Internalization and Sorting of a Fluorescent Analogue of Glucosylceramide to the Golgi Apparatus of Human Skin Fibroblasts: Utilization of Endocytic and Nonendocytic Transport Mechanisms

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Abstract. We examined the uptake and intracellular transport of the fluorescent glucosylceramide analogue N-[5-(5,7-dimethyl BODIPY™)-1-pentanoyl]-glucosyl sphingosine (C₅-DMB-GlcCer) in human skin fibroblasts, and we compared its behavior to that of the corresponding fluorescent analogues of sphingomyelin, galactosylceramide, and lactosylceramide. All four fluorescent analogues were readily transferred from defatted BSA to the plasma membrane during incubation at 4°C. When cells treated with C₅-DMB-GlcCer were washed, warmed to 37°C, and subsequently incubated with defatted BSA to remove fluorescent lipid at the cell surface, strong fluorescence was observed at the Golgi apparatus, as well as weaker labeling at the nuclear envelope and other intracellular membranes. Similar results were obtained with C₅-DMB-galactosylceramide, except that labeling of the Golgi apparatus was weaker than with C₅-DMB-GlcCer. Internalization of C₅-DMB-GlcCer was not inhibited by various treatments, including ATP depletion or warming to 19°C, and biochemical analysis demonstrated that the lipid was not metabolized during its internalization. However, accumulation of C₅-DMB-GlcCer at the Golgi apparatus was reduced when cells were treated with a nonfluorescent analogue of glucosylcera-

mide, suggesting that accumulation of C₅-DMB-GlcCer at the Golgi apparatus was a saturable process. In contrast, cells treated with C₅-DMB-analogues of sphingomyelin or lactosylceramide internalized the fluorescent lipid into a punctate pattern of fluorescence during warming at 37°C, and this process was temperature and energy dependent. These results with C₅-DMB-sphingomyelin and C₅-DMB-lactosylceramide were analogous to those obtained with another fluorescent analogue of sphingomyelin in which labeling of endocytic vesicles and plasma membrane lipid recycling were documented (Koval, M., and R. E. Pagano. 1990. *J. Cell Biol.* 111:429–442). Incubation of perforated cells with C₅-DMB-sphingomyelin resulted in prominent labeling of the nuclear envelope and other intracellular membranes, similar to the pattern observed with C₅-DMB-GlcCer in intact cells. These observations are consistent with the transbilayer movement of fluorescent analogues of glucosylceramide and galactosylceramide at the plasma membrane and early endosomes of human skin fibroblasts, and suggest that both endocytic and nonendocytic pathways are used in the internalization of these lipids from the plasma membrane.

G LYCOSPHINGOLIPIDS (GSLs)[1] are constituents of the plasma membranes of all eukaryotic cells and play important roles in cell–cell interactions, modu-

lation of cell growth and differentiation, and intracellular signaling. The backbone of GSLs is synthesized at the endoplasmic reticulum and subsequently transported to the Golgi

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1. *Abbreviations used in this paper:* BODIPY™, boron dipyrromethene difluoride; C₂-GalCer, N-acetyl-galactosyl sphingosine; C₂-GlcCer, N-acetyl-glucosyl sphingosine; C₅-DMB-Cer, N-[5-(5,7-dimethyl BODIPY™)-1-pentanoyl]D-*erythro*-sphingosine; C₅-DMB-GalCer, C₅-DMB-galactosyl sphingosine; C₅-DMB-GlcCer, C₅-DMB-glucosyl sphingosine; C₅-DMB-

LacCer, C₅-DMB-lactosyl sphingosine; C₅-DMB-SM, C₅-DMB-sphingosylphosphorylcholine; C₆-GalCer, N-hexanoyl-galactosyl sphingosine; C₆-GlcCer, N-hexanoyl-glucosyl sphingosine; C₆-NBD-GlcCer, N-(N-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl])glucosyl sphingosine; Cer, ceramide; GlcCer, glucosylceramide; DF-BSA, defatted BSA; GSL, glycosphingolipid; HMEMB, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered MEM, pH 7.4, without indicator, and containing 0.5 mM each choline chloride, ethanolamine, serine, and (myo)inositol; LacCer, lactosylceramide; SM, sphingomyelin.

apparatus, where it is sequentially glycosylated. The GSLs are then transported to the cell surface in a manner analogous to movement of newly synthesized membrane proteins along the secretory pathway. Once delivered to the cell surface, GSLs are thought to undergo multiple rounds of recycling between the plasma membrane and endosomes, and are slowly transported along the endocytic pathway to lysosomes, where they are hydrolyzed to sphingosine and fatty acids. The reader is referred to recent reviews for further discussion of various aspects of GSL metabolism, transport, and function (Hannun and Bell, 1989; Merrill and Stevens, 1989; Hakomori, 1990; Schwarzmann and Sandhoff, 1990; Pagano, 1990a; Kolesnick, 1991; Hoekstra and Kok, 1992; van Echten and Sandhoff, 1993; van Meer, 1993; Rosenwald and Pagano, 1993).

In the present study, we examined the intracellular transport of a fluorescent glycolipid analogue, glucosylceramide (N-[5-(5,7-dimethyl BODIPY™)-1-pentanoyl]-glucosyl sphingosine [C_5-DMB-GlcCer]), following its insertion into the plasma membrane bilayer of human skin fibroblasts to learn more about the possible mechanisms by which endogenous GSLs may be transported between the cell surface and intracellular membranes. In contrast to sphingomyelin, which is internalized via endocytic vesicles and undergoes plasma membrane lipid recycling (Koval and Pagano, 1989, 1990, 1991; Mayor et al., 1993), we found that C_5-DMB-GlcCer was internalized by both endocytic and nonendocytic pathways, resulting in labeling of various intracellular membranes and accumulation at the Golgi apparatus. These data and complementary experiments with C_5-DMB-GalCer and C_5-DMB-lactosyl sphingosine (C_5-DMB-LacCer) suggest that different pathways for the transport of endogenous GSLs from the plasma membrane to intracellular membranes may be used depending on the GSL headgroup. Our data also provide a plausible mechanism for the sorting of fluorescent analogues of sphingomyelin and glucosylceramide from each other during endocytosis, which was previously observed using HT29 cells (Kok et al., 1991).

Materials and Methods

Cells and Cell Culture

Normal (GM5659) and Gaucher's type II disease (GM1260) human skin fibroblasts were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown in Eagle's MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM each nonessential amino acids.

Cells were grown on no. 1 thickness glass coverslips for fluorescence microscopy or in 35-mm diameter tissue culture dishes for biochemical studies. All cells were grown at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Lipids

C_5-DMB-sphingosylphosphorylcholine (C_5-DMB-SM), C_5-DMB-D-erythro-sphingosine (C_5-DMB-Cer), and C_5-DMB-fatty acid were obtained from Molecular Probes, Inc. (Eugene, OR). C_5-DMB-GlcCer, C_5-DMB-GalCer, and C_5-DMB-LacCer were prepared by N-acylation (Schwarzmann and Sandhoff, 1987) of glucopsychosine, psychosine, and "lyso-LacCer" (a generous gift from Dr. G. Schwarzmann, University of Bonn, Bonn, Germany), respectively, using the N-hydroxysuccinimide ester of the C_5-DMB-fatty acid. Nonfluorescent, short-chain analogues of glucosylceramide (GlcCer) and galactosylceramide (GalCer) were prepared in a similar manner using the N-hydroxysuccinimidyl esters of acetic acid and hexanoic acid. The sphingolipids were purified by preparative thin-layer chromatog-

raphy using $CHCl_3$/MeOH/15 mM $CaCl_2$ (60:35:8, vol/vol/vol) as the developing solvent.

The concentration of the C_5-DMB-SM stock solution was determined by phosphorus analysis (Rouser et al., 1981). The concentrations of C_5-DMB-GlcCer, C_5-DMB-GalCer, C_5-DMB-LacCer, and C_5-DMB-Cer stock solutions were determined by measurement of their fluorescence intensities relative to C_5-DMB-SM, using $CHCl_3$/MeOH (2:1, vol/vol) as the solvent. (The ratio of fluorescence to absorbance at 508 nm was the same for all five lipids.) Fluorescence measurements were performed using an SLM 8000C spectrophotofluorimeter (SLM Instruments, Inc., Urbana, IL) ($\lambda_{ex} = 480$ nm; $\lambda_{em} = 516$ nm).

Sphingolipid/BSA Complexes

Complexes of C_5-DMB-Cer, C_5-DMB-GlcCer, C_5-DMB-GalCer, C_5-DMB-LacCer, and C_5-DMB-SM were prepared as described (Pagano and Martin, 1988), except that the complexes contained 25 μM each of fluorescent lipid and BSA. Briefly, 250 nmol of the desired C_5-DMB-lipid in a $CHCl_3$/MeOH stock solution was dried, first under a stream of nitrogen and then in vacuo. The dried lipid was dissolved in 200 μl of ethanol and injected into 10 ml of HMEMB containing 1.7 mg defatted BSA (DF-BSA)/ml while being vortex mixed. The complex was dialyzed overnight at 4°C against 500 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid–buffered MEM, pH 7.4, without indicator and containing 0.5 mM each choline, chloride, ethanolamine, serine, and (myo)inositol (HMEMB) and then frozen at −20°C in plastic tubes. Before use, the complexes were diluted with HMEMB as required. Complexes of N-acetyl-glucosyl sphingosine (C_2-GlcCer) or N-acetyl-galactosyl sphingosine (C_2-GalCer) and N-hexanoyl-glucosyl sphingosine (C_6-GlcCer) or N-hexanoyl-galactosyl sphingosine (C_6-GalCer) were prepared in a similar manner, except that the ratio of lipid to BSA was 2:1 (mol/mol).

Incubation of Fluorescent Lipids with Cells

Cultures were washed in HMEMB, incubated with 2.5, 5, or 10 μM (as indicated) C_5-DMB-sphingolipid/DF-BSA in HMEMB for 30 min at 2°C, washed, and further incubated in HMEMB for 0–30 min at 37°C. In some experiments, the cultures were chilled and incubated at 11°C (six times, 10 min each) with HMEMB containing 2% DF-BSA ("back exchange") or with HMEMB alone before photography or biochemical analysis.

In biochemical experiments, cells were harvested after the appropriate incubation using a rubber policeman, washed by centrifugation, resuspended, and aliquots were removed for protein content determination (Lowry et al., 1951) and for lipid extraction (Bligh and Dyer, 1959). The relative fluorescence of the lipid extracts was determined ($\lambda_{ex} = 495$ nm;

Table I. Uptake and Back Exchange of C_5-DMB-GlcCer, C_5-DMB-GalCer, and C_5-DMB-SM from Human Skin Fibroblasts*

Lipid labeling concentration‡	Uptake	Percent remaining after back exchange
	pmol C_5-DMB-lipid/μg protein	
C_5-DMB-GlcCer		
5 μM	0.148 ± 0.017	25.7 ± 3.5
10 μM	0.208 ± 0.012	27.9 ± 4.2
C_5-DMB-GalCer		
5 μM	0.227 ± 0.016	5.3 ± 1.3
C_5-DMB-SM		
5 μM	0.262 ± 0.020	5.3 ± 0.5

* Cells were incubated with the indicated concentration of C_5-DMB-GlcCer, C_5-DMB-GalCer, or C_5-DMB-SM/DF-BSA complex for 30 min at 2°C, washed, and the lipids were extracted, quantified, and normalized to cellular protein. Some of the labeled cells were back exchanged by incubating with 2% DF-BSA (6 × 10 min at 11°C; see Materials and Methods) before lipid extraction and analysis. (A sufficient amount of C_5-DMB-LacCer was not available for quantitative studies.) Data represent means ± SD ($n = 3$).
‡ Parallel cultures incubated with 10 μM C_5-DMB-GlcCer, 5 μM C_5-DMB-GalCer, or 5 μM C_5-DMB-SM/DF-BSA for 30 min at 2°C were washed and observed under the fluorescence microscope in the green and red channel (see Materials and Methods). The color of the plasma membrane was orange in all three cases, and these labeling concentrations were used for Fig. 2.
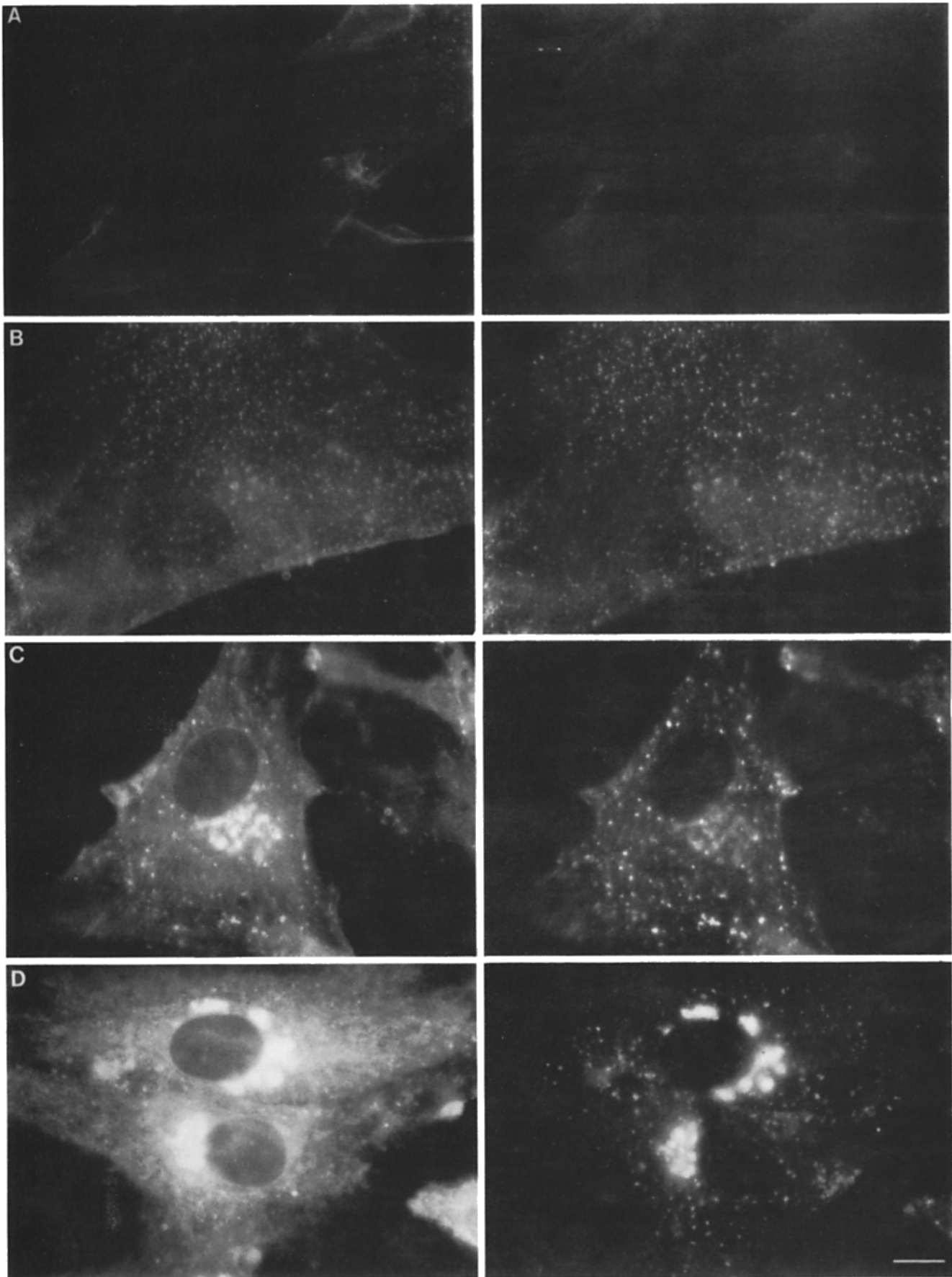
Figure 1. Redistribution of C5-DMB-GlcCer from the plasma membrane to intracellular membranes with time at 37°C. Cells were incubated with C5-DMB-GlcCer/BSA complex for 30 min at 2°C, washed, warmed for (A) 0, (B) 5, (C) 15, or (D) 30 min at 37°C, and subsequently "back exchanged" (see Materials and Methods). Separate photomicrographs of a given field were obtained at green + red wavelengths (*left panels*, ≥520 nm) and red wavelengths (*right panels*, ≥590 nm). Note the redistribution of fluorescence to numerous intracellular membranes including the Golgi apparatus in (C) and (D). *Bar,* 10 μm.

λ_{em} = 515 nm) and normalized to the protein content. The amounts of fluorescent C_5-DMB-lipid present in the cell extracts were determined by standard curves generated from lipid extracts prepared from various amounts of each C_5-DMB-lipid/BSA complex. Lipid extracts were also analyzed qualitatively by thin-layer chromatography.

Miscellaneous Procedures

For experiments using perforated cells, cells were washed with HMEMB, perforated by filter stripping (Simons and Virta, 1987; Kobayashi and Pagano, 1988), labeled with 5 μM C_5-DMB-lipid/BSA in transport medium (see Gravotta et al., 1990) for 15 min at 2°C, washed in transport medium, and photographed.

Cells were treated with inhibitors as described in the second footnote of Table II.

Control cells or ATP-depleted cells (see Table II, second footnote) were incubated for 30 min at 37°C with 1 mg/ml Texas red–labeled dextran (10 kD; Molecular Probes, Inc.) in the absence (control) or presence (ATP depleted) of inhibitors, washed, and observed by fluorescence microscopy.

Fluorescence microscopy was performed with an inverted microscope (IM-35; Carl Zeiss, Inc., Thornwood, NY) equipped with a Planapo 100X (1.3 NA) objective and epifluorescence optics. Samples were excited at 450–490 nm, and the fluorescence observed at either $\geqslant$520 nm (green + red wavelengths) or at $\geqslant$590 nm (red wavelengths) (see Pagano et al., 1991). Photomicrographs representative of observations of large numbers of cells in each sample were obtained either in color using Kodak Ektachrome 400 film (4-s exposure), which was pushed one stop during processing, or black and white using Kodak Tri-X film (4-s exposure) and processed at ASA 1600 with a developer (Diafine; Acufine, Chicago, IL). All micrographs from a given experiment were printed identically.

Results

Uptake and Back Exchangeability of C_5-DMB–labeled Lipids

We first examined the uptake of C_5-DMB-GlcCer, C_5-DMB-GalCer, and C_5-DMB-SM using equal concentrations of the fluorescent lipid/DF-BSA complexes. Cells were incubated with 5 μM C_5-DMB-lipid/BSA for 30 min at 2°C, washed, and the lipids were extracted and quantified. The amount of cell-associated C_5-DMB-SM and C_5-DMB-GalCer were nearly equal, but were considerably greater than the amount of cell-associated C_5-DMB-GlcCer. This difference in uptake was diminished by increasing the concentration of C_5-DMB-GlcCer/BSA to 10 μM (Table I). When cells treated under these conditions were examined under the fluorescence microscope, only plasma membrane fluorescence was detected, and the fluorescence emission was orange in each case. Since the fluorescence emission spectrum of C_5-DMB-lipids is concentration dependent (Pagano et al., 1991), these observations suggest that the different C_5-DMB-lipid species had similar "local" concentrations in the plasma membrane bilayer.

We also examined the ability of each of the C_5-DMB-lipids to be removed from the plasma membrane by incubation with a nonfluorescent acceptor (back exchange). Both C_5-DMB-GalCer and C_5-DMB-SM were almost completely removed by incubation with DF-BSA, while significantly more of the C_5-DMB-GlcCer taken up by cells at 2°C remained cell associated after back exchange. This result was confirmed qualitatively by fluorescence microscopy, in which plasma membrane labeling was readily observed after back exchange of cells treated with C_5-DMB-GlcCer, but was not seen with C_5-DMB-SM or C_5-DMB-GalCer. Lipid extraction and analysis confirmed that the cell-associated fluorescence was from C_5-DMB-GlcCer, and not from a fluorescent metabolite or breakdown product (data not shown).

Time Course for Internalization of C_5-DMB-GlcCer at 37°C

Fig. 1 shows a time course for redistribution of C_5-DMB-fluorescence from the plasma membrane to intracellular membranes. Cells were incubated with C_5-DMB-GlcCer/BSA for 30 min at 2°C, washed, warmed to 37°C for various times, back exchanged at 10°C, and observed at either $\geqslant$520 nm (left panels, green + red wavelengths) or at $\geqslant$590 nm (right panels, red wavelengths) (see Pagano et al., 1991). When cells were observed at $\geqslant$520 nm, fluorescence was initially seen only at the plasma membrane (Fig. 1 A). After 5 min at 37°C the fluorescence was also seen in "dotlike" structures scattered throughout the cytoplasm (Fig. 1 B). By 15–30 min of incubation, almost all of the cell-associated fluorescence was seen at the Golgi apparatus, nuclear envelope, and other intracellular membranes (Fig. 1, C and D). When the same cells were observed at red wavelengths so that only membranes containing high concentrations of the C_5-DMB-lipids were seen (see Pagano et al., 1991), redistribution of fluorescence from the plasma membrane to endosomes, and later to the Golgi apparatus, was seen (Fig. 1, right panels). However, no labeling of the ER or plasma membrane was detected in the red microscope channel, suggesting that the concentration of the C_5-DMB-lipids in these membranes was relatively low.

We next compared the uptake and distribution of C_5-DMB-GlcCer, C_5-DMB-GalCer, C_5-DMB-SM, and C_5-DMB-LacCer in human skin fibroblasts (Fig. 2). Cells were incubated with these lipids for 30 min at 2°C, washed, warmed to 37°C for 15 min, back exchanged, and then observed under the fluorescence microscope at green + red wavelengths. C_5-DMB-SM was distributed in a punctate pattern throughout the cytoplasm (Fig. 2 A), and, by analogy to previous studies using another fluorescent analogue of sphingomyelin (Koval and Pagano, 1990), labeled the endosomes and Golgi apparatus. C_5-DMB-LacCer gave a similar labeling pattern to that seen with C_5-DMB-SM (Fig. 2 B). In contrast, treatment with C_5-DMB-GlcCer resulted in strong labeling of the Golgi apparatus, as well as weaker labeling of the plasma membrane, nuclear envelope, and other intracellular membranes (Fig. 2 C). Some weak labeling of endosomes was also observed. Interestingly, cells treated with C_5-DMB-GalCer exhibited labeling of both vesicle-like structures, as well as numerous other intracellular membranes, including the Golgi apparatus (Fig. 2 D). However, labeling of the Golgi apparatus was considerably weaker than when C_5-DMB-GlcCer was used (compare Fig. 2, C vs D).

Lipid extraction and analysis of cells that were treated with C_5-DMB-GlcCer/BSA for 30 min at 2°C, washed, and warmed for 30 min at 37°C showed that $\geqslant$98% of the cell-associated fluorescence was from C_5-DMB-GlcCer (data not shown). In addition, when C_5-DMB-GlcCer/BSA was incubated with mutant (Gaucher's) fibroblasts lacking glucocerebrosidase (Brady et al., 1965), an identical pattern of labeling to that seen in normal fibroblasts was observed, demonstrating that the intracellular fluorescence seen with C_5-DMB-GlcCer was not caused by its hydrolysis.

Finally, we note that when cells treated with BSA complexes of C_5-DMB-GalCer, C_5-DMB-LacCer, or C_5-DMB-SM were observed at $\geqslant$520 nm (green + red wavelengths), an interesting pattern of fluorescence labeling of the endosomes was seen. Namely, within the same cell, some endo-
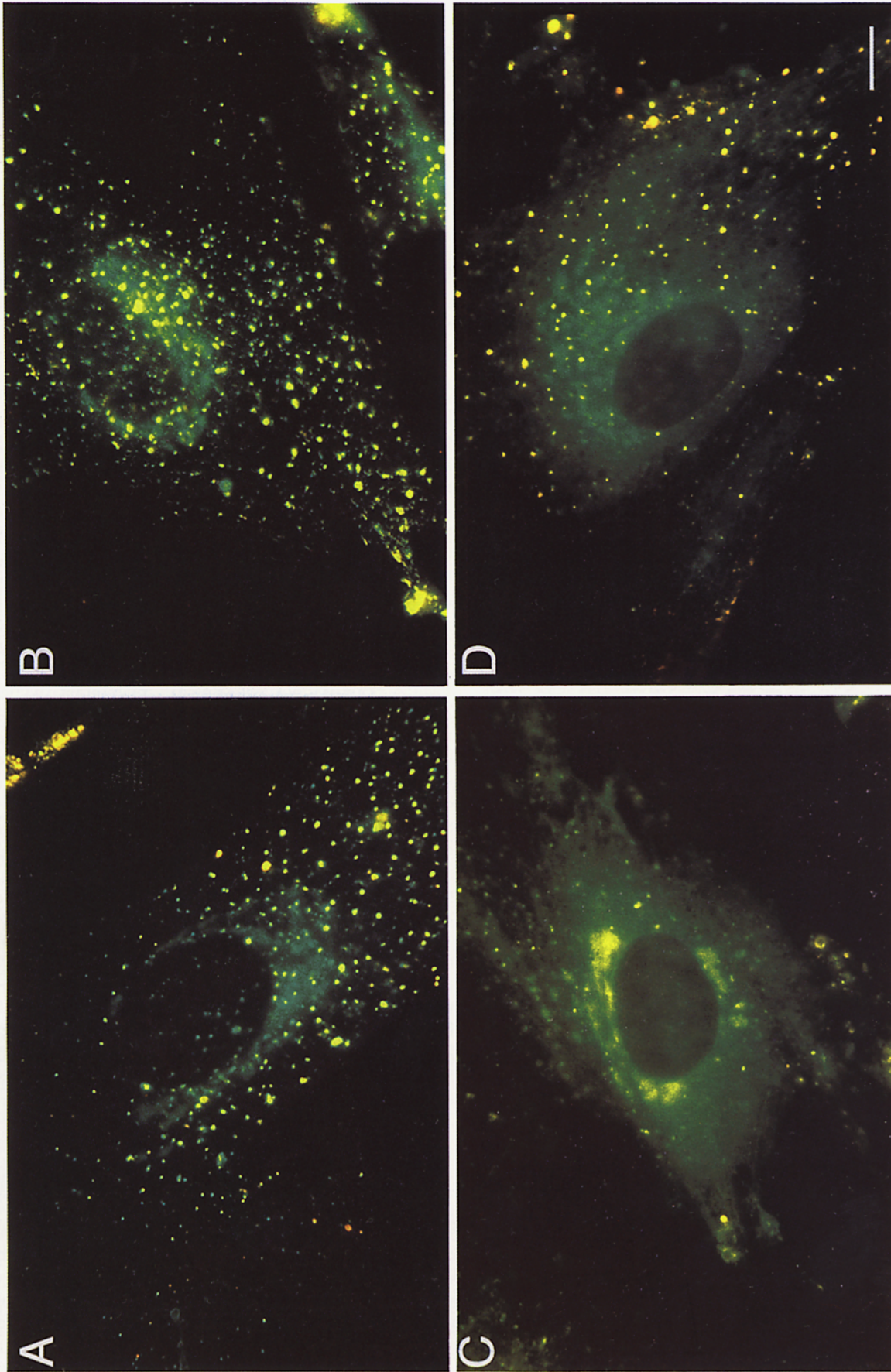
Figure 2. Comparison of C$_5$-DMB-SM, C$_5$-DMB-LacCer, C$_5$-DMB-GlcCer, and C$_5$-DMB-GalCer redistribution from the plasma membrane at 37°C. Cells were incubated with BSA complexes of (*A*) C$_5$-DMB-SM, (*B*) C$_5$-DMB-LacCer, (*C*) C$_5$-DMB-GlcCer, or (*D*) C$_5$-DMB-GalCer for 30 min at 2°C, washed, warmed for 15 min at 37°C, back exchanged, and photographed at green + red wavelengths. Note the presence of both orange and green endosomes, particularly in (*A*), (*B*), and (*D*), and the labeling of the Golgi apparatus in (*C*). *Bar,* 10 μm.
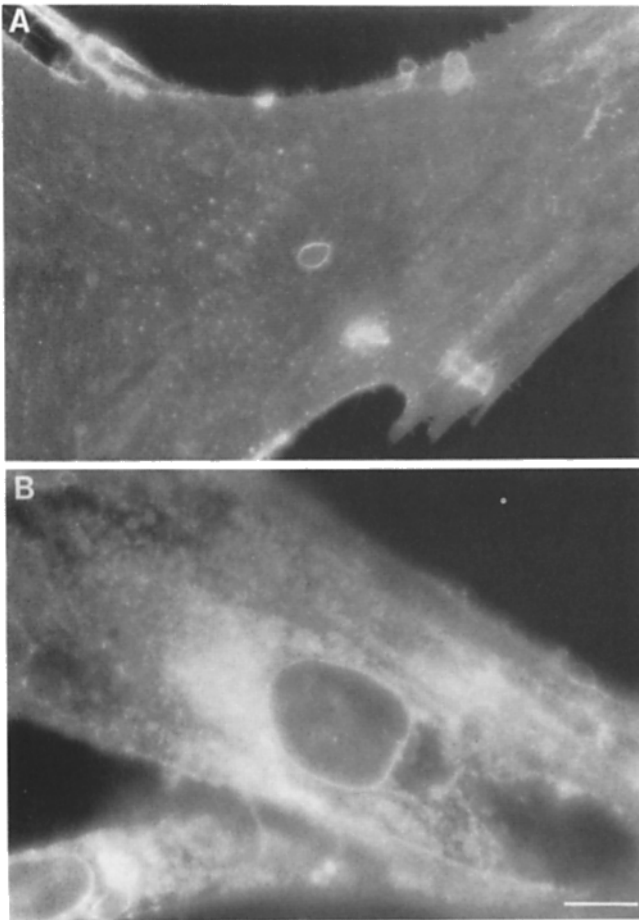
Figure 3. Effect of perforation on the intracellular distribution of C₅-DMB-fluorescence. Intact (*A*) or perforated (*B*) cells were incubated with C₅-DMB-SM/BSA at 2°C, washed, and photographed at green + red wavelengths. Note that C₅-DMB-SM prominently labeled the plasma membrane of intact cells, but resulted in strong intracellular labeling when perforated cells were used. Similar results to those shown in (*B*) were obtained when cells were pretreated with C₅-DMB-SM/BSA at 2°C and subsequently perforated (data not shown). *Bar*, 10 μm.

somes exhibited green fluorescence, while others emitted orange fluorescence. In addition, "orange endosomes" were sometimes seen at the leading edge of cells. These results suggest that different populations of endosomes within the same cell may contain different concentrations of the fluorescent lipid analogues (Pagano, R. E., and O. C. Martin, manuscript in preparation).

Distribution of C₅-DMB-lipids in Perforated Cells

We next examined the intracellular distribution of C₅-DMB-SM in intact vs perforated cells. C₅-DMB-SM labeled the plasma membrane of intact cells at low temperature (Fig. 3 *A*) and distributed into endocytic vesicles upon warming the cells to 37°C (Fig. 2 *A*). However, when cells were perforated before (Fig. 3 *B*) or after (data not shown) incubation with the fluorescent lipid at low temperature, numerous intracellular membranes, including the ER, mitochondria, and nuclear envelope, were labeled. Similar ex-

periments were performed at low temperature using C₅-DMB-GlcCer and C₅-DMB-GalCer, and, in both cases (data not shown), the fluorescent lipids labeled the plasma membrane of intact cells and the intracellular membranes of perforated cells. These results demonstrate that labeling of intracellular membranes by C₅-DMB-lipids can occur once the lipids cross the plasma membrane bilayer. Indeed, both intact and perforated cells incubated with C₅-DMB-Cer gave identical patterns of intracellular labeling (data not shown), presumably because ceramide analogues undergo spontaneous transbilayer movement (Pagano and Martin, 1988; Pagano, 1989) and readily cross the plasma membrane bilayer of intact cells.

Effects of ATP Depletion and Temperature on the Intracellular Distribution of C₅-DMB-GlcCer

The effect of ATP depletion on the internalization of C₅-DMB-SM (Fig. 4) and C₅-DMB-GlcCer (Fig. 5) was examined. Control or ATP-depleted cells were incubated with the fluorescent lipid for 30 min at 2°C, washed, warmed to 37°C for 30 min, back exchanged with DF-BSA, and observed at either ≥520 nm (*left panels, green + red wavelengths*) or at ≥590 nm (*right panels, red wavelengths*). All steps were performed in the absence or presence of inhibitors. When C₅-DMB-SM was used, punctate labeling corresponding to labeled endosomes was readily seen in control cells (Fig. 4 *A*), but intracellular labeling was inhibited in ATP-depleted cells (Fig. 4 *B*). These ATP-depletion conditions also inhibited the internalization of Texas red dextran (see Materials and Methods), a fluid phase endocytic marker (Koval and Pagano, 1990) (data not shown). In contrast, when C₅-DMB-GlcCer (Fig. 5) or C₅-DMB-GalCer were used (data not shown), punctate labeling was inhibited by ATP depletion (compare Fig. 5, *B* vs *A*), but labeling of the endoplasmic reticulum, mitochondria, and nuclear envelope were not affected. As in control cells (see above), ≥98% of the cell-associated fluorescence in lipid extracts of ATP-depleted cells was from C₅-DMB-GlcCer (data not shown). Interestingly, ATP depletion appeared to reduce the accumulation of C₅-DMB-GlcCer at the Golgi apparatus (Fig. 5 *B, right panel*).

We also examined the effect of temperature on C₅-DMB-GlcCer internalization (Fig. 6). Cells were incubated with C₅-DMB-GlcCer for 30 min at 2°C, washed, and warmed to 10°C (2 h), 19°C (2 h), or 37°C (15 min) before back exchange with DF-BSA. While treatment of cells at 10°C inhibited internalization (Fig. 6 *A*), labeling of intracellular membranes was readily seen at 19°C (Fig. 6 *B*), as well as 37°C (Fig. 6 *C*).

Evaluation of Potential Inhibitors of C₅-DMB-GlcCer Transport from the Plasma Membrane to Intracellular Membranes

Finally, we evaluated the effects of numerous reagents on the redistribution of C₅-DMB-GlcCer from the plasma membrane to intracellular membranes (Table II). Cells were treated with various (*a*) inhibitors, (*b*) structural analogues of glucose or glucose transport inhibitors, (*c*) sphingolipid analogues, (*d*) protein modification reagents, (*e*) enzymes, (*f*) modifiers of cellular cholesterol, or (*g*) other reagents. With one exception, none of these reagents had an effect on
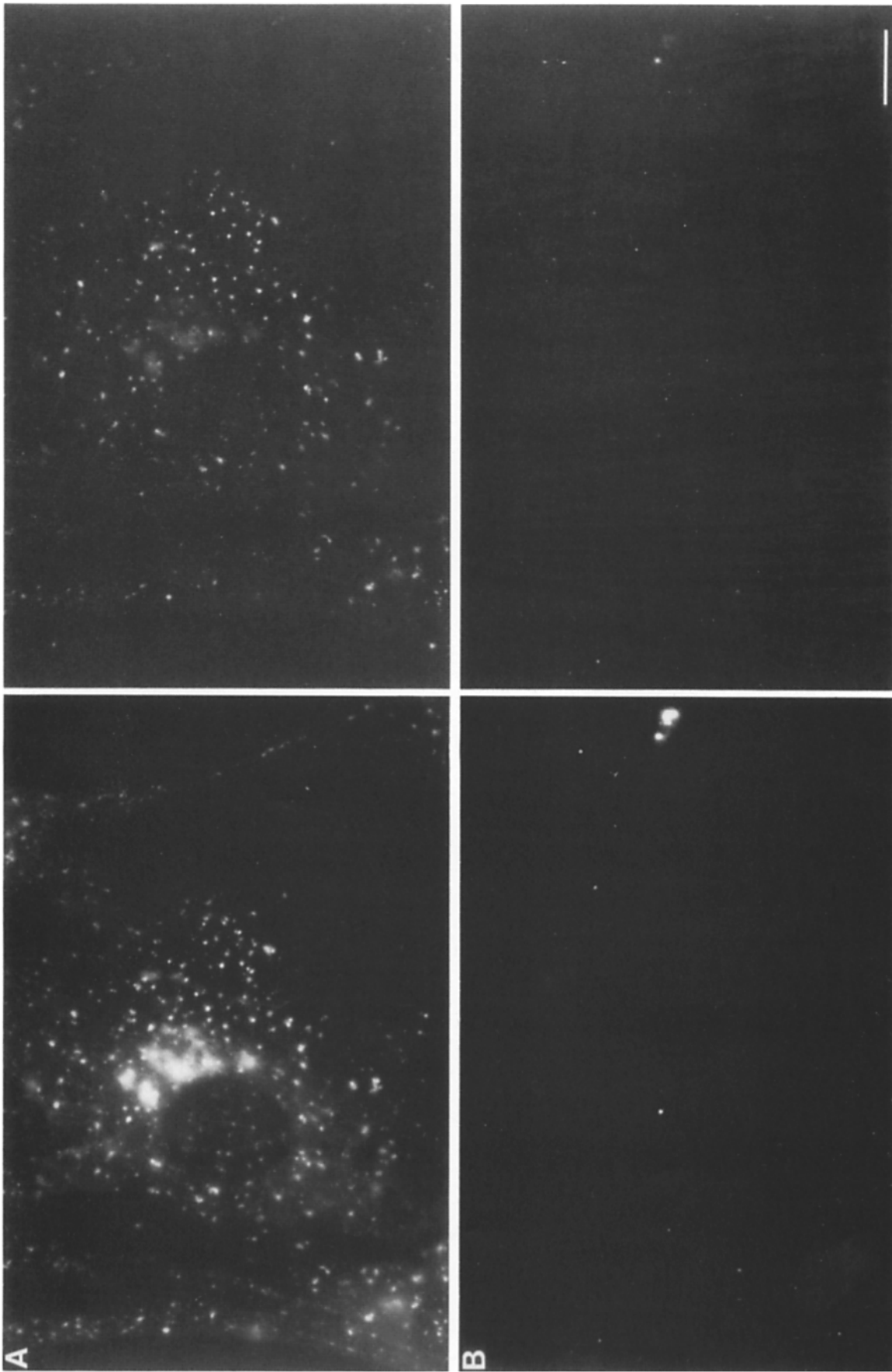
Figure 4. Effect of ATP depletion on the intracellular redistribution of C₅-DMB-SM. (*A*) Control or (*B*) ATP-depleted cells were incubated with C₅-DMB-SM/BSA for 30 min at 2°C, washed, warmed to 37°C for 30 min, back exchanged, and photographed at green + red wavelengths (*left panels*) or red wavelengths (*right panels*). *Bar*, 10 µm.
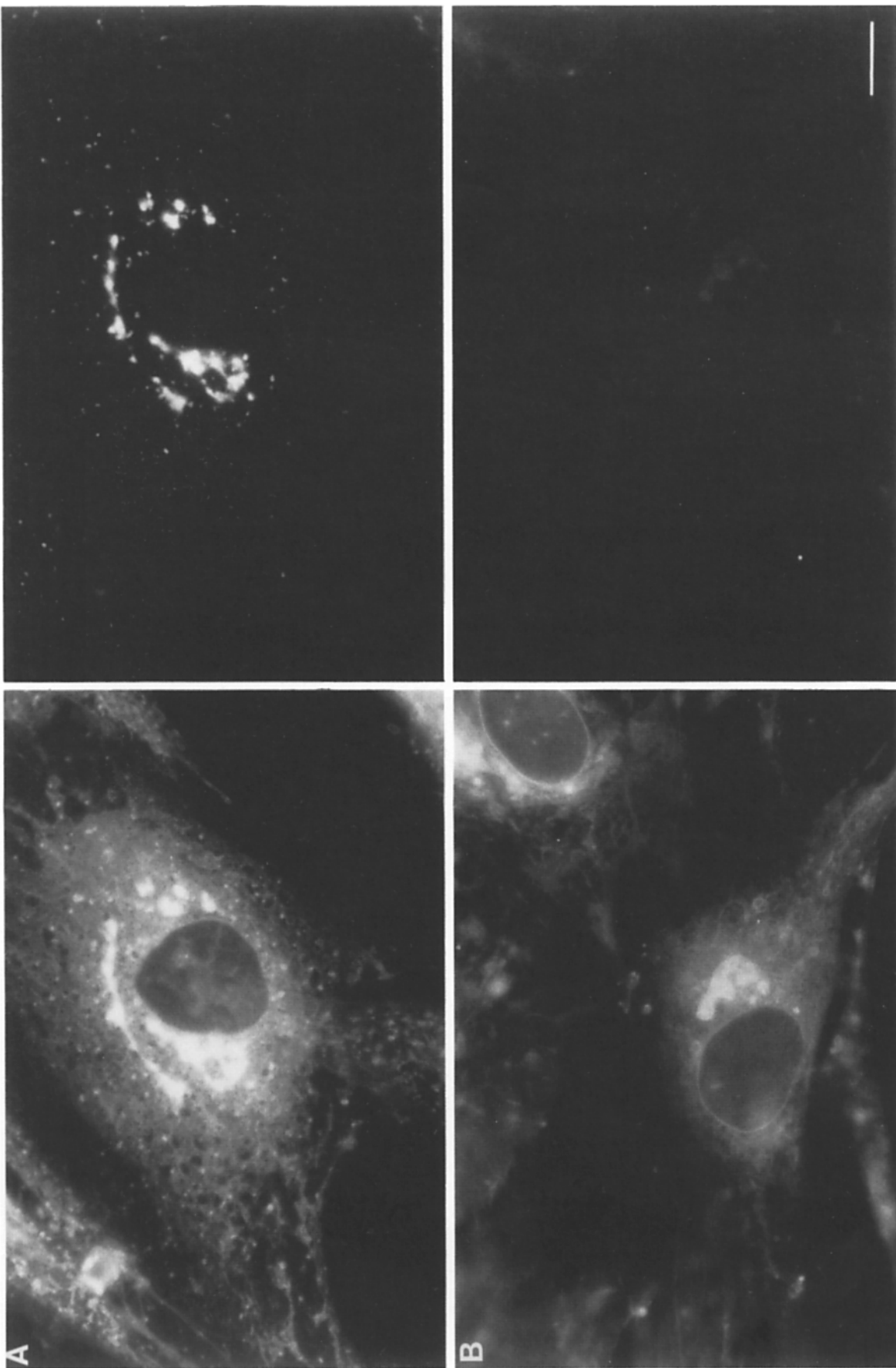
Figure 5. Effect of ATP-depletion on the intracellular redistribution of C₅-DMB-GlcCer. (A) Control or (B) ATP-depleted cells were incubated with C₅-DMB-GlcCer/BSA for 30 min at 2°C, washed, warmed to 37°C for 30 min, back exchanged, and photographed at green + red wavelengths (*left panels*) or red wavelengths (*right panels*). *Bar*, 10 µm.
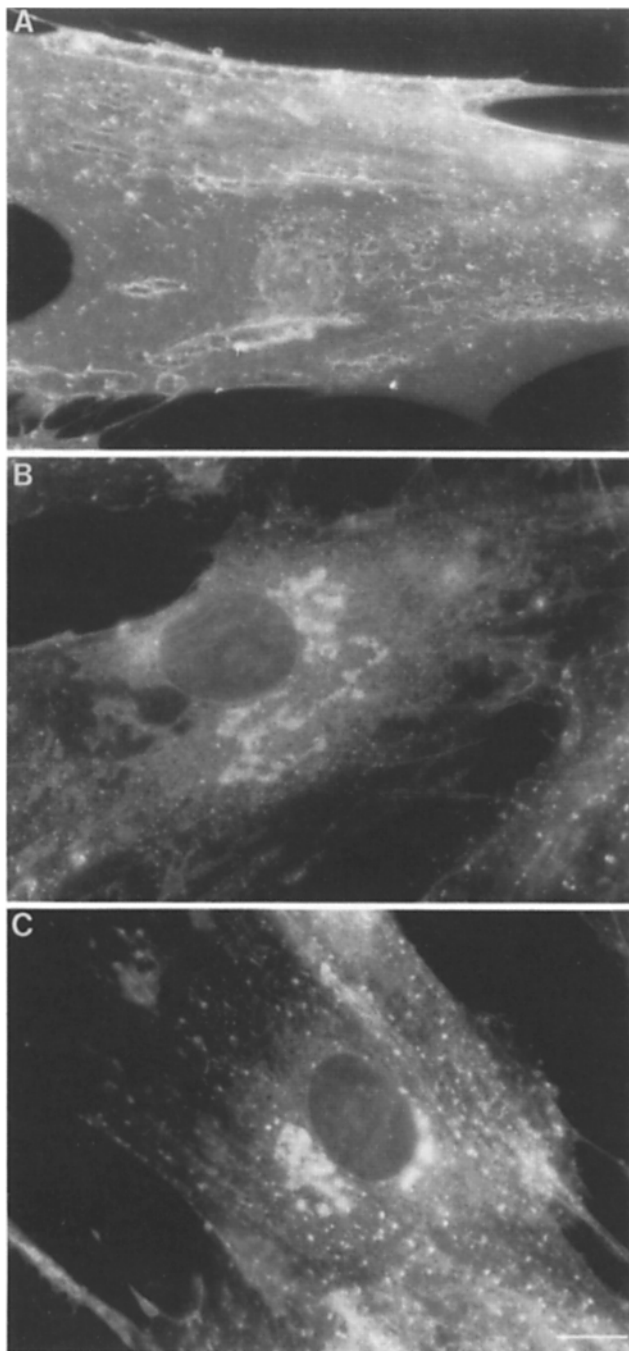
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Figure 6. Effect of temperature on the intracellular redistribution of C5-DMB-GlcCer. Cells were incubated with C5-DMB-GlcCer/BSA for 30 min at 2°C, washed, warmed for (*A*) 2 h at 10°C, (*B*) 2 h at 19°C, or (*C*) 15 min at 37°C before back exchange with DF-BSA. The samples were then photographed at green + red wavelengths. *Bar*, 10 μm.

the intracellular labeling seen with C5-DMB-GlcCer. Treatment of cells with a nonfluorescent GlcCer (Fig. 7 *B*) (but not GalCer; Fig. 7 *C*) analogue at a high concentration reduced labeling of the Golgi apparatus by C5-DMB-GlcCer compared to control cells (Fig. 7 *A*). (In this experiment, C5-DMB-GlcCer was added before addition of the nonfluorescent GlcCer and GalCer analogues so that both control

cells and cells treated with the nonfluorescent glycolipid analogues would contain the same amount of fluorescent lipid [see legend of Fig. 7].) This result suggests that accumulation of C5-DMB-GlcCer at the Golgi apparatus may be a saturable process.

Discussion

In this paper, we investigated the uptake and intracellular transport of a fluorescent analogue of glucosylceramide, C5-DMB-GlcCer, and compared it to the behavior of three other fluorescent sphingolipid analogues (C5-DMB-GalCer, C5-DMB-LacCer, and C5-DMB-SM), which also label the plasma membrane of human skin fibroblasts during incubations at low temperatures. We provide evidence that C5-DMB-GlcCer and C5-DMB-GalCer are internalized by a combination of endocytosis and transbilayer movement ("flip flop") at the plasma membrane and endosomes. This latter process results in labeling of intracellular membranes and accumulation of the fluorescent lipids at the Golgi apparatus. In contrast, C5-DMB-SM and C5-DMB-LacCer appear to be internalized primarily by endocytosis, producing a punctate fluorescence pattern that is readily distinguished from that obtained with the C5-DMB-GlcCer and C5-DMB-GalCer analogues.

Intracellular Transport of C5-DMB-SM and C5-DMB-LacCer

Fig. 8 *A* shows a model for the movement of C5-DMB-SM and C5-DMB-LacCer from the plasma membrane to intracellular membranes. These lipids are integrated into the plasma membrane bilayer during incubation at low temperature. During subsequent warming to 37°C, the fluorescent lipid is internalized into numerous fluorescent vesicles that are scattered throughout the cytoplasm (Fig. 2, *A* and *B*), and, by analogy to previous studies with another sphingomyelin analogue, correspond to labeled endosomes (Koval and Pagano, 1990). This internalization process was ATP dependent (Fig. 4) and inhibited at low temperature (data not shown). C5-DMB-SM could be almost completely removed from the cell surface after the low temperature labeling (see Table I) by incubation with defatted BSA at low temperature (back exchange), suggesting that the fluorescent lipid was present in the outer leaflet of the plasma membrane bilayer. The presence of discrete "punctate dots" of fluorescence, rather than a more generalized labeling of many different intracellular membranes, suggests that C5-DMB-SM and C5-DMB-LacCer were restricted to the lumenal leaflet of endocytic vesicles (topologically equivalent to the outer leaflet of the plasma membrane bilayer) during internalization and, therefore, unable to equilibrate and label other cellular membranes. This idea is consistent with our finding that C5-DMB-SM (as well as other C5-DMB-lipids) labeled many different intracellular membranes in perforated cells where the plasma membrane was disrupted and a barrier to transmembrane movement of lipids no longer existed (Fig. 3 *B*).

In addition to labeling of endosomes, some labeling of the Golgi apparatus was also seen when C5-DMB-SM or C5-DMB-LacCer were used; (e.g., Fig. 2, *A* and *B*). This most likely results from hydrolysis of these lipids at the plasma membrane and/or lysosomes and subsequent transport of the resulting fluorescent ceramide to the Golgi apparatus (Koval

*Table II. Effects of Various Reagents on the Accumulation of C₅-DMB-GlcCer at the Golgi Apparatus of Human Skin Fibroblasts**

Treatment	Concentration	Pretreatment conditions Temp. °C	Time min	Present throughout experiment	Accumulates at Golgi apparatus
Inhibitors					
Sodium azide +	5 mM				
2-deoxyglucose‡	50 mM	37	30	+	±§
NH₄Cl	20 mM	None		+	+
Chloroquine	20 μM	None		+	+
Structural analogues of glucose; glucose transport inhibitors					
Glucose	5 mM	37	30	+	+
Galactose	5 mM	37	30	+	+
Glucose-1-phosphate	5 mM	37	30	+	+
Glucosamine	5 mM	37	30	+	+
β-D-Glucuronic acid	5 mM	37	30	+	+
X-Glu, X-Gal	1 mM	37	30	+	+
Cytochalasin B	50 μM	37	30	+	+
Sphingolipid analogues					
PDMP	20 μM	None		+	+
C₂-GalCer‖	100 μM	None		+‖	+
C₂-GlcCer‖	100 μM	None		+‖	+
C₆-GalCer‖	100 μM	None		+‖	+
C₆-GlcCer‖	100 μM	None		+‖	−
Protein modification reagents					
NEM¶	0.5 mM	2	30	−	+
Fixation**		22	30	−	+
DIDS	1 mM	2	30	−	+
SITS	1 mM	2	30	−	+
Enzymatic treatments					
Trypsin	12.5 μg/ml	2	30	−	+
Neuraminidase	2.5 μg/ml	2	30	−	+
Endoglycoceramidase	1.5 mU/ml	37	30	−	+
Sphingomyelinase	0.13 mU/ml	37	15	−	+
Modification of cellular cholesterol					
Cholesterol oxidase	10 IU/ml	37	10	−	+
Lovastatin	10 μM	37	24 hr	−	+
Other reagents					
Brefeldin A	5 μg/ml	37	30	+	+
Nocodazole	10 μg/ml	37	90	+	+
Verapamil	200 μM	37	30	+	+

* Except for treatment with some of the sphingolipid analogues (see fourth footnote), cell cultures on glass coverslips were washed with HMEMB and preincubated (if indicated) with each reagent at the indicated concentration, temperature, and time. The cells were then washed, incubated with 10 μM C₅-DMB-GlcCer/BSA for 30 min at 2°C, washed, warmed to 37°C for 15-30 min, back exchanged, and examined for intracellular fluorescence. Where indicated, the reagents were also present throughout the experiment; i.e., during incubation with C₅-DMB-GlcCer, during warming and back exchange, and during the microscopic observation. Parallel control cultures showed intracellular fluorescence similar to that in Fig. 1, C and D.

‡ ATP depletion was performed as described (Martin and Pagano, 1987) by pretreating cells with 5 mM NaN₃ and 50 mM 2-deoxyglucose in glucose-free HMEMB for 30 min at 37°C, and performing all subsequent incubations and washes in glucose-free HMEMB containing these inhibitors.

§ +, Prominent labeling of the Golgi apparatus, e.g., as in Fig. 5 A; ±, reduced accumulation at the Golgi apparatus as in Fig. 5 B; −, minimal labeling of the Golgi apparatus as in Fig. 7 B.

‖ Cell cultures on glass coverslips were washed with HMEMB, incubated with 2.5 μM C₅-DMB-GlcCer/BSA for 30 min at 2°C, washed, and warmed for 15 min at 37°C in the presence of each sphingolipid analogue/BSA complex (see Materials and Methods). The cells were then both back exchanged and observed in the presence of each sphingolipid analogue/BSA complex.

¶ N-ethylmaleimide treatment was followed by a 5-min treatment on ice with 0.5 mM DTT in HMEMB and washing with HMEMB before incubation with C₅-DMB-GlcCer/BSA.

** Fixative contained 3% paraformaldehyde and 0.02% glutaraldehyde in PBS.
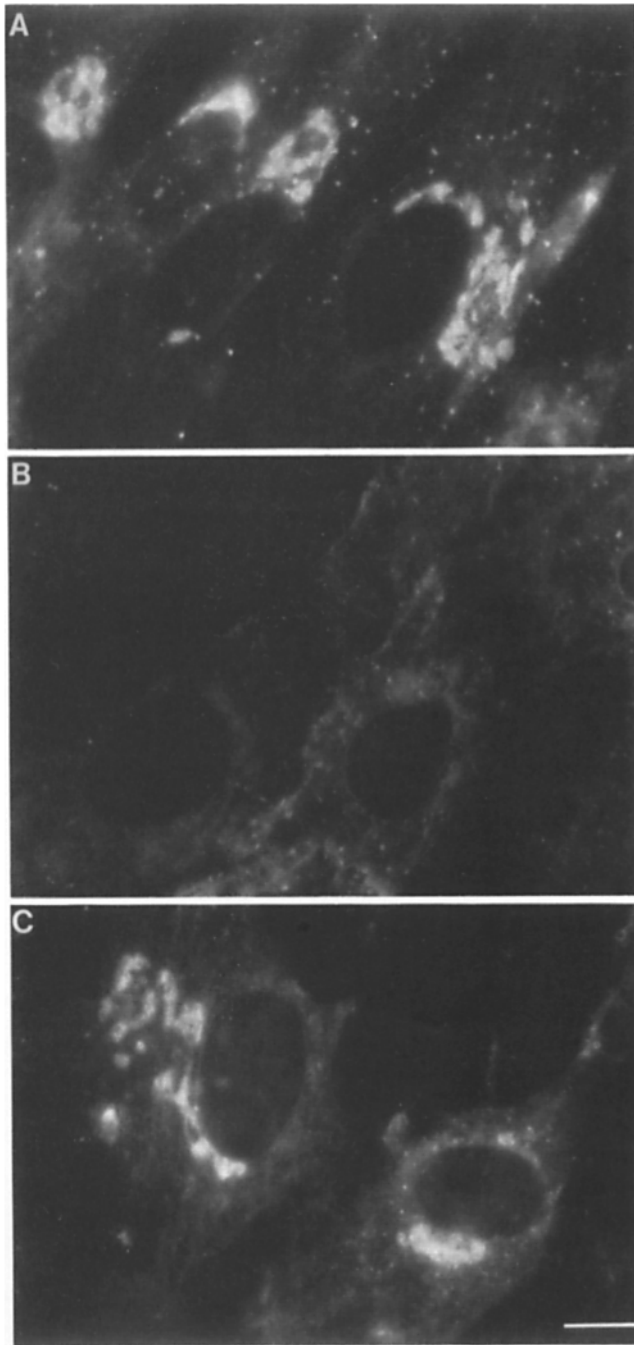
Figure 7. A nonfluorescent GlcCer analogue reduces accumulation of C_5-DMB-GlcCer at the Golgi apparatus. Cells were incubated with 2.5 μM C_5-DMB-GlcCer/BSA for 30 min at 2°C, washed, and warmed for 15 min at 37°C in the presence of (*A*) BSA alone, (*B*) 100 μM C_6-GlcCer/BSA, or (*C*) 100 μM C_6-GalCer/BSA. The samples were then back exchanged in the absence (*A*) or presence (*B* and *C*), of nonfluorescent C_6-sphingolipid/BSA, and photographed at green + red wavelengths. Note the prominent labeling of the Golgi apparatus in (*A*) and (*C*) relative to that in (*B*). Bar, 10 μm.

and Pagano, 1990), which has a high affinity for ceramide (Lipsky and Pagano, 1985; Pagano et al., 1989, 1991; Pagano, 1990*b*; Rosenwald and Pagano, 1993).

Intracellular Transport of C_5-DMB-GlcCer

Incubation of cells with C_5-DMB-GlcCer/BSA resulted in a markedly different pattern of intracellular fluorescence than that seen with C_5-DMB-SM and C_5-DMB-LacCer. When cells were treated with C_5-DMB-GlcCer/BSA and subsequently warmed to 37°C, the fluorescent lipid redistributed into punctate dots of fluorescence throughout the cytoplasm and rapidly into other intracellular membranes; e.g., the nuclear envelope and Golgi apparatus. A working model for the intracellular transport of C_5-DMB-GlcCer is given in Fig. 8 *B* and discussed below.

Transbilayer Movement of C_5-DMB-GlcCer at the Plasma Membrane and Subsequent Labeling of Intracellular Membranes. During low temperature incubation, C_5-DMB-GlcCer labeled the plasma membrane. However, 25–30% of the C_5-DMB-GlcCer incorporated into the plasma membrane at low temperature was resistant to back exchange with defatted BSA (Table I). One possible explanation for this observation is that a portion of the C_5-DMB-GlcCer was transported to the inner leaflet of the plasma membrane bilayer, where it became inaccessible to back exchange with defatted BSA (Fig. 8 *B*, *step I*). Such a mechanism is the case for fluorescent analogues of phosphatidylethanolamine and phosphatidylserine, which label the plasma membrane of Chinese hamster fibroblasts and undergo transbilayer movement at low temperature (Martin and Pagano, 1987). Alternatively, a portion of the C_5-DMB-GlcCer at the cell surface might interact with other cell surface constituents, reducing the size of the back-exchangeable pool of this lipid. Once present on the cytoplasmic leaflet of the plasma membrane, C_5-DMB-GlcCer could move by spontaneous or protein-mediated diffusion to label other intracellular membranes (Fig. 8 *B*, *step II*). As discussed above for C_5-DMB-SM, such an explanation is consistent with our observations of C_5-DMB-GlcCer distribution in perforated cells after low temperature incubations.

Transbilayer Movement of C_5-DMB-GlcCer during Endocytosis and Subsequent Labeling of Intracellular Membranes. During warming to 37°C, some C_5-DMB-GlcCer also enters the cell by an endocytic route (Fig. 8 *B*, *step III*). Indeed, inhibition of endocytosis by ATP depletion eliminated the presence of punctate fluorescence in the cytoplasm, which most likely corresponds to endosomes, but otherwise had little effect on labeling of other intracellular membranes (compare Fig. 5, *A* and *B*, *left panels*).

It is likely that transbilayer movement of C_5-DMB-GlcCer also occurs in endosomes (Fig. 8 *B*, *step IV*), since in the time course experiment of C_5-DMB-GlcCer distribution (Fig. 1), fluorescence was initially seen primarily in endosomes, while at later times, fluorescence was widely distributed in other intracellular membranes and reduced in vesiclelike structures. However, transport of C_5-DMB-GlcCer from endosomes to lysosomes was not required for labeling of the nuclear envelope, Golgi apparatus, and other intracellular membranes, since labeling of these membranes also occurred at 19°C (Fig. 6 *B*), a temperature at which vesicular transport between endosomes and lysosomes is blocked (Dunn et al., 1980; Aulinskas et al., 1982).
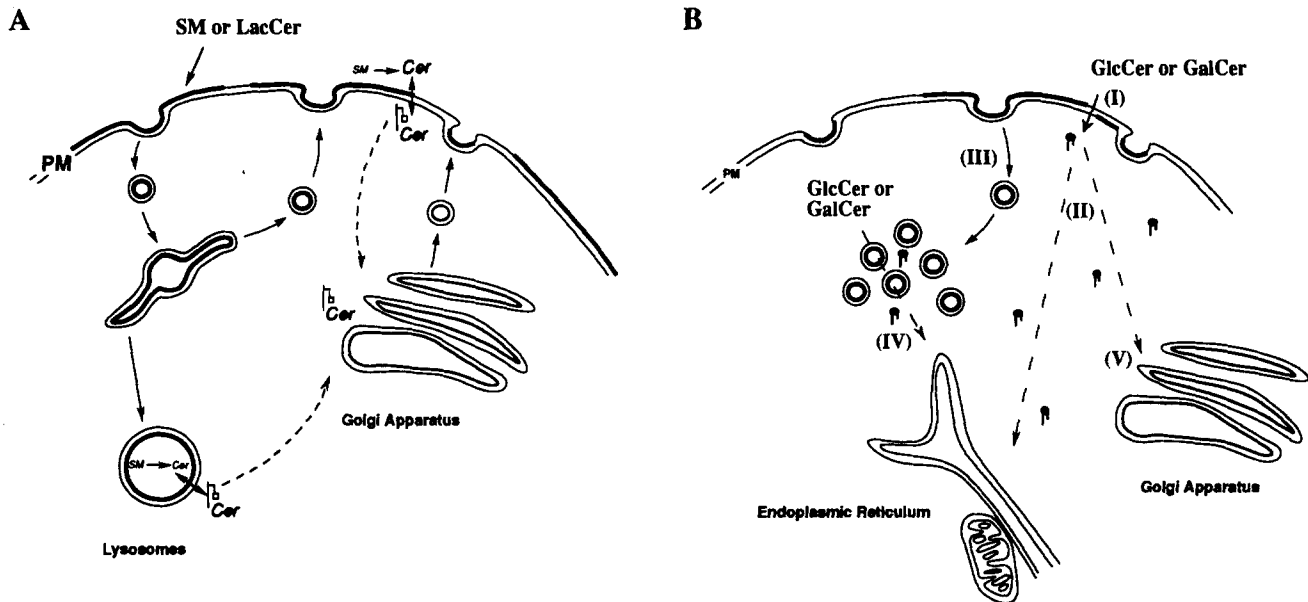
A SM or LacCer

B

Figure 8. Working model for internalization of fluorescent sphingolipid analogues from the plasma membrane to intracellular membranes. Thick lines represent portions of the bilayer containing fluorescent lipid. (A) C₅-DMB-SM and C₅-DMB-LacCer, initially present in the outer leaflet of the plasma membrane bilayer, are restricted to the lumenal leaflet of endocytic vesicles and produce a punctate pattern of intracellular fluorescence during internalization. These lipids are also slowly transported to the lysosomes along the degradative pathway (see Koval and Pagano, 1990). A portion of the fluorescent sphingomyelin is degraded by a neutral sphingomyelinase at the plasma membrane and an acidic sphingomyelinase in the lysosomes. The ceramide formed by this process subsequently stains the Golgi apparatus. (B) C₅-DMB-GlcCer and C₅-DMB-GalCer, initially present in the outer leaflet of the plasma membrane bilayer, undergo transbilayer movement to the cytosolic surfaces of the plasma membrane and endosomes. These fluorescent lipids are then able to label numerous intracellular membranes including the Golgi apparatus, where they accumulate. Roman numerals, steps of the internalization process discussed in text.

The process of C₅-DMB-GlcCer transport from the plasma membrane and endosomes to label other intracellular membranes was inhibited at 10°C (Fig. 6), but not by a wide variety of drugs, inhibitors, and enzymatic treatments (Table II). Thus, it seems likely that the transbilayer movement of C₅-DMB-GlcCer at the plasma membrane and endosomes and subsequent labeling of intracellular membranes by this lipid was not the result of a protein-mediated process, although we cannot completely exclude this possibility.

It is interesting to note that the intracellular distribution of C₅-DMB-GalCer was similar to that seen with C₅-DMB-GlcCer, except that more vesicle-like structures and weaker labeling of the Golgi apparatus were seen when C₅-DMB-GalCer was used (compare Fig. 2, C vs D). This observation, along with our finding that C₅-DMB-GalCer is efficiently removed from the cell surface by back exchange (Table I), suggests that C₅-DMB-GalCer undergoes transbilayer movement at the plasma membrane and endosomes more slowly than C₅-DMB-GlcCer.

Accumulation of C₅-DMB-GlcCer at the Golgi Apparatus. The mechanism for accumulation of C₅-DMB-GlcCer at the Golgi apparatus (Fig. 8 B, step V) is unknown. Interestingly, incubation of cells with a non-fluorescent analogue of GlcCer (but not GalCer) reduced the labeling of the Golgi apparatus by C₅-DMB-GlcCer (compare Fig. 7, A vs B), although it is not clear whether this analogue directly inhibited the accumulation of C₅-DMB-GlcCer at the Golgi apparatus, or reduced the labeling there because transport of

C₅-DMB-GlcCer from the plasma membrane and endosomes to intracellular membranes was slowed. In addition, labeling of the Golgi apparatus by C₅-DMB-GlcCer was reduced in ATP-depleted cells (compare Fig. 5, A vs B, right panels), suggesting that accumulation of the GlcCer analogue at this organelle might be an energy-dependent process. The accumulation of a fluorescent analogue of phosphatidylserine at the Golgi apparatus is also ATP-dependent (Kobayashi and Arakawa, 1991).

One intriguing possibility for the accumulation of C₅-DMB-GlcCer at the Golgi apparatus is related to the synthesis of endogenous GlcCer. Previous studies have demonstrated that GlcCer synthase is enriched at the Golgi complex, and that synthesis of GlcCer occurs on the cytosolic surface of membrane vesicles derived from this organelle (Coste et al., 1985, 1986; Futerman and Pagano, 1991; Jeckel et al., 1992; Trinchera et al., 1991). These findings imply that after its synthesis, GlcCer must undergo transbilayer movement to the lumenal surface to account for the known topology of higher order glycosphingolipids within the Golgi apparatus and plasma membrane. Thus, it is tempting to speculate that C₅-DMB-GlcCer accumulates at the Golgi apparatus because it uses the same molecular machinery present at the Golgi apparatus for transbilayer movement of newly synthesized endogenous GlcCer.

Sorting of Sphingolipids in the Endocytic Pathway

The intracellular transport of N-(N-[6-[(7-nitrobenz-2-oxa-

1,3-diazol-4-yl)amino] hexanoyl]) glucosyl sphingosine (C$_6$-NBD-GlcCer) has been examined in several cell types and its internalization from the plasma membrane compared with the corresponding analogue of sphingomyelin, C$_6$-NBD-SM (Kok et al., 1989, 1991, 1992). Evidence is presented that in the human colon adenocarcinoma cell line HT29, C$_6$-NBD-GlcCer and C$_6$-NBD-sphingosylphosphorylcholine are sorted during internalization along the endocytic pathway (Kok et al., 1991); i.e., while C$_6$-NBD-GlcCer is transported to the Golgi apparatus, C$_6$-NBD-sphingosylphosphorylcholine moves predominantly to the lysosomes. Although the mechanism for the sorting of these sphingolipid analogues is unknown, it was suggested that these lipids separate from one another by sequestration into different membrane domains that enter different populations of endosomes (Hoekstra and Kok, 1992). While the present study was carried out using different fluorescent analogues of GlcCer and sphingomyelin and a different cell type, it raises the possibility that these lipids may segregate from one another upon internalization from the plasma membrane because they use endocytic and nonendocytic uptake mechanisms to different extents.

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