detected by fluorography, and quantitated by scraping the bands into vials followed by scintillation counting.

Cell line	Conditions	$OAcG_{D3}$	G_{D3}	$OAcG_{D3}$	G_{D3}	$OAcG_{D3}$, G_{D3}
		cpm		%		
M21	Control	271	1236	18	82	0.219
	BFA	401	1416	22	78	0.283
M14	Control	160	753	17	83	0.212
	BFA	225	894	20	80	0.252

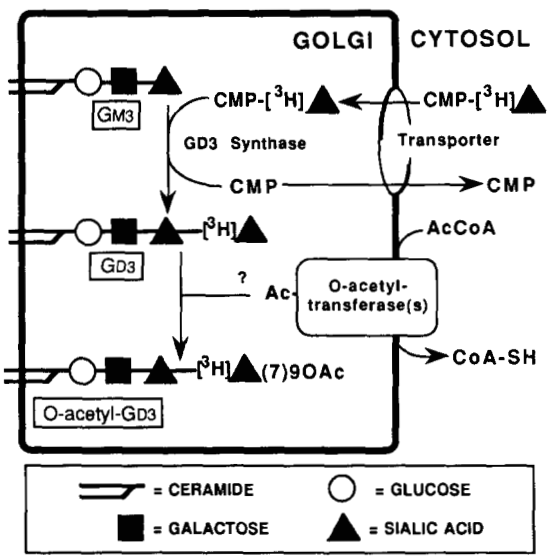


FIG. 3. **Schematic view of the components required for biosynthesis of G_{D3} and (7)9-*O*-acetyl-G_{D3} in a single intact Golgi compartment.** The donors are synthesized in the cytosol, whereas the acceptors and the transferases are oriented toward the lumen of the intact compartment. The acetate group from acetyl-coenzyme A is depicted as being transferred via an acetyl intermediate, such as previously reported in the rat liver Golgi apparatus (86). However, transport of Ac-CoA into the lumen has not been ruled out in this system.

perchloric acid-precipitable radioactivity was protected from this treatment, decreasing to 20% upon disruption of the membranes with detergent. This confirms the intactness and correct topology of the compartments being labeled.

Sialic acid addition to *N*-linked glycoproteins is thought to occur mainly in the *trans*-Golgi cisternae and *trans*-Golgi network (81, 82), compartments which "straddle" the transport block of BFA (83). Since the current experiments with BFA indicate that *O*-acetylation of G_{D3} might occur in compartments proximal to the *trans*-Golgi network, it was also necessary to examine the intactness and correct topology of earlier Golgi subcompartments. Since at least part of the addition of GlcNAc to *N*-linked glycoproteins is thought to be an early biosynthetic event taking place in the *medial*-Golgi (84, 85), we carried out analogous experiments with Golgi-enriched fractions labeled by UDP-[^{3}H]GlcNAc. Fig. 4, *bottom*, shows that >95% of the perchloric acid-precipitable radioactivity in this case is also protected from proteinase K digestion, decreasing to 9% upon detergent addition.

Further evidence for the maintenance of membrane topology and integrity was provided by analyzing ganglioside products from a Golgi-enriched fraction incubated with CMP-[4-

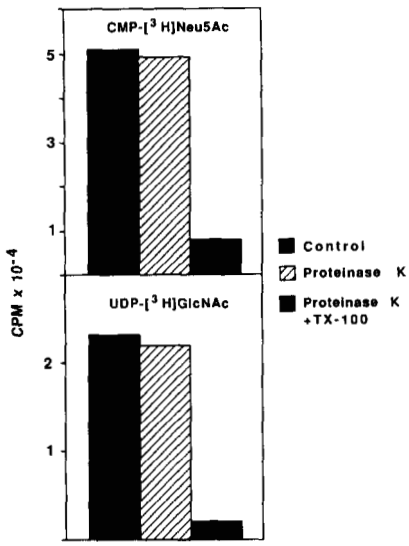


FIG. 4. **Protection from proteolysis of the radiolabeled glycoproteins generated in the Golgi labeling reaction.** Aliquots (1200 μl) of freshly isolated M21 Golgi-enriched fractions were labeled with UDP-[^{3}H]GlcNAc or CMP-[^{3}H]Neu5Ac as described under "Experimental Procedures." Three equivalent aliquots were removed and treated with or without proteinase K (1:50 protease:endogenous protein) or Triton X-100 (0.15% final) as indicated. After incubation at room temperature for 30 min, perchloric acid was added to 8% (v/v), and the precipitated radioactivity quantitated by scintillation counting.

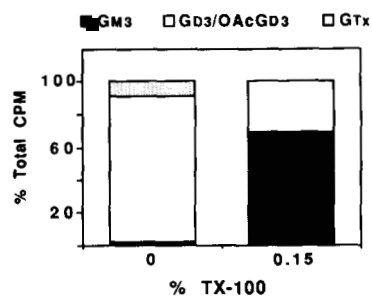


FIG. 5. **Effects of detergent on the incorporation of radiolabel into gangliosides in Golgi-enriched fractions.** Freshly prepared Golgi-enriched fractions from M21 cells were labeled with CMP-[4-^{3}H]Neu5Ac in the presence or absence of 0.15% Triton X-100 (*TX-100*). Lipids were extracted by addition of chloroform:methanol, 1:1 (v/v), to chloroform:methanol:H_2O, 10:10:1 (v/v) final. The extracts were dried, desalted by Sep-Pak, and analyzed by DEAE-HPLC as described under "Experimental Procedures." Peaks corresponding to G_{M3}, G_{D3}/$OAcG_{D3}$, and G_{Tx} were quantitated directly by scintillation counting. Since G_{D3} and $OAcG_{D3}$ co-elute from the DEAE column they were quantitated together.

^{3}H]Neu5Ac in the absence or presence of 0.15% Triton X-100 (a concentration typically used to assay ganglioside glycosyltransferases (23, 78)). As shown in Fig. 5, in the absence of detergent, the majority of [^{3}H]Neu5Ac is incorporated into G_{D3} (70%) and unidentified trisialoganglioside(s) (G_{Tx}) (26%), while only 4% of the label is incorporated into G_{M3}. If Triton X-100 is added prior to labeling, the ratio of [^{3}H]Neu5Ac incorporated into the three gangliosides changes dramatically, with G_{M3} representing 80% of the incorporated label, G_{D3} only 20%, and G_{Tx} becoming undetectable. All ganglioside biosynthetic enzymes should be markedly activated by detergents provided that adequate acceptors are available. Since total incorporation of [^{3}H]Neu5Ac into gangliosides was not very different in the presence or absence of detergent, the dramatic decrease in G_{D3} and G_{Tx} synthesis with detergent indicates that the enzymes have been separated from their normal

proximity to endogenous acceptors in the intact compartments, where sugar nucleotides can be concentrated. In contrast, the marked increase in G_{M3} synthesis may be explained by the exposure of a large latent or cryptic pool of lactosyl ceramide co-purifying with the Golgi-enriched fractions, which gains access to G_{M3} synthase only when membrane integrity is compromised. These results also provide indirect evidence that biosynthesis of G_{D3} and G_{Tx} in the Golgi labeling reaction requires concentration of sugar nucleotide within the lumen of the isolated Golgi compartments by the nucleotide transporter. Taken together, the experiments presented in Figs. 4 and 5 provide good evidence for the maintenance of membrane integrity and correct topological orientation in the Golgi labeling reactions described below. Thus, the reactions studied with the labeled sugar nucleotides should reflect the normal compartmentalization of the Golgi apparatus in the intact cell.

O-Acetyltransferase Activity Is Co-localized in Golgi Subcompartments with G_{D3} Biosynthesis—As illustrated above, treatment with BFA indicates that G_{D3} biosynthesis and *O*-acetylation of G_{D3} may occur within the same Golgi subcompartment(s). An alternative explanation is that *O*-acetylation of sialic acid actually takes place at the sugar nucleotide level. CMP-Neu5,(7)9-Ac_2 could then act as a donor for synthesis of $OAcG_{D3}$ from G_{M3}, by the action of G_{D3} synthase. To address this possibility, M21 Golgi-enriched preparations were labeled with CMP-[4-^{3}H]Neu5Ac in the presence or absence of 20 μM unlabeled Ac-CoA. Sugar nucleotide pools from 90% ethanol supernatants of washed pelleted vesicles were purified by descending paper chromatography. Free sialic acids were released from the sugar nucleotides by mild acid hydrolysis, purified, and analyzed by HPLC. Under these conditions of analysis, *O*-acetyl esters should be stable (57). However, even in the presence of added Ac-CoA, CMP-Neu5,(7)9-Ac_2 was undetectable (<2%, data not shown). Thus, it is highly unlikely that *O*-acetylation occurs at the sugar nucleotide level. Rather acetate groups appear to be added directly to endogenous acceptor G_{D3} molecules.

To provide direct evidence for the co-localization of G_{D3} biosynthesis and G_{D3} *O*-acetylation, Golgi-enriched fractions from M21 cells were labeled with CMP-[4-^{3}H]Neu5Ac in the presence and absence of nonlabeled Ac-CoA, and the radiolabeled ganglioside products were examined. If *O*-acetyltransferase and G_{D3} biosynthesis occur in the same Golgi subcompartment(s), an *O*-acetyl ester could be added to radiolabeled G_{D3}, forming $OAcG_{D3}$ (see Fig. 3). Fig. 6 and Table III illustrate the results of this experiment. Remarkably, $OAcG_{D3}$ represents approximately 10% of newly synthesized [^{3}H]Neu5Ac-labeled G_{D3}, even without exogenously added Ac-CoA (*i.e.* an endogenously available *O*-acetyl group is being added directly to the newly incorporated sialic acid residue). Upon addition of 20 μM Ac-CoA, the amount of $OAcG_{D3}$ increases about 2-fold, to 20–25% of the newly synthesized G_{D3}. Addition of 200 μM Ac-CoA increased this level very modestly, indicating that the ganglioside *O*-acetyltransferase reaction is close to saturation at 20 μM Ac-CoA. A possible explanation for synthesis of $OAcG_{D3}$, even in the absence of exogenous Ac-CoA, is that the melanoma-associated ganglioside *O*-acetyltransferase functions through an acetyl intermediate analogous to the sialate:*O*-acetyltransferase previously described by us in rat liver (86). Thus, a subset of *O*-acetyltransferase molecules may have been occupied with pre-existing acetyl donors derived from cytosolic Ac-CoA at the time of homogenization. These data also indicate that the G_{M3} acceptor is co-localized with G_{D3} synthase, CMP-Neu5Ac sugar nucleotide transporter, and at least a portion of ganglioside *O*-acetyltransferase biosynthetic activity.

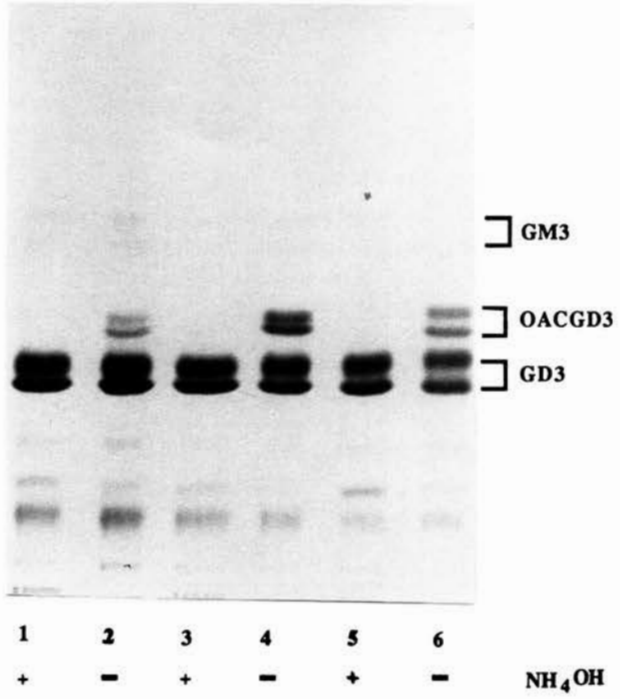


FIG. 6. **Effects of acetyl-CoA on the labeling of Golgi-enriched fractions from M21 cells with CMP-[4-^{3}H]Neu5Ac.** Aliquots of freshly prepared Golgi-enriched fractions from M21 were labeled with CMP-[4-^{3}H]Neu5Ac with or without added acetyl-CoA. Total lipids were extracted, divided into two equal aliquots, loaded alongside one another on an aluminum-backed HPTLC plate, and developed. After development and fluorography, bands corresponding to G_{D3} and $OAcG_{D3}$ were cut from the plates and quantitated by scintillation counting (see Table III). *Lanes 1* and *2*, no acetyl-CoA; *lanes 3* and *4*, 20 μM acetyl-CoA; *lanes 5* and *6*, 200 μM acetyl-CoA. As indicated, *lanes 1, 3,* and *5* were treated with ammonium hydroxide vapors prior to development of the TLC to saponify *O*-acetyl esters.

TABLE III

Incorporation of label from CMP-[4-^{3}H]Neu5Ac into G_{D3} and $OAcG_{D3}$ when Golgi-enriched preparations are labeled in the presence or absence of acetyl-CoA

Golgi-enriched preparations from M2 cells were labeled with CMP-[4-^{3}H]Neu5Ac with or without exogenously added acetyl-CoA. Equivalent aliquots were labeled, lipids extracted and analyzed by HPTLC. After fluorography (see Fig. 5) the bands corresponding to G_{D3} and $OAcG_{D3}$ were cut out from the plate and quantitated by scintillation counting.

Ac-CoA	$OAcG_{D3}$	G_{D3}	$OAcG_{D3}$	G_{D3}	$OAcG_{D3}$, G_{D3}
	cpm		%		
None	365	2464	13	87	0.15
20 μM	576	1794	24	76	0.32
200 μM	399	902	31	69	0.44

We have previously labeled similar Golgi-enriched fractions from M21 cells with [*acetyl*-^{3}H]Ac-CoA and demonstrated the biosynthesis of both *O*-[*acetyl*-^{3}H]acetyl-G_{D3} and *O*-[*acetyl*-^{3}H]acetyl-G_{D2} (36, 37). Additional evidence for co-localization of *O*-acetyltransferase and G_{D3} biosynthetic machinery could therefore be provided by labeling with [*acetyl*-^{3}H]Ac-CoA in the presence and absence of unlabeled CMP-Neu5Ac. If the two biosynthetic activities are co-localized, the relative amount of *O*-[*acetyl*-^{3}H]G_{D3} synthesized in these preparations should increase when unlabeled CMP-Neu5Ac is added. As shown in Fig. 7, addition of 10 μM CMP-Neu5Ac gave a 2-fold increase in the amount of *O*-[*acetyl*-^{3}H]acetyl-G_{D3}, both in absolute counts and relative to *O*-[*acetyl*-^{3}H]acetyl-G_{D2}.

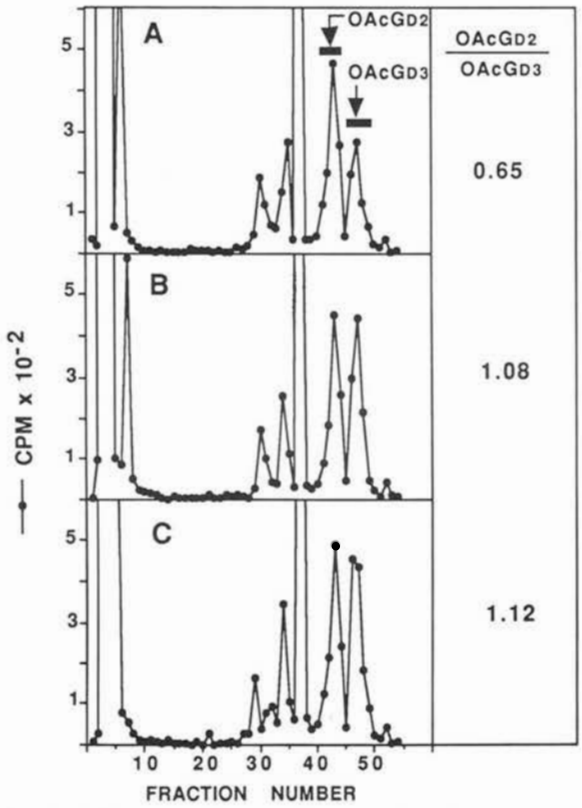


FIG. 7. **Effects of CMP-Neu5Ac on the labeling of Golgi-enriched fractions from M21 cells with [*acetyl*-^{3}H]acetyl-CoA.** Aliquots of freshly prepared M21 Golgi-enriched fractions were labeled with [*acetyl*-^{3}H]acetyl-CoA in the presence or absence of CMP-Neu5Ac. Total lipids were extracted and fractionated by DEAE-HPLC. Fractions (1 ml) were collected and 50 μl of each studied by lipid ELISA using mAbs JONES ($OAcG_{D3}$) and 14.G2A ($G_{D2}/OAcG_{D2}$). The *bars* indicate all mAb-reactive fractions, and peak elution positions are indicated by *arrows*. Fractions corresponding to $OAcG_{D3}$ and $OAcG_{D2}$ were confirmed by HPTLC analysis (data not shown). *Panel A*, no CMP-Neu5Ac; *panel B*, 10 μM CMP-Neu5Ac; *panel C*, 100 μM CMP-Neu5Ac.

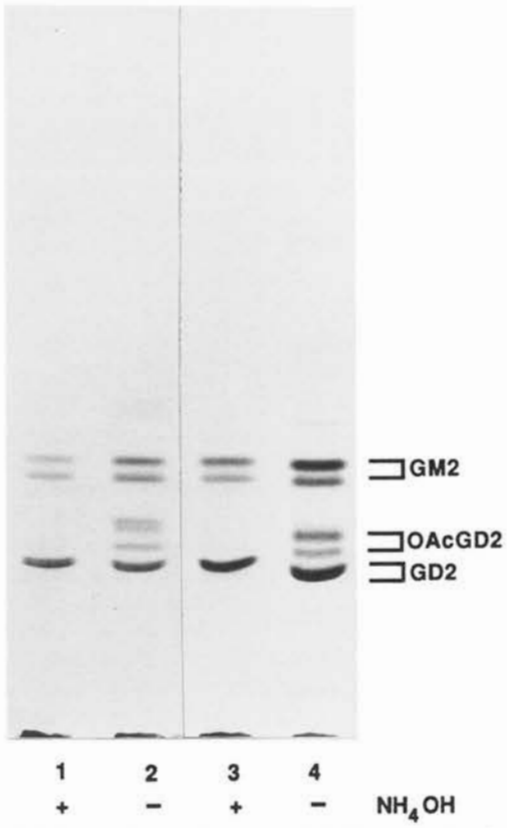


FIG. 8. **Effect of acetyl-CoA on labeling of M21 Golgi-enriched fractions with UDP-[6-^{3}H]GalNAc.** Aliquots of freshly prepared Golgi-enriched fractions from M21 cells were labeled with UDP-[6-^{3}H]GalNAc in the presence or absence of acetyl-CoA. Total lipids were extracted, fractionated by HPTLC plate, and subjected to fluorography. Bands corresponding to G_{M2}, G_{D2}, and $OAcG_{D2}$ were scraped from plates and quantitated by scintillation counting (see Table IV). *Lanes 1* and *2*, no acetyl-CoA; *lanes 3* and *4*, 20 μM acetyl-CoA. *Lanes 1* and *3* were treated with ammonium hydroxide vapors prior to development as indicated.

Addition of 100 μM CMP-Neu5Ac provided no further increase, indicating that the process is saturated at 10 μM CMP-Neu5Ac. In this regard, it should be noted that the K_m of G_{D3} synthase for CMP-Neu5Ac is approximately 200 μM (23, 78), while that of the CMP-Neu5Ac transporter is ~2–4 μM (74, 76). This indicates that transport and concentration of CMP-Neu5Ac into the lumen of intact topologically correct compartments is rate-limiting for the reaction. Alternatively, it is possible that all endogenous G_{M3} acceptors were expended upon addition of 10 μM CMP-Neu5Ac. Taken together with the BFA experiments described above, these results provide evidence for the co-localization of G_{M3} acceptor, G_{D3} synthase, CMP-Neu5Ac sugar nucleotide transporter, and ganglioside *O*-acetyltransferase within the same Golgi subcompartment(s).

O-Acetyl-G_{D3} Acts as an Endogenous Acceptor for G_{D2} Synthase in Intact Golgi-enriched Fractions—The M21 cell line is known to express 9(7)-$OAcG_{D2}$ as well as 9(7)-$OAcG_{D3}$ (36). Since G_{D3} is the direct precursor of G_{D2} (23), it is possible that $OAcG_{D3}$ is a precursor of $OAcG_{D2}$. To address this, M21 Golgi-enriched fractions were labeled with UDP-[^{3}H]GalNAc. As illustrated by Fig. 8 (*lanes 1* and *2*), ganglioside products formed in the UDP-[^{3}H]GalNAc Golgi labeling reaction co-migrate with G_{M2}, G_{D2}, and $OAcG_{D2}$. Treatment of the total lipid extract with ammonia vapors prior to TLC development eliminates the base-labile doublet co-migrating with authentic $OAcG_{D2}$, supporting the identity of the labeled $OAcG_{D2}$. These products were further analyzed by DEAE-HPLC, monitoring the elution profile of tritiated products by specific mAbs as described above. Each radioactive peak was re-analyzed by HPTLC alongside standard $OAcG_{D2}$, and by HPTLC immuno-overlay with mAbs 14.G2A and JONES as described under "Experimental Procedures." These data confirmed the identity of the disialoganglioside products G_{D2} and $OAcG_{D2}$. As quantitated in Table IV, newly synthesized G_{D2} and $OAcG_{D2}$ are formed in approximately equal proportions. In fact, the amount of $OAcG_{D2}$ synthesized ranged from 35 to 50% in various preparations.

TABLE IV

Distribution of radiolabel in gangliosides from M21 Golgi preparations labeled with UDP-[^{3}H]GalNAc in the presence and absence of Ac-CoA

Golgi-enriched preparations from M21 cells were labeled with UDP-[6-^{3}H]GalNAc in the presence or absence of Ac-CoA. Gangliosides were separated by HPTLC, sprayed with EN^{3}HANCE, and exposed to film. The fluorogram illustrated in Fig. 8 was quantitated by densitometry as described under "Experimental Procedures."

Ganglioside	No Ac-CoA	20 μM Ac-CoA
	%	
G_{D2}	27	28
OAc-G_{D2}	32	32
G_{M2}	41	40

It should be noted that no exogenous addition of acetyl-CoA is required for this high level of $OAcG_{D2}$ synthesis to occur. As described below, other evidence indicates that ganglioside *O*-acetyltransferase does not overlap G_{D2} biosynthetic compartments. Thus, it is improbable that *O*-acetylation of *newly synthesized* G_{D2} occurs through endogenous acetyl intermediates, as appears to be the case for *O*-acetylation of G_{D3}. Instead, the best explanation is that $OAcG_{D3}$ is co-localized within the compartment for G_{M2}/G_{D2} biosynthesis and acts as an efficient acceptor for G_{M2}/G_{D2} synthase (or possibly a distinct *N*-acetylgalactosaminyl transferase).

While G_{D2} Is an Acceptor for Ganglioside O-Acetyltransferase, this Activity Does Not Overlap with G_{D2} Biosynthetic Compartment(s)—To determine if ganglioside *O*-acetyltransferase activity is co-localized with G_{D2} biosynthetic machinery in the same Golgi subcompartment, Golgi-enriched fractions were labeled with UDP-[^{3}H]GalNAc in the presence and absence of exogenous acetyl-CoA. As shown in Fig. 8 and Table IV, the amount of $OAcG_{D2}$ relative to G_{D2} is not increased when 20 μM Ac-CoA is added to the Golgi labeling reaction (200 μM Ac-CoA was also without effect, data not shown). This indicates that G_{D2} biosynthetic activity and ganglioside *O*-acetyltransferase may not overlap. Corroborating evidence for this conclusion was obtained by labeling Golgi-enriched fractions with [*acetyl*-^{3}H]Ac-CoA in the presence or absence of manganese (required for G_{D2} synthase) and with increasing concentrations of UDP-GalNAc. If ganglioside *O*-acetyltransferase is co-localized with G_{D2} biosynthetic machinery, addition of UDP-GalNAc and managanese should increase the amount of $OAcG_{D2}$ formed relative to $OAcG_{D3}$. However, as shown in the example in Table V, the amount of $OAcG_{D2}$ synthesized remains constant even when the Golgi labeling reaction is carried out in the presence of 200 μM UDP-GalNAc. Thus, although an endogenous pool of G_{D2} is known to be a substrate for ganglioside *O*-acetyltransferase when labeling with [*acetyl*-^{3}H]Ac-CoA (36), the ganglioside *O*-acetyltransferase does not seem to be co-localized with the compartments for G_{D2} biosynthesis. One possibility is that the *O*-acetyltransferase that *O*-acetylates G_{D3} (which appears to be in early compartments) may be distinct from the *O*-acetyltransferase that can *O*-acetylate G_{D2} (presumably in a very distal Golgi compartment). Another possibility is that the M21 Golgi-enriched preparation contains other organelles such as endosomes in which endogenous G_{D2} acceptor and ganglioside *O*-acetyltransferase are co-localized, but in which sugar nucleotide transporters and/or G_{D3} acceptors are absent.

Biosynthesis of G_{D3} and G_{D2} Occur in Partially Overlapping Compartments—Recent observations suggest that several oligosaccharide biosynthetic steps can occur within a single Golgi cisternae (87, 88).[2] To determine if G_{D3} and G_{D2} biosynthetic activities overlap in melanoma cells, M21 Golgi-enriched fractions were labeled with CMP-[4-^{3}H]Neu5Ac in the presence or absence of UDP-GalNAc, and conversely with UDP-[^{3}H]GalNAc in the presence or absence of unlabeled CMP-Neu5Ac. A shown in Fig. 9*A*, the synthesis of G_{D2} is not observed when the Golgi-enriched fractions are labeled with CMP-[^{3}H]Neu5Ac alone. This is not surprising since G_{M2} is not a substrate for G_{D3} synthase (77, 78). However, upon addition of 10 μM UDP-GalNAc, the formation of G_{D2} is observed in a ratio of G_{D2}/G_{D3} of 0.12 (Fig. 9*B*). These results indicate that compartments exist in which G_{M3} is accessible to both G_{D3} and G_{D2} biosynthetic activities. Thus, [^{3}H]Neu5Ac is added to endogenous G_{M3} forming [^{3}H]G_{D3}, a portion of which can form [^{3}H]G_{D2} if UDP-GalNAc is provided. Upon addition of 0.15% Triton X-100 to the labeling reaction, this biosynthesis of G_{D2} is abrogated. This indicates that transfer of unlabeled GalNAc to radiolabeled G_{D3} occurs within the lumen of intact compartments, and that disruption of vesicle integrity may prevent the concentration of UDP-GalNAc required for biosynthesis of radiolabeled G_{D2}.

In the converse experiment, Golgi-enriched fractions were also labeled with UDP-[^{3}H]GalNAc in the presence or absence of CMP-Neu5Ac. Fig. 10 clearly demonstrates a increase in the ratio of G_{D2} to G_{M2} from 1.4 to 5.2 with the addition of 10 μM CMP-Neu5Ac. This ratio does not increase further upon

TABLE V

Effects of UDP-GalNAc and manganese on incorporation of label from [^{3}H]acetyl-CoA into $OAcG_{D3}$ and $OAcG_{D2}$

Golgi-enriched preparations from M21 cells were labeled with [^{3}H] acetyl-CoA in the presence or absence of 1 mM $MnCl_2$ with different concentrations of UDP-GalNAc. Lipids were extracted and fractionated by DEAE-HPLC, monitoring 10% of each fraction eluted by lipid ELISA with mAbs directed against G_{D2}, $OAcG_{D2}$, and $OAcG_{D3}$. Fractions were dried to remove chloroform and 90% of each was quantitated by scintillation counting. Fractions corresponding to $OAcG_{D2}$ and $OAcG_{D3}$ as indicated by lipid ELISA are quantitated above.

	$OAcG_{D2}$	$OAcG_{D3}$	$OAcG_{D2}/OAcG_{D3}$
	cpm		
[^{3}H]Ac-CoA	309	521	0.59
[^{3}H]Ac-CoA + Mn	346	542	0.64
[^{3}H]Ac-CoA + Mn + 20 μM UDP-GalNAc	251	606	0.41
[^{3}H]Ac-CoA + Mn + 200 μM UDP-GalNAc	295	485	0.61

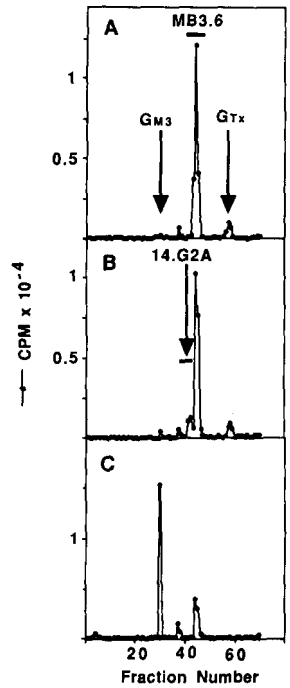


FIG. 9. **Effect of UDP-GalNAc on labeling of Golgi-enriched fractions with CMP-[4-^{3}H]Neu5Ac.** Aliquots of freshly prepared Golgi-enriched fractions from M21 cells were labeled with CMP-[4-^{3}H]Neu5Ac in the presence or absence of 10 μM UDP-GalNAc or 0.15% Triton X-100. Lipids were extracted by direct addition of chloroform:methanol to the reaction mixture, desalted, and analyzed by DEAE-HPLC collecting 1-ml fractions. Following fractionation, 50-μl aliquots were re-analyzed for reactivity to mAbs MB3.6 (G_{D3}) and 14.G2A ($OAcG_{D2}/G_{D2}$) by lipid ELISA. The remainder of each fraction was dried and quantitated by scintillation counting. *Panel A*, no additions; *panel B*, with 10 μM UDP-GalNAc; *panel C*, with 10 μM UDP-GalNAc and 0.15% Triton X-100.

[2] Hayes, B. K., and Varki, A., *J. Biol. Chem.*, in press.

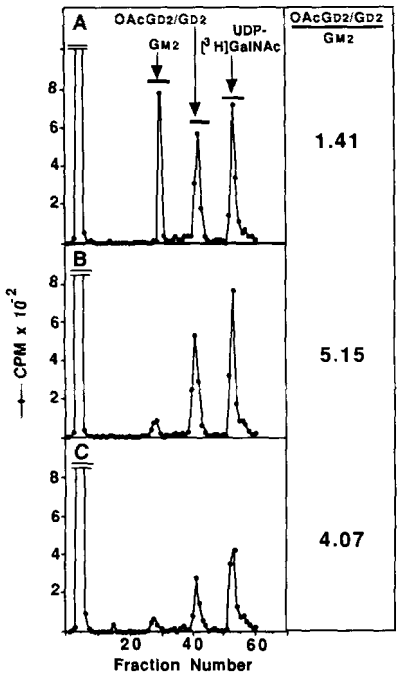


FIG. 10. **Effects of CMP-Neu5Ac on labeling of Golgi-enriched fractions with UDP-[6-^{3}H]GalNAc.** Aliquots of freshly prepared Golgi-enriched fractions from M21 cells were labeled with UDP-[6-^{3}H]GalNAc with or without added CMP-Neu5Ac. Total lipids were extracted and half of each sample was fractionated by DEAE-HPLC, collecting 1-ml fractions. Aliquots (50 μl) of each fraction were tested for reactivity to mAbs 14.G2A and MB3.6 directed against $OAcG_{D2}/G_{D2}$ and G_{D3}, respectively, by lipid ELISA. The remaining half of each sample was analyzed by HPTLC to confirm the products (data not shown). *Panel A*, no added CMP-Neu5Ac; *panel B*, 10 μM CMP-Neu5Ac added; *panel C*, 100 μM CMP-Neu5Ac added.

addition of 100 μM CMP-Neu5Ac, indicating the reaction is saturated at 10 μM, a concentration 20-fold below the K_m of G_{D3} synthase. This again provides indirect evidence for the maintenance of membrane integrity and topology. Kinetic experiments have previously shown that GalNAc is added to G_{M3} and G_{D3} by the same glycosyltransferase, forming G_{M2} and G_{D2}, respectively (23). The decrease in the ratio of G_{M2}/G_{D2} is therefore best explained by endogenous G_{M3} being co-localized within a compartment containing partially overlapping G_{D3} and G_{M2}/G_{D2} biosynthetic activities. Thus, upon addition of unlabeled CMP-Neu5Ac, more nonlabeled G_{D3} would be synthesized from G_{M3}, providing more substrate for G_{D2} synthesis and less for G_{M2} synthesis. Taken together, the experiments shown in Figs. 9 and 10 provide evidence that two sequentially acting ganglioside biosynthetic activities reside in compartments that partially overlap.

DISCUSSION

In contrast to *N*-linked oligosaccharide biosynthesis, the organization of ganglioside biosynthesis in the Golgi apparatus has remained somewhat obscure. In particular, little information is available regarding the spatial localization or sequential interdependence of ganglioside *O*-acetyltransferase(s) relative to other ganglioside biosynthetic activities. Recent reports indicate a sequential distribution, from *cis* to *trans* of ganglioside glycosyltransferase activities in the Golgi apparatus of rat liver (87, 89) and cultured cerebellar neurons (90). These conclusions were based on subcellular fractionation studies in which sucrose gradient fractions were examined for ganglioside biosynthetic enzymes using conventional assays with detergents. For the reasons discussed below, and because the ganglioside *O*-acetyltransferase is detergent labile,[3] such assays were not used in the current study. Instead, we explored these questions by BFA treatment and with *in vitro* labeling of intact Golgi-enriched compartments of correct topology.

Table VI summarizes all of the findings made in this study using this *in vitro* Golgi labeling reaction. It is interesting to compare this labeling reaction with more conventional methods for studying the organization of the Golgi apparatus. Localization of glycosyltransferase activities by density gradients or immunocytochemical techniques is informative. However, any given step in ganglioside biosynthesis requires the co-localization of the appropriate acceptor, sugar nucleotide transporter, and glycosyltransferase in the same Golgi compartment. Thus, localizing transferase activities by immunological or density gradient techniques does not conclusively demonstrate that biosynthesis occurs at the locations identified. Furthermore, the separation of enzyme activity peaks on density gradients is usually not complete, and the overlapping regions could represent either overlapping densities of distinct vesicles or overlap in the localization of the enzymes themselves. Likewise, immunolocalization studies only highlight the area of maximum concentration of the enzyme in question. The Golgi labeling reaction described here is dependent upon the co-localization of appropriate acceptors, sugar nucleotide transporters, and glycosyltransferases in intact topologically correct preparations, thereby mimicking *in vivo* ganglioside biosynthesis. For this reason, we refer throughout this paper to the functional localization of a given reaction rather than to the name of the enzyme involved, *e.g.* "G_{D3} biosynthesis" or "G_{D3} biosynthetic machinery" rather than G_{D3} synthase. Another useful feature is that the subcellular fractions used do not require a high level of purification. This is because only those compartments that are intact can transport the appropriate sugar nucleotide, and contain the correct transferases and acceptors will give a signal, *i.e.* the system defines itself. This is of value particularly when subcellular fractionation is difficult, as can be the case when studying certain cell types grown in culture. We have also recently applied this same principle towards studying the biosynthesis of *N*-linked oligosaccharides in the rat liver Golgi apparatus.[2]

In theory, the same approach could be applied to unbroken cells, which could be permeabilized, washed free of cytosol, and incubated directly with sugar nucleotides (91). However, the permeabilizing agents used could disrupt the membrane integrity of the Golgi apparatus as well. In the current study we chose to partially purify Golgi fractions because label from [^{3}H]Ac-CoA is incorporated into many other lipids in the crude extract, confusing subsequent analysis. A disadvantage of the Golgi labeling reaction is its inability to determine the precise physical location of a particular ganglioside biosynthetic reaction in the intact cell, relative to others. In the current study, we partially dealt with this limitation by separating ganglioside biosynthetic activities into compartments that are distal or proximal to a transport block imposed by BFA.

Brefeldin A has been used by two other groups to explore the organization of ganglioside biosynthetic activities in the Golgi apparatus (71, 72). In both instances, the biosynthetic steps leading up to G_{D3} biosynthesis were unaffected by the drug, while steps beyond this, *e.g.* G_{M2}/G_{D2} synthesis, were found to be severely inhibited. The current study confirms that G_{M2}/G_{D2} synthase is localized in compartments distal to the BFA transport block. However, two of three melanoma

[3] E. R. Sjoberg and A. Varki, unpublished data.

TABLE VI

Summary of findings with labeling of Golgi-enriched preparations

This table provides a summary of the results of all the Golgi labeling reactions described in this article. The table is arranged in groups based on the radiolabeled high energy donor used. The effects of exogenous unlabeled high energy donors (all at 20 μM) are indicated, *e.g.* CMP-[^{3}H]Sia + Ac-CoA is compared directly with CMP-[^{3}H]Sia alone.

Nucleotide used		Results
Labeled	Unlabeled	
CMP-[^{3}H]Neu5Ac	None	$OAcG_{D3}$ and G_{D3} are synthesized in 1:9 ratio
CMP-[^{3}H]Neu5Ac	Acetyl-CoA	Ratio of $OAcG_{D3}$:G_{D3} increases to 1:4
CMP-[^{3}H]Neu5Ac	UDP-GalNAc	Along with G_{D3} and $OAcG_{D3}$, G_{D2} is also synthesized
[^{3}H]Acetyl-CoA	None	$OAcG_{D2}$ and $OAcG_{D3}$ are synthesized in 3:1 ratio
[^{3}H]Acetyl-CoA	CMP-Sia	$OAcG_{D2}$ is unaltered, while $OAcG_{D3}$ increases 2-fold
[^{3}H]Acetyl-CoA	UDP-GalNAc	No change (no increase in $OAcG_{D2}$ synthesized)
UDP-[^{3}H]GalNAc	None	G_{M2}, G_{D2}, and $OAcG_{D2}$ are synthesized in ~equal ratios
UDP-[^{3}H]GalNAc	CMP-Sia	Relative amount of G_{M2} decreases markedly
UDP-[^{3}H]GalNAc	Acetyl-CoA	No change (no increase in $OAcG_{D2}$ synthesized)

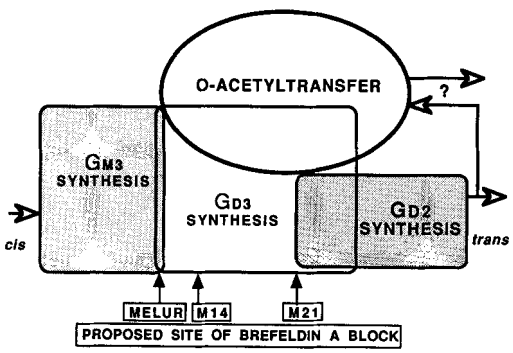


FIG. 11. **Proposed organization of the late biosynthetic reactions of gangliosides in human melanoma cells.** The compartments of biosynthesis shown are defined as those that are functionally capable of carrying out a particular reaction, *i.e.* which have the necessary acceptor, transferase, and donor available. The figure also indicates the proposed sites of the brefeldin A-induced block in each of the three melanoma cell lines studied. See text for discussion.

cell lines examined showed marked reduction of G_{D3} biosynthesis in the presence of BFA. It is unlikely that BFA negatively influences G_{D3} biosynthesis by direct effects on the enzyme since a third melanoma cell line, M21, showed a slight buildup of G_{D3} in the presence of BFA. Instead, it would appear that the majority of G_{D3} biosynthetic activity in two of the cell lines examined was rendered inaccessible to its G_{M3} acceptor by BFA. Thus, the organization of G_{D3} biosynthetic activities throughout the Golgi apparatus may vary between very similar cell lines (see Fig. 11). Indeed, increasing evidence supports a tissue- and cell-specific organization of oligosaccharide biosynthesis in the Golgi apparatus. For example, using immunocytochemical localization, α2–6 sialyltransferase is found in *trans*-cisternae exclusively in intestinal goblet cells and hepatocytes. However, when the same approach is applied to intestinal absorptive cells, α2–6 sialyltransferase is found scattered throughout the Golgi apparatus (82). This is the first report indicating that ganglioside biosynthetic activities are also differentially organized between different cell lines, even of the same type. Thus, cells may at least partially control ganglioside biosynthesis by differentially regulating the spatial organization of biosynthetic activities throughout the Golgi apparatus.

It is well accepted that the Golgi apparatus is both functionally and topologically polarized, with proteins entering the Golgi apparatus at the *cis* face and exiting at the *trans* face (92–94). Somewhat less clear is the extent to which oligosaccharide processing activities are compartmentalized within cisternae between the entry and exit points of the Golgi apparatus. From cell fractionation and immunocytochemical experiments it is clear that both protein and ganglioside biosynthetic activities are generally organized in a polarized fashion, corresponding roughly to the sequential order of monosaccharide addition (87, 89, 90, 95–99). Current dogma indicates that such sequentially interdependent biosynthetic steps are uniquely compartmentalized (75). However, there is evidence for the compartmental overlap of at least some sequentially interdependent *N*-linked biosynthetic activities (81, 88). The current study provides two examples of compartmental overlap between ganglioside processing activities: ganglioside *O*-acetyltransferase with G_{D3} synthesis and G_{D3} synthesis with G_{D2}synthesis (see Table VI).

Previous data indicate that G_{M2}, G_{D2}, and G_{T2} are synthesized by the same enzyme, G_{M2}/G_{D2} synthase (23, 100), *i.e.* G_{M3} is a direct acceptor for both G_{D3} and G_{M2}/G_{D2} synthases. Although the levels of enzyme activities in a cell generally correlate with the types of gangliosides found, significant inconsistencies are found (101). Our observation that the G_{D3} and G_{M2}/G_{D2} biosynthetic activities at least partially overlap in melanoma cells indicates that they can compete for acceptor substrate even in a compartmentalized system. By altering the extent of such co-localization the cell could conceivably fine tune the relative amounts of G_{M2}, G_{D3}, and G_{D2} expressed on the cell surface without changing the amount of each enzyme. Since the M21 cell line expresses both $OAcG_{D3}$ and $OAcG_{D2}$ (36), we also used the Golgi labeling reaction to explore precursor-product relationships between these two gangliosides. While ganglioside *O*-acetyltransferase overlaps the G_{D3} biosynthetic compartment, no evidence was found for compartmental overlap between G_{D2} biosynthesis and ganglioside *O*-acetyltransferase. Instead, some $OAcG_{D3}$ is co-localized with G_{D2} biosynthetic activity and acts as an efficient acceptor for G_{D2} synthase.

It has been noted that addition of *O*-acetyl esters to B-series gangliosides occur specifically on a terminal α2–8-

linked sialic acid originating from the internal core β1–4-linked Gal of lactosylceramide (36). Thus, while *O*-acetylation of terminal α2–8-linked sialic acids precludes further addition of monosaccharides to the branch carrying the *O*-acetyl ester, it does not attenuate further biosynthesis along other branches of the molecule. This finding supports a hypothesis we recently proposed for the regulation of *O*-acetylated ganglioside biosynthesis in the developing nervous system (36). We suggested that the developmental loss of $OAcG_{D3}$ expression noted by many investigators using mAbs (41, 46, 47, 102, 103) is not due to loss of *O*-acetylation, but to conversion from $OAcG_{D3}$ to more complex *O*-acetylated gangliosides such as $OAcG_{T1b}$ and $OAcG_{Q1b}$, that are not recognized by the mAbs used. In this scenario, ganglioside *O*-acetyltransferase would be constitutively expressed throughout development and the developmental loss of $OAcG_{D3}$ expression would be regulated by the increased action of G_{D2} synthase (36). The demonstration that *O*-acetylation of gangliosides occurs early in biosynthesis and does not attenuate further extension of these molecules contrasts with the *O*-acetylation of *N*-linked oligosaccharides in rat liver Golgi. In the latter case, *O*-acetylation of α2–6-linked sialic acids is a terminal biosynthetic event and shows no compartmental overlap with the sialylation reaction (57).[2]

We previously reported that *O*-acetyl groups on melanoma-associated G_{D3} turn over more rapidly than core sialic acid molecules on G_{D3} (37). From the current studies, two possibilities could account for this metabolic fate of the *O*-acetyl ester. First, G_{D3} could be endocytosed from the plasma membrane and returned to the Golgi apparatus to acquire *O*-acetyl esters. Indeed, such retrograde trafficking is not without precedent. Studies using biotinylated ganglioside derivatives and double radiolabeling indicate that plasma membrane gangliosides can be endocytosed and targeted to the Golgi apparatus for biosynthesis of more complex gangliosides (104). Second, G_{D3} molecules might be endocytosed from the plasma membrane to endosomes, where they may acquire *O*-acetyl esters from a distinct ganglioside *O*-acetyltransferase localized in such compartments. In a recent report (36), we showed that G_{D2} can also be an acceptor for a ganglioside *O*-acetyltransferase forming $OAcG_{D2}$. The data presented here supports this finding, yet paradoxically, ganglioside *O*-acetyltransferase activity cannot be shown to directly overlap the compartment(s) in which G_{D2} biosynthetic activity resides. Several plausible explanations can be considered (see Fig. 11). First, it is possible that the Golgi-enriched preparations studied here are contaminated with other organelles such as endosomes in which G_{D2} acceptor and ganglioside *O*-acetyltransferase are co-localized, but which cannot transport sugar nucleotides. Thus, G_{D2} could be endocytosed from the cell surface into endosomes for addition of *O*-acetyl esters. If this is the case, it could represent a rapid control mechanism for regulating the level of *O*-acetylation on gangliosides. Second, G_{D2} may be endocytosed from the plasma membrane and targeted to the Golgi subcompartment in which *O*-acetyltransferase activity resides, co-localized with G_{D3} biosynthetic activity. Third, G_{D2} may be first synthesized in the *trans*-Golgi network region, and then by retrograde vesicular transport gain access to the *O*-acetylation compartment. Finally, it is possible that the *O*-acetyltransferase that *O*-acetylates G_{D3} (which appears to be in an early compartment) is distinct from the *O*-acetyltransferase that can *O*-acetylate G_{D2} (presumably in a very distal Golgi compartment, beyond that where G_{D2} is synthesized). Possibilities two and three dictate that G_{D2} acceptor would be found in the same compartment with ganglioside *O*-acetyltransferase and G_{D3} biosynthetic activities. Further experiments are necessary to elucidate which of these mechanisms is functional in melanoma cells. Resolution of these issues also requires the isolation and characterization of the ganglioside *O*-acetyltransferase(s), a task that has proven difficult because of the extreme lability of the enzyme(s) to solubilization.

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