Molecular Trapping of a Fluorescent Ceramide Analogue at the Golgi Apparatus of Fixed Cells: Interaction with Endogenous Lipids Provides a *trans*-Golgi Marker for Both Light and Electron Microscopy

Richard E. Pagano, Michael A. Sepanski, and Ona C. Martin

Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210-3301

Abstract. We have previously shown that a fluorescent derivative of ceramide, *N*-(ε-7-nitrobenz-2-oxa-1,3-diazol-4-yl-aminocaproyl)-D-*erythro*-sphingosine (C_6-NBD-Cer), vitally stains the Golgi apparatus of cells (Lipsky, N. G., and R. E. Pagano. 1985. *Science (Wash. DC)*. 228:745–747). In the present paper we demonstrate that C_6-NBD-Cer also accumulates at the Golgi apparatus of fixed cells and we explore the mechanism by which this occurs. When human skin fibroblasts were fixed with glutaraldehyde and then incubated with C_6-NBD-Cer at 2°C, the fluorescent lipid spontaneously transferred into the cells, labeling the Golgi apparatus as well as other intracellular membranes. Subsequent incubations with defatted BSA at 24°C removed excess C_6-NBD-Cer from the cells such that fluorescence was then detected only at the Golgi apparatus. Similar results were obtained using other cell types. A method for visualizing the fluorescent lipid at the electron microscopic level, based on the photoconversion of a fluorescent marker to a diaminobenzidine product (Sandell, J. H., and R. H. Masland. 1988. *J. Histochem. Cytochem.* 36:555–559), is described and evidence is presented that C_6-NBD-Cer was localized to the *trans* cisternae of the Golgi apparatus. While accumulation occurred in cells fixed in various ways, it was inhibited when fixation protocols that extract or modify cellular lipids were used. In addition, Filipin, which forms complexes with cellular cholesterol, labeled the Golgi apparatus of fixed cells and inhibited accumulation of C_6-NBD-Cer at the Golgi apparatus. These results are discussed in terms of a simple model based on the physical properties of C_6-NBD-Cer and its interactions with endogenous lipids of the Golgi apparatus. Possible implications of these findings for metabolism and transport of (fluorescent) sphingolipids in vivo are also presented.

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We have developed a series of fluorescent lipid derivatives that are useful in studying the synthesis, molecular sorting, and intracellular transport of lipids in animal cells (for review see Pagano and Sleight, 1985). The metabolism of these lipids in cells can be studied by conventional lipid biochemical procedures, and these data can then be correlated with the intracellular distribution of these molecules within living cells by fluorescence microscopy. One lipid – a fluorescent analogue of ceramide, *N*-(ε-7-nitrobenz-2-oxa-1,3-diazol-4-yl-aminocaproyl)-D-*erythro*-sphingosine (C_6-NBD-Cer)[1] – is particularly interesting because it prominently labels the Golgi apparatus of cells (Lipsky and Pagano, 1983, 1985*a*,*b*), providing a means for studying the dynamics of the Golgi apparatus (Cooper, M. S., A. H. Cornell-Bell, A. Chernjavsky, and S. J. Smith, unpublished observations) and the traffic of sphingolipids through this organelle (Lipsky and Pagano, 1983, 1985*a*; van Meer et al., 1987).

When living cells are treated with C_6-NBD-Cer at low temperature and washed, the endoplasmic reticulum, nuclear envelope, and mitochondria become fluorescently labeled. Upon warming the cells to 37°C, the fluorescent ceramide is metabolized to fluorescent sphingomyelin and glucosylceramide, and, concomitantly, the Golgi apparatus becomes intensely fluorescent. With increasing time at 37°C, the plasma membrane also becomes visibly labeled as fluorescent sphingomyelin and glucosylceramide are transported to the cell surface. Transport of the newly synthesized fluorescent sphingolipids to the plasma membrane is inhibited by the ionophore Monensin (Lipsky and Pagano, 1985*a*) and during mitosis (Kobayashi and Pagano, 1989), as is the movement of newly synthesized glycoproteins to the cell surface under these conditions (Griffiths et al., 1983; Quinn et al., 1983;

1. *Abbreviations used in this paper*: BHK, baby hamster kidney; C_6-NBD-Cer, *N*-(ε-7-nitrobenz-2-oxa-1,3-diazol-4-yl-aminocaproyl)-D-*erythro*-sphingosine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CHO, Chinese hamster ovary; DAB, diaminobenzidine; DF-BSA, defatted bovine serum albumin; HCMF, 10 mM Hepes-buffered Ca- and Mg-free Puck's saline, pH 7.4; HMEM, 10 mM Hepes-buffered Dulbecco's minimum essential medium without indicator; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; TPPase, thiamine pyrophosphatase.

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Warren, 1985). These results suggest that the fluorescent sphingomyelin and glucosylceramide analogues are synthesized intracellularly and then transported from the Golgi apparatus to the plasma membrane by a vesicle-mediated process analogous to the well-documented "secretory pathway" for plasma membrane and secretory proteins (Burgess and Kelly, 1987). (The topology of fluorescent sphingomyelin and glucosylceramide at the Golgi apparatus has not been determined directly, but since the lumenal elements of the Golgi cisternae are topologically equivalent to the external leaflet of the plasma membrane and since the fluorescent sphingomyelin and glucosylceramide delivered to the plasma membrane can be back-exchanged from the cell surface by incubation with nonfluorescent liposomes or defatted BSA [DF-BSA] [Lipsky and Pagano, 1985*a*; van Meer et al., 1987], it is assumed that fluorescent sphingomyelin and glucosylceramide are restricted to the lumenal leaflet of the Golgi apparatus.)

While transport of the endogenously synthesized fluorescent sphingolipids from the Golgi apparatus to the plasma membrane appears to be vesicle mediated, the mechanism whereby C_6-NBD-Cer initially accumulates at the Golgi apparatus is unclear. One possibility is that the fluorescent lipid, and perhaps its metabolites, are transported to the Golgi apparatus by an energy- and temperature-dependent process analogous to that seen for the delivery of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus. Alternatively, C_6-NBD-Cer labeling of the Golgi apparatus might be due to binding of the fluorescent lipid to enzymes involved in ceramide metabolism or to the lipid's possible preferential partitioning into this organelle as a result of some special "physical property" of the Golgi membrane. In our initial attempts to distinguish among these possibilities, we were unable to inhibit C_6-NBD-Cer labeling of the Golgi apparatus at low temperatures or in the presence of various metabolic inhibitors. Surprisingly, fluorescent labeling of the Golgi apparatus even occurred in cells that had been fixed before incubation with the fluorescent lipid. In the present paper we characterize C_6-NBD-Cer labeling of the Golgi apparatus of fixed cells and present evidence that accumulation of the fluorescent lipid is restricted to the *trans*-Golgi cisternae, most likely through interactions with endogenous lipids of the Golgi apparatus.

Materials and Methods

Cells and Cell Culture

Monolayer cultures of BHK-21 (C-13) (CCL10; American Type Culture Collection, Rockville, MD) fibroblasts were grown in Dulbecco's minimum essential medium supplemented with 10% FBS, 10% tryptose phosphate broth, and 50 μg/ml gentamicin. Chinese hamster ovary (CHO-K1) fibroblasts (CCL61; American Type Culture Collection) were grown in Dulbecco's minimum essential alpha medium (410-2000; Gibco Laboratories, Grand Island, NY) supplemented with 5% FBS and 50 μg/ml gentamicin. Normal (GM0041B; 41B) human skin fibroblasts (Coriell Institute, Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Dulbecco's minimum essential medium supplemented with 20% FBS and 50 μg/ml gentamicin. All cells were grown at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Cells were grown on 25-mm-diameter (No. 1 thickness) glass coverslips for fluorescence microscopy and in 35-mm-diameter tissue culture dishes for other analyses.

Inhibitor Treatment of Cells

For ATP depletion (Martin and Pagano, 1987) or treatment with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), cells were washed in 10 mM Hepes-buffered Dulbecco's minimum essential medium without indicators (HMEM), pH 7.4, lacking glucose and incubated 30 min at 37°C with HMEM lacking glucose but containing (*a*) 5 mM sodium azide and 50 mM 2-deoxyglucose or (*b*) 10 μM CCCP. All subsequent incubations were carried out in the absence of glucose and in the presence of the indicated inhibitors. Aqueous solutions of CCCP were made by dilution of a 10 mM stock solution of CCCP in ethanol that was stored at −70°C.

Fixation Protocols

Monolayer cultures were rinsed in HMEM and fixed as follows. For glutaraldehyde fixation, cells were incubated for 10–30 min at room temperature in 0.5% glutaraldehyde in either (*a*) calcium-free Hanks' balanced salt solution containing 2 mM $MgCl_2$, 2 mM EGTA, 1 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.1; (*b*) 5% sucrose and 100 mM Pipes, pH 7; or (*c*) 0.1 M cacodylate buffer, pH 7.4. In some cases, the fixed cultures were then transferred to an ice-water bath and incubated (three times for 5 min) with freshly prepared $NaBH_4$ (0.5 mg/ml; Weber et al., 1978) in ice-cold 10 mM Hepes-buffered calcium- and magnesium-free Puck's saline (HCMF), pH 7.4. The cells were then rinsed several times over a 20–30-min period in cold HCMF. For formaldehyde fixation, cells were incubated for 10–30 min at room temperature in 2–3% formaldehyde in PBS. For methanol/acetone fixation, cells were washed in HMEM at room temperature and plunged into −20°C methanol for 5 min followed by −20°C acetone for 2 min. The cells were then rinsed several times in HCMF at room temperature.

Incubation of Fixed Cells with Fluorescent Lipids

Fluorescent (7-nitrobenz-2-oxa-1,3-diazol-4-yl [NBD]) *N*-acyl-sphingosines were synthesized, purified, and then complexed with DF-BSA as previously described (Pagano and Martin, 1988). The complexes were ~5 μM in both the fluorescent lipid and DF-BSA. 1,2-(acyl, NBD-aminocaproyl) phosphatidic acid was synthesized as described (Pagano and Longmuir, 1985).

Fixed cells were washed in HCMF and incubated for 30 min at 2°C with a fluorescent *N*-acyl-sphingosine–DF-BSA complex. The cells were then washed in HCMF and incubated with 3.4 mg DF-BSA/ml HCMF ("back-exchanged") to remove excess C_6-NBD-Cer from the preparation (see Results). Unless otherwise stated, four 30-min back-exchanges at room temperature were performed. The glass coverslips were then mounted on depression slides and observed by fluorescence microscopy. In one experiment, lipid vesicles (Pagano and Longmuir, 1985) containing 1,2-(acyl, NBD-aminocaproyl) phosphatidic acid were used in place of C_6-NBD-Cer–DF-BSA.

Filipin Labeling of Fixed Cells

Cells were fixed in glutaraldehyde or formaldehyde as described above, washed in HCMF, and incubated for 15 min at room temperature with 125 μg Filipin/ml HCMF (Blanchette-Mackie et al., 1988). The cells were then washed in HCMF and observed by fluorescence microscopy using a 100× Neofluar objective and ultraviolet excitation (filter pack 487702; Carl Zeiss, Inc., Thornwood, NY). Aqueous solutions of Filipin were prepared immediately before use from fresh stock solutions containing 5 mg Filipin (Sigma Chemical Co., St. Louis, MO)/ml of dimethylformamide or ethanol.

C_6-NBD-Cer Labeling of Cells Stained for Thiamine Pyrophosphatase (TPPase)

In some studies, samples were stained for TPPase (Novikoff and Goldfisher, 1961; Novikoff, 1976; Lewis and Knight, 1977) before incubation with C_6-NBD-Cer as follows. Cells were fixed for 60 min at 2°C with 0.5% glutaraldehyde, 5% sucrose, 100 mM Pipes, pH 7, washed, and incubated overnight at 4°C in 10% sucrose, 100 mM Pipes, pH 7. The samples were then rinsed in 80 mM Tris-maleate, pH 7.2, and incubated for 90 min at 37°C in a freshly prepared solution of 4 mM $Pb(NO_3)_2$, 5 mM $MnCl_2 \cdot 4H_2O$, 2.5 mM cocarboxylase (TPPase), 80 mM Tris-maleate, pH 7.2, and 146 mM sucrose that was centrifuged for 5 min at 15,000 *g* immediately before use. The cells were then washed with 80 mM Tris-maleate, pH 7.2, and incubated for 2 min at room temperature with freshly prepared 1% ammonium sulfide. Control samples were treated identically, except that incubations were carried out in the absence of either $Pb(NO_3)_2$, cocarboxylase, or ammonium sulfide treatment. After staining for TPPase, the cells were washed in HCMF and treated with C_6-NBD-Cer as described above.

Lipid Analysis

Fixed cells were harvested for lipid extraction using a rubber policeman and

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then extracted by the procedure of Bligh and Dyer (1959) using 0.9% NaCl and 10 mM HCl in the aqueous phase. Lipid extracts were analyzed qualitatively by thin layer chromatography using $CHCl_3/CH_3OH/H_2O$ (65:25:4) or $CHCl_3/CH_3OH/28\%$ NH_4OH/H_2O (160:40:1:3) as the developing solvent.

Electron Microscopic Studies of C_6-NBD-Cer in Fixed Cells

Cells grown on 35-mm-diameter tissue culture dishes were fixed for 30 min at room temperature in 1% glutaraldehyde, incubated with C_6-NBD-Cer–DF-BSA at 2°C, and back-exchanged four times (30 min each) with 3.4 mg DF-BSA/ml HCMF at room temperature. The samples were then washed in 0.1 M Tris buffer, pH 7.6, and 0.9 ml of a fresh solution of 1.5 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA)/ml 0.1 M Tris buffer, pH 7.6, was added to the culture dish. After 10 min, an area of the culture dish was irradiated for 30 min at room temperature using the 476.5-nm line of a tunable argon laser operating at 50 mW power. To obtain a relatively large area of irradiated cells, the laser beam was expanded to a line ~1 mm wide by 1 cm long using a cylindrical lens. Similar results were obtained by irradiating the specimen under a conventional fluorescence microscope at low magnification (6.3×) using a filter combination appropriate for excitation of the NBD fluorophore (filter pack 487717; Carl Zeiss, Inc.; λ_{ex} = 485 nm), except that a much smaller area of the culture dish could be irradiated by this method. After irradiation, the sample was washed in 0.1 M Tris, pH 7.6, and observed under the light microscope using a 40× water immersion objective for evidence of a DAB reaction product. The sample was then rinsed in 0.1 M cacodylate buffer, pH 7.4, treated with 1% OsO_4 in 0.1 M cacodylate buffer for 60 min at room temperature, washed in cacodylate buffer, and processed for electron microscopy as described below. Control samples were treated as described above, except that treatment with C_6-NBD-Cer was omitted.

Labeled samples were processed for electron microscopy as follows. After osmication, the samples were washed in 0.1 M cacodylate buffer and rinsed in distilled water. The cells were then stained for 0.5–1 h in 0.5% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon. To minimize heat-induced distortion of the culture dishes, polymerization was initiated at 40°C for 24 h and the plates were then transferred to 57°C for 24–48 h. The culture dishes were split away from the embedding plastic, which was then further polymerized at 70°C for 24 h. Sections were obtained with an ultramicrotome (Porter-Blum MT-2; Sorvall Instruments Div., Newton, CT), mounted on formvar-coated grids, and stained with lead citrate before examination. Electron micrographs were obtained with an electron microscope (100S; JEOL USA, Peabody, MA) operating at 80 kV.

Other Procedures

Fluorescence microscopy was performed using an inverted microscope (IM-35; Carl Zeiss, Inc.) equipped with a Planapo 100× (1.3 NA) and a Neofluar 100× objective. For observation of C_6-NBD-Cer–treated cells after photooxidation in the presence of DAB, a microscope (Universal; Carl Zeiss, Inc.) equipped with a 40× water immersion objective was used. All photomicrographs were obtained using Tri-X film (Eastman Kodak Co., Rochester, NY) and processed at ASA 1,600 with developer (Diafine; Accufine, Inc., Chicago, IL).

Concentrations of C_6-NBD-Cer stock solutions were determined by measurements of relative fluorescence compared with a fluorescent standard containing a known amount of NBD lipid. All measurements were made in 2% Triton X-100 or chloroform/methanol (2:1 [vol/vol]) using a spectrophotofluorimeter (8000C; SLM-Aminco, Urbana, IL) (λ_{ex} = 470 nm; λ_{em} = 530 nm).

Other Chemicals

Tissue culture media were from Gibco Laboratories. Specially purified aqueous detergent solutions of Triton X-100 and NP-40 were from Pierce Chemical Co. (Rockford, IL). Unless otherwise noted, all other chemicals were purchased from Sigma Chemical Co.

Results

C_6-NBD-Cer Labels the Golgi Apparatus of Living Cells at Low Temperature or in the Presence of Inhibitors

When human skin fibroblasts were incubated with C_6-NBD-Cer for 30 min at 2°C, washed, and observed under the fluorescence microscope, intracellular fluorescence was observed as shown in Fig. 1 *a*. This pattern of fluorescence was similar to that seen in other cell types in which the mitochondria, endoplasmic reticulum, and nuclear envelope were labeled under these incubation conditions (Lipsky and Pagano, 1983, 1985*a*,*b*). If the washed cells were maintained at 2°C for 1–2 h, they developed prominent fluorescence in a perinuclear reticular network (Fig. 1 *b*), previously shown to be the Golgi apparatus in human skin fibroblasts (Lipsky and Pagano, 1985*b*). Accumulation of fluorescent lipid at the Golgi apparatus was not inhibited when similar incubations were carried out in the presence of CCCP, an agent which blocks the transport of secretory proteins from the endoplasmic reticulum to the Golgi complex (Jamieson and Palade, 1968; Kruse and Bornstein, 1975; Tartakoff and Vassalli, 1977, 1979) or in cells treated with NaN_3 and 2-deoxyglucose to deplete cellular ATP levels (data not shown). Similar results were obtained using baby hamster kidney (BHK) and Chinese hamster ovary (CHO)–K1 fibroblasts, except that pronounced labeling of the Golgi apparatus was seen as early as 30 min after incubation at 2°C (data not shown). These findings suggested that transport of C_6-NBD-Cer to the Golgi apparatus was temperature and energy independent.

Incubation of C_6-NBD-Cer with Fixed Cells

When cells were fixed with glutaraldehyde and subsequently incubated with C_6-NBD-Cer for 30 min at 2°C, labeling of the Golgi apparatus could be detected, but was difficult to see clearly because of a "high background" from other fluorescently labeled intracellular membranes within the treated cells (Fig. 2 *a*). This background fluorescence was markedly reduced when the labeled cells were incubated with an appropriate acceptor (e.g., lipid vesicles, 10% FBS, or DF-BSA) in a process referred to as back-exchange. Most of this background fluorescence was removed after back-exchange, producing a preparation of fixed cells in which only the Golgi apparatus was prominently stained (Fig. 2 *b*). When the fluorescent lipids from the back-exchanged cells were extracted and analyzed, only C_6-NBD-Cer was detected. Similar results were obtained using BHK (Fig. 2 *c*) and CHO-K1 fibroblasts (Fig. 2 *d*). When fixed cells were treated with 1,2-(acyl, NBD-aminocaproyl) phosphatidic acid (Pagano and Longmuir, 1985), the pattern of intracellular fluorescence was nearly identical to that shown in Fig. 2 *a*, except that no labeling of the Golgi apparatus was seen. This suggested that the NBD labeling of the Golgi apparatus was specific for ceramide (see below) and not simply due to the presence of the fluorophore on an exchangeable (Nichols and Pagano, 1981, 1982) lipid.

The amount of C_6-NBD-Cer remaining in the fixed cells after varying the number of back-exchange treatments with DF-BSA was quantified by extracting the fluorescent lipid from the cells with detergent and determining the relative fluorescence intensity of the extracts (Fig. 3). Complete extraction of NBD fluorescence from the back-exchanged cells was obtained since the extracted cells, which remain attached to the culture dish, were no longer visible by fluorescence microscopy. Increasing the number of back-exchanges resulted in decreasing amounts of cell-associated NBD fluorescence. As shown in Fig. 2, initial back-exchange treatments highlighted staining of the Golgi apparatus by removing NBD fluorescence from other intracellular membranes, while additional back-exchange treatments more gradually decreased

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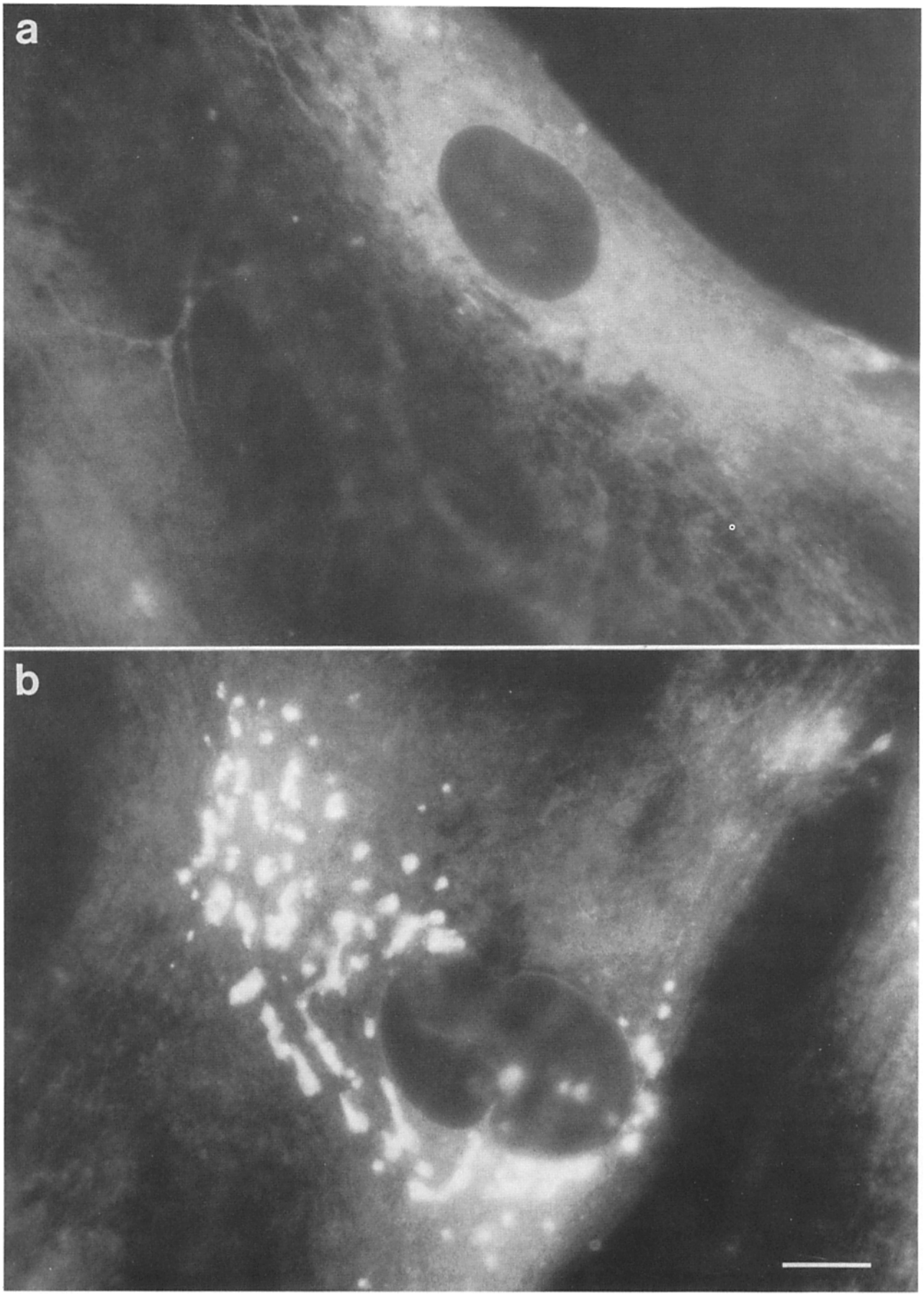

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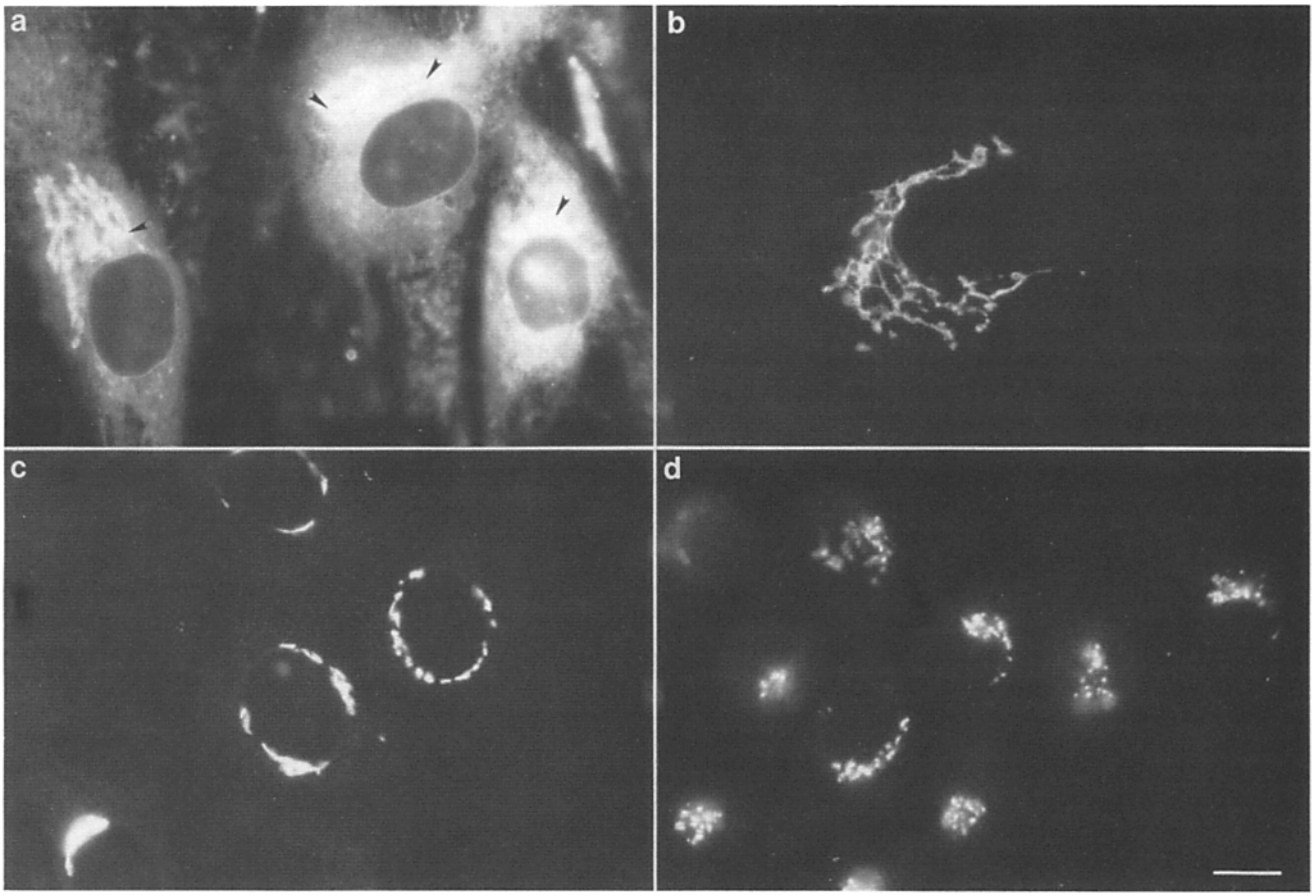

Figure 2. Accumulation of C_6-NBD-Cer at the Golgi apparatus of fixed cells. Cells were fixed with 0.5% glutaraldehyde (see Materials and Methods) for 10 min at room temperature, washed, incubated for 30 min at 2°C with 5 nmol C_6-NBD-Cer–DF-BSA/ml HCMF, and (*a*) washed in HCMF and photographed immediately or (*b–d*) incubated with 3.4 mg DF-BSA/ml HCMF (back-exchanged) at room temperature (four times; 30 min each) before washing in HCMF and photography. (*a* and *b*) Human skin fibroblasts; (*c*) BHK cells; (*d*) CHO-K1 cells. Note that before back-exchange, fluorescence at the Golgi apparatus (*a*, *arrows*) is partially obscured by fluorescence in other regions of the cell. All micrographs were exposed and printed under identical conditions. Bar, 10 μm.

the intensity of staining of the Golgi apparatus (not shown). We found that optimal staining of the Golgi apparatus was obtained after four 30-min back-exchanges at 24°C using 3.4 mg DF-BSA/ml HCMF. The amount of cell-associated C_6-NBD-Cer remaining in cells treated in this manner varied somewhat depending on the cell type (1 and 4.2 × 10^8 molecules per BHK and 41B cell, respectively). Similar results were obtained when a single 120-min back-exchange treatment was substituted for four 30-min back-exchanges (data not shown). Finally, we note that extraction of C_6-NBD-Cer from cells could be accelerated by performing the back-exchanges at 37 or 44°C and that, after overnight back-exchange at these temperatures, essentially all fluorescence was removed from the cells (data not shown).

Electron Microscopic Distribution of C_6-NBD-Cer in Fixed Cells

When C_6-NBD-Cer–treated cells were incubated with DAB and irradiated at wavelengths appropriate for excitation of the NBD fluorophore, either under the fluorescence microscope or using a laser, the NBD fluorescence was photobleached, and a black reaction product formed at the Golgi apparatus as shown in Fig. 4 *a* for CHO-K1 cells. No reaction product was detected in adjacent areas of the culture dish that were not irradiated. In control experiments, cells that had not been treated with C_6-NBD-Cer were irradiated in the presence of DAB under standard conditions (see Materials and Methods) and no reaction product was seen. However, prolonged irradiation (45–60 min) of these unlabeled cells resulted in formation of a diffuse, nonspecific DAB product throughout the cell. Additional control experiments established that when DAB was omitted or when labeled cells were not irradiated, no reaction product was seen.

Cells that had been treated with C_6-NBD-Cer and DAB as described above were further processed and examined under the electron microscope. In these cells, a black reaction product was readily visible in only one or two Golgi cister-

Figure 1. C_6-NBD-Cer accumulates at the Golgi apparatus of living cells at low temperature. Human skin fibroblasts were incubated for 30 min at 2°C with C_6-NBD-Cer, washed, and further incubated in HMEM for (*a*) 0 or (*b*) 60 min at 2°C before photography. Note bright labeling of the Golgi apparatus in *b*. Bar, 10 μm.

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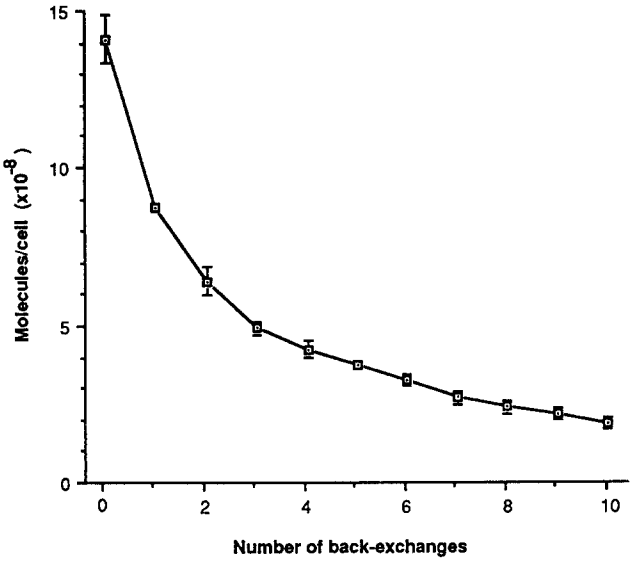

Figure 3. Back-exchange of C_6-NBD-Cer from fixed cells. Monolayer cultures of human skin fibroblasts were fixed, incubated with C_6-NBD-Cer–DF-BSA for 30 min at 2°C, washed, and back-exchanged for the indicated number of times (30 min each) with 3.4 mg DF-BSA/ml HCMF at 24°C. The cells were then extracted with 2% Triton X-100 and the NBD fluorescence of the extracts was quantified in a fluorimeter (see Materials and Methods). Data points are the mean ± SD of three determinations.

nae (Fig. 4, *b* and *c*), but labeling of all of the Golgi stacks was never seen, even when longer irradiation times were used. In some sections, numerous small vesicles were also labeled, often in close proximity to the labeled Golgi stacks (Fig. 4 *c*). Similar results to those shown in Fig. 4 were also obtained using either BHK cells or human skin fibroblasts; however, the human skin fibroblasts were often damaged by the irradiation process.

Effect of TPPase Staining on C_6-NBD-Cer Labeling of the Golgi Apparatus

We stained the *trans*-Golgi cisternae of human skin fibroblasts for TPPase (Novikoff and Goldfisher, 1961; Novikoff, 1976) and examined the effect of this procedure on subsequent labeling of the Golgi apparatus by C_6-NBD-Cer. We first confirmed that under the conditions used to obtain optimal TPPase staining at the light microscope level, only a single Golgi stack was labeled at the electron microscopic level (Fig. 5). We then examined the effect of TPPase staining on the accumulation of C_6-NBD-Cer fluorescence at the Golgi apparatus. In these experiments, TPPase-stained cells were incubated with C_6-NBD-Cer for 30 min at 2°C, washed, and observed, either immediately or after back-exchange, under the fluorescence microscope. In control samples for the TPPase reaction in which lead (Fig. 6, *a* and *c*), TPPase (not shown), or ammonium sulfide treatment (not shown) were omitted, no black deposition product was seen at the Golgi apparatus by phase microscopy, while prominent NBD fluorescence was readily seen at the Golgi apparatus, either before (Fig. 6 *a*) or after (Fig. 6 *c*) back-exchange. In contrast, when all substrates required for TPPase staining were used, the expected black deposition product was readily seen at the Golgi apparatus (Fig. 6, *b* and *d*, *arrows*), while essentially no NBD fluorescence was detected at this organelle.

Effect of Filipin Staining on C_6-NBD-Cer Labeling of the Golgi Apparatus

We studied the labeling of the Golgi apparatus of fixed cells by C_6-NBD-Cer using cells that had been prelabeled with Filipin, a fluorescent polyene antibiotic that forms complexes with cholesterol (for reviews see Miller, 1984; Yeagle, 1985). When human skin fibroblasts were fixed and incubated with Filipin, prominent labeling of the plasma membrane and cytoplasmic vesicles and weaker labeling of the Golgi apparatus (Blanchette-Mackie et al., 1988) were observed (Fig. 7 *a*). In control experiments, repetitive back-exchanges with DF-BSA had no effect on the pattern of Filipin fluorescence. When fixed cells were incubated with Filipin, washed, and subsequently incubated with C_6-NBD-Cer and back-exchanged, a weak cytoplasmic NBD fluorescence was detected, but no NBD labeling of the Golgi apparatus was seen (Fig. 7 *b*). However, if cells were first incubated with C_6-NBD-Cer, incubated with Filipin, and then back-exchanged, very faint NBD labeling of the Golgi apparatus was seen (Fig. 7 *c*). The diminished labeling shown in Fig. 7, *b* and *c*, was not due to the presence of small amounts of organic solvents that were required for Filipin labeling of cells since prominent labeling of the Golgi apparatus was seen in cells treated with C_6-NBD-Cer and back-exchanged with DF-BSA when these procedures were performed in the presence of these solvents (Fig. 7 *d*).

Other Factors Affecting C_6-NBD-Cer Labeling of the Golgi Apparatus of Fixed Cells

Fixation Conditions. We surveyed various fixation procedures to determine their effects on C_6-NBD-Cer labeling of the Golgi apparatus (Table I). Increasing the glutaraldehyde concentration or fixation time or varying the fixation temperature had no effect on labeling of the Golgi apparatus by C_6-NBD-Cer. Formaldehyde-fixed cells gave identical results to those shown in Fig. 2 using glutaraldehyde fixation. Treatment of glutaraldehyde-fixed cells with $NaBH_4$ to reduce autofluorescence (Weber et al., 1978) further reduced background fluorescence, but had no apparent effect on subsequent labeling of the Golgi apparatus by C_6-NBD-Cer (not shown). Labeling of the Golgi apparatus by C_6-NBD-Cer was also not affected by treatment of fixed cells with 0.5–10

Figure 4. Electron microscopic localization of C_6-NBD-Cer at the Golgi apparatus of fixed cells. CHO-K1 cells were fixed, incubated with C_6-NBD-Cer, and back-exchanged to give prominent labeling of the Golgi apparatus as shown in Fig. 2 *d*. The cells were then photobleached in the presence of DAB, washed, treated with OsO_4, and processed for electron microscopy (see Materials and Methods). (*a*) Light micrograph obtained from an 0.5-μm section. Note perinuclear black deposition product (*arrows*). (*b* and *c*) Electron micrographs obtained from thin sections. Note that only one or two Golgi stacks were labeled by this procedure, although numerous small vesicles near the Golgi apparatus were also labeled in some sections (*c*). *N*, nucleus. Bars: (*a*) 10 μm; (*b* and *c*) 0.5 μm.

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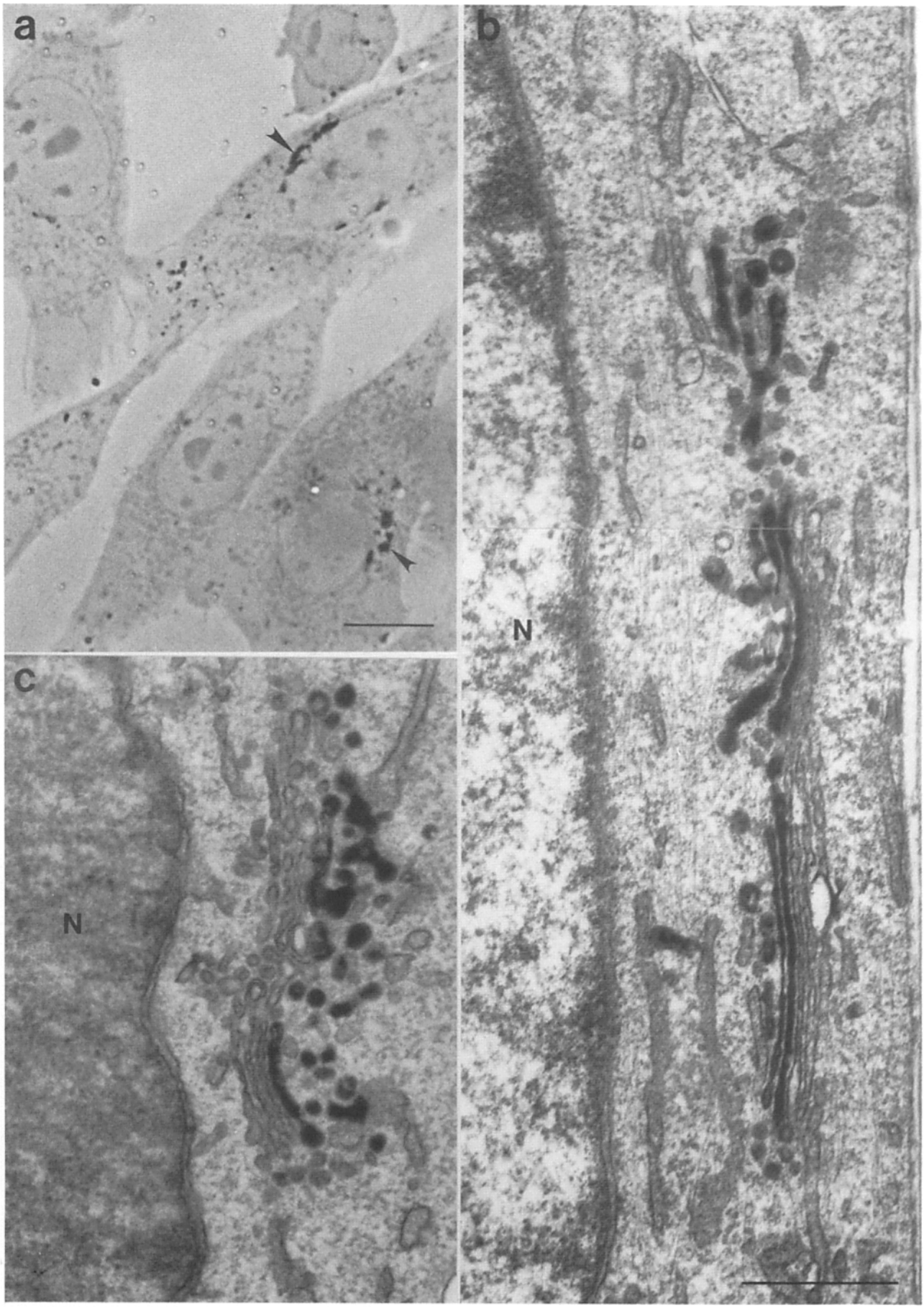

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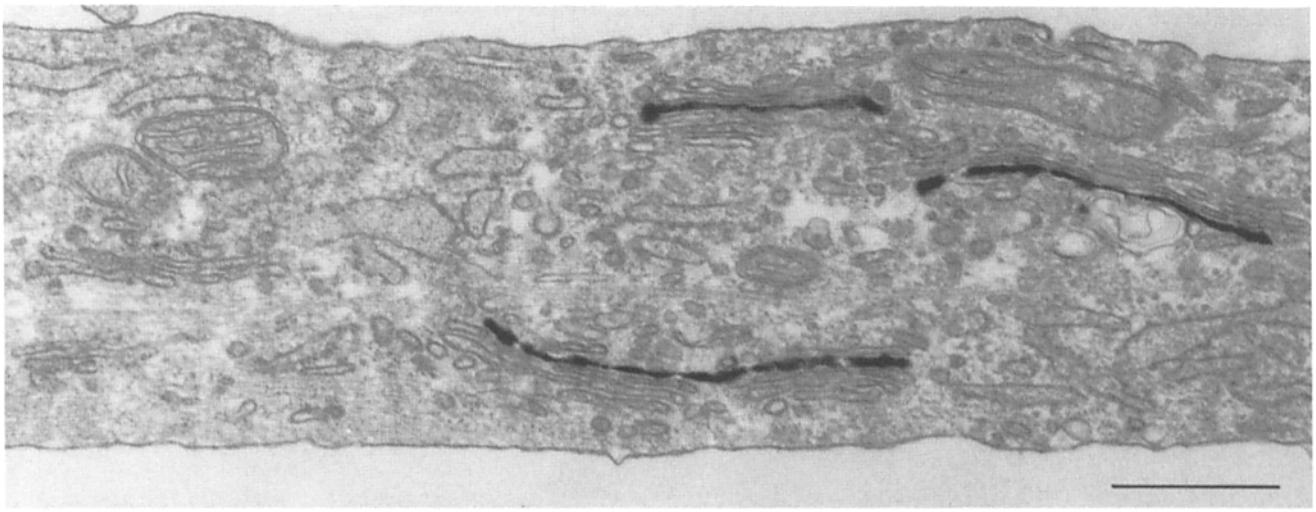

Figure 5. TPPase staining of the *trans*-Golgi cisternae of human skin fibroblasts. Human skin fibroblasts were fixed, stained for TPPase activity (Novikoff, 1976), and processed for electron microscopy. Bar, 0.5 μm.

mM *N*-ethylmaleimide (30 min at 24°C; pH 8.5) before incubation with the fluorescent lipid. However, several other treatments completely eliminated the postfixation staining of the Golgi apparatus by C_6-NBD-Cer. These included osmication of glutaraldehyde-fixed cells, brief treatment of glutaraldehyde-fixed cells with detergents, or fixation of cells with methanol/acetone at −20°C. In these cases, a weak diffuse fluorescence throughout the cells was visible, but no evidence of Golgi apparatus labeling was seen (not shown).

Structure of the Fluorescent N-(Acyl)-Sphingosine. The molecular basis for labeling of the Golgi apparatus of fixed cells by C_6-NBD-Cer was further explored using a structurally related series of *N*-(acyl)-sphingosines synthesized from various long chain bases and different fluorescent fatty acids (Table II). Accumulation of fluorescence at the Golgi apparatus of fixed cells was not seen using *N*-(ε-NBD-aminohexanoyl)-3-keto-sphingosine or *N*-(L-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine, but prominent labeling was seen using *N*-(ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine and *N*-(D-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine under identical incubation conditions (Fig. 8). These results are identical to those reported for this series of molecules in living cells (Pagano and Martin, 1988) and suggest that two regions of the fluorescent *N*-(acyl)-sphingosines, defined by the three-OH group of sphingosine and the D-α-NBD group of the fluorescent fatty acid, are critical for accumulation of these compounds at the Golgi apparatus of fixed cells.

Discussion

In this paper we demonstrate that C_6-NBD-Cer, a vital stain for the Golgi apparatus (Lipsky and Pagano, 1985*b*), also labels this organelle in fixed cells and show that this labeling is specific for the *trans*-Golgi stacks. We explore possible mechanisms for this labeling and present evidence that it most likely results from interactions between C_6-NBD-Cer and endogenous lipids of the Golgi apparatus.

C_6-NBD-Cer Accumulates in the trans-Golgi Stacks

To study the intracellular distribution of C_6-NBD-Cer in fixed cells at the electron microscopic level, we adapted the procedure originally described by Moranto (1982), and elaborated upon by Sandell and Masland (1988), in which a fluorescently labeled specimen is irradiated in the presence of DAB. This procedure produces an electron-opaque osmiophilic polymer at the site of the fluorescent label within the specimen that can then be studied by electron microscopy. Although the precise mechanism of this photoconversion is not known, it has been suggested that the photoexcited molecules react to form oxygen or hydroxyl radicals that then oxidize DAB (Sandell and Masland, 1988), resulting in a reaction product similar to that produced by the peroxidase-catalyzed reaction of DAB with H_2O_2. We extended this method to C_6-NBD-Cer–labeled cells and studied the distribution of the fluorescent lipid within the Golgi apparatus of fixed cells at the electron microscopic level. As shown in Fig. 4, the fluorescent lipid was localized to only one or two stacks of the Golgi apparatus, although numerous small vesicles were also labeled in some sections, possibly resulting from tangential sections through the Golgi cisternae.

To determine which Golgi cisternae were labeled by C_6-NBD-Cer in fixed cells, we first stained the *trans*-Golgi region for TPPase activity and then examined the effect of this procedure on fluorescence labeling of the Golgi apparatus by

Figure 6. TPPase staining inhibits C_6-NBD-Cer labeling of the Golgi apparatus. Human skin fibroblasts were fixed and stained for TPPase. The cells were then incubated with C_6-NBD-Cer and examined under the fluorescence microscope before (*a* and *b*) or after (*c* and *d*) back-exchange with DF-BSA. In *b* and *d*, all substrates required for TPPase staining were present. Note positive TPPase staining in *b* and *d* at arrows and absence of NBD fluoresence at the Golgi apparatus. In *a* and *c*, lead was omitted from the reaction. Note absence of TPPase staining and presence of NBD fluorescence at the Golgi apparatus under these conditions. Fluorescence micrographs were exposed and printed under identical conditions. Bar, 10 μm.

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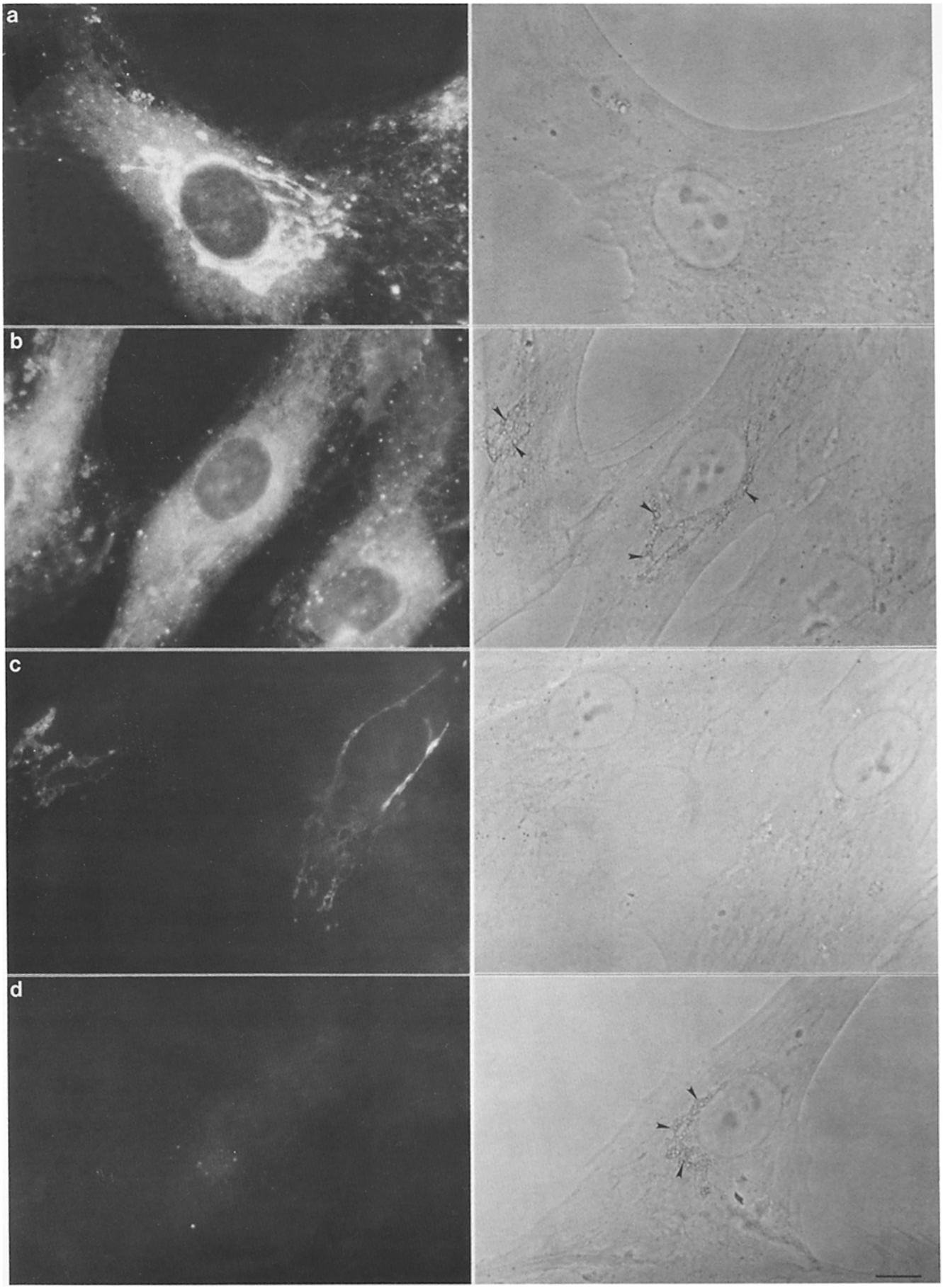

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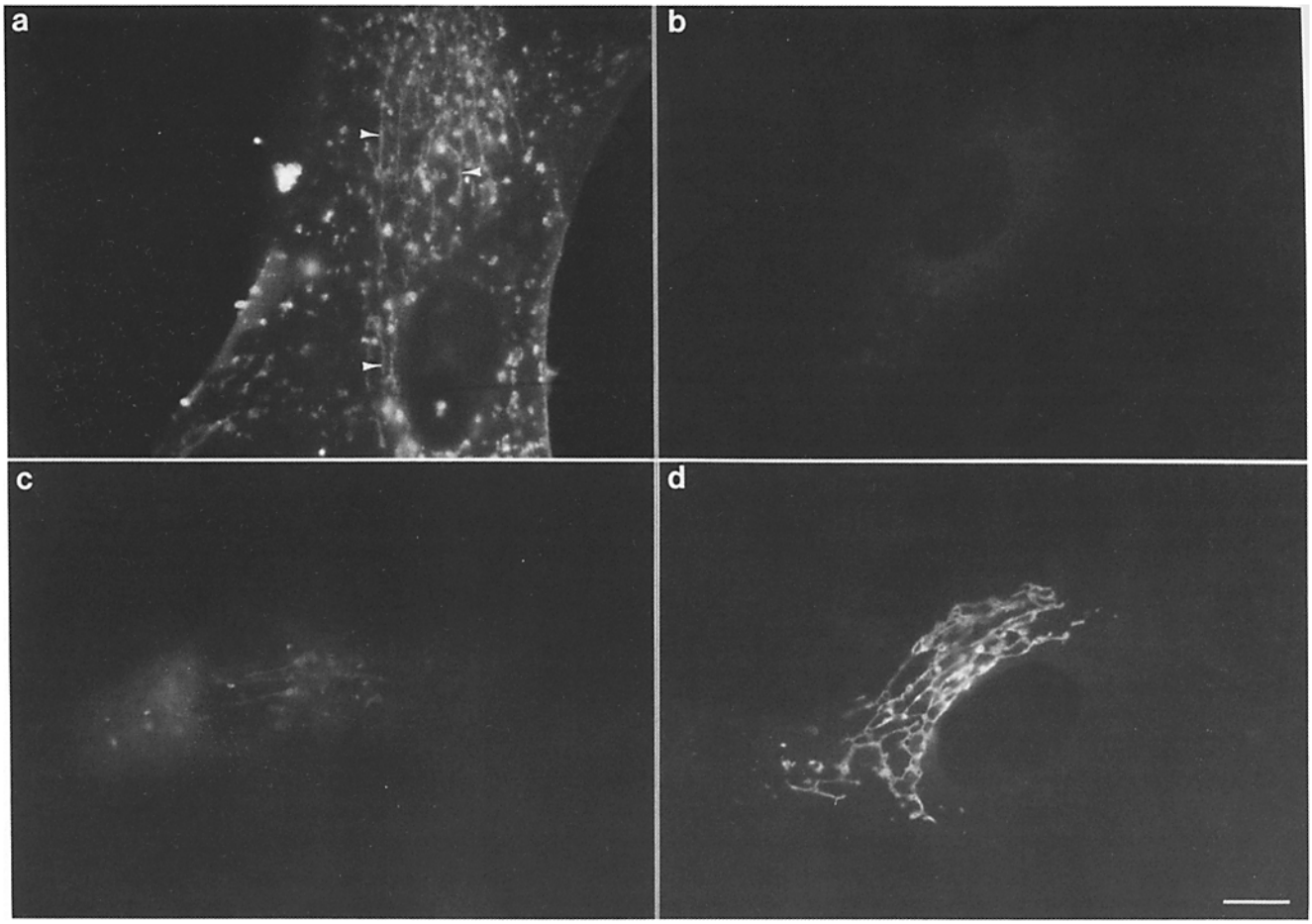

Figure 7. Effect of Filipin on labeling of the Golgi apparatus of fixed cells by C_6-NBD-Cer. Human skin fibroblasts were fixed and stained with (*a*) Filipin alone; (*b*) Filipin followed by C_6-NBD-Cer; (*c*) C_6-NBD-Cer followed by Filipin; or (*d*) C_6-NBD-Cer alone. All samples were then back-exchanged with 3.4 mg/ml DF-BSA (four times; 30 min each at 24°C) before observation and photography. In *d*, C_6-NBD-Cer incubation was performed in the presence of 2.5% ethanol. (*a*) Filipin fluorescence; (*b–d*) NBD fluorescence. In *b–d*, micrographs were exposed and printed under identical conditions. Bar, 10 μm.

*Table I. Effects of Various Fixation Protocols and Permeabilization Procedures on Labeling of the Golgi Apparatus by C_6-NBD-Cer**

Fixation protocol	Golgi labeled‡
Glutaraldehyde§	+
Glutaraldehyde/$NaBH_4$‖	+
Formaldehyde‖	+
Methanol/acetone‖	−
Glutaraldehyde/OsO_4¶	−
Glutaraldehyde/detergents**	−

* Cells (41B, BHK, or CHO-K1) were fixed according to the indicated protocol, washed in HCMF, and incubated for 30 min at 2°C with 5 nmol C_6-NBD-Cer–DF-BSA/ml HCMF. The cells were then washed in HCMF, back-exchanged (four times; 30 min each at 24°C) with 3.4 mg DF-BSA/ml HCMF, washed in HCMF, and examined under the fluorescence microscope.

‡ (+) Golgi labeling as in Fig. 2, *b–d*; (−) no labeling.

§ Cells were fixed with 0.5% glutaraldehyde (10–30 min at 24°C) in various buffers (see Materials and Methods). In some experiments, 1% glutaraldehyde was used.

‖ See Materials and Methods for fixation protocols.

¶ Cells were fixed with 0.5% glutaraldehyde (15 min at 24°C), washed, and incubated with 1% OsO_4 (30 min at 24°C) in 0.1 M cacodylate buffer, pH 7.4.

** Cells were fixed with 0.5% glutaraldehyde, 5% sucrose, 100 mM Pipes, pH 7, (20 min at 24°C), washed, and treated with 0.1% Triton X-100, 0.1% NP-40, or 0.5% Saponin for 3 min at 24°C. The cells were then washed repeatedly in 10% sucrose, 100 mM Pipes for a total of 30 min at 24°C before incubation with C_6-NBD-Cer.

C_6-NBD-Cer. In TPPase-stained cells, C_6-NBD-Cer labeling of the Golgi apparatus was virtually eliminated (Fig. 6, *b* and *d*), while in control experiments in which various components required for a positive TPPase reaction were omitted, C_6-NBD-Cer was readily seen at the Golgi apparatus (Fig. 6, *a* and *c*). In addition, when TPPase-stained cells were subsequently labeled with C_6-NBD-Cer but not back-exchanged, NBD fluorescence was dramatically reduced at the Golgi apparatus, but not in other regions of the cell (compare Fig. 6, *b* and *a*). This control established that the loss of NBD fluorescence at the Golgi apparatus was not due to a nonspecific reduction in NBD fluorescence resulting from the reagents used for TPPase staining. Loss of NBD fluorescence at the Golgi apparatus might be due to the opaque TPPase reaction product blocking transmission of light required for NBD fluorescence excitation. Alternatively, the presence of the reaction product in the *trans*-Golgi stacks might inhibit C_6-NBD-Cer uptake in those stacks by altering the properties of those Golgi membranes. In either case, we conclude that C_6-NBD-Cer labeling of the Golgi apparatus was restricted to the *trans*-most cisternae which are labeled by TPPase staining.

Our finding that treatment of cells with Filipin eliminated staining of the Golgi apparatus by C_6-NBD-Cer (Fig. 7) is

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*Table II. Labeling of the Golgi Apparatus in Fixed Cells by Various Fluorescent N-(Acyl)-Sphingosines**

Fluorescent (NBD) fatty acid	Long-chain base	Golgi labeled
ε-NBD-aminohexanoic acid	D-*Erythro*-sphingosine	+
"	L-*Erythro*-sphingosine	+
"	D-*Threo*-sphingosine	+
"	L-*Threo*-sphingosine	+
"	D-*Erythro*-dihydro sphingosine	+
"	L-*Threo*-dihydro sphingosine	+
	Phytosphingosine	+
"	3-Keto-sphingosine	−
L-α-OH, ε-NBD-amino hexanoic acid	D-*Erythro*-sphingosine	+
D-α-OH, ε-NBD-amino hexanoic acid	"	+
L-α-NBD-aminohexanoic acid	"	−
D-α-NBD-aminohexanoic acid	"	+

* Human skin fibroblasts were fixed with 0.5% glutaraldehyde (10–15 min at 24°C) as described under Materials and Methods, washed, and subsequently incubated with a DF-BSA complex of the indicated fluorescent *N*-(acyl)-sphingosine as described in Table I. The cells were then back-exchanged as described in Table I and examined under the fluorescence microscope for labeling of the Golgi apparatus.

also consistent with localization of C_6-NBD-Cer to the *trans*-Golgi region since Filipin–cholesterol complexes are highly enriched in the *trans*-Golgi stacks (Orci et al., 1981). We speculate that the presence of these complexes could disrupt the structure of this subset of Golgi membranes and inhibit labeling with C_6-NBD-Cer.

Model for C_6-NBD-Cer Labeling of the Fixed Cells

In considering a model for C_6-NBD-Cer labeling of fixed cells, it is important to recall that C_6-NBD-Cer can undergo rapid and spontaneous monomeric transfer (Nichols and Pagano, 1981, 1982) between membranes and transbilayer movement across a given membrane (Pagano and Martin, 1988; Pagano, 1989). As a result of these properties, the most plausible model for C_6-NBD-Cer labeling of fixed cells is that during incubation at low temperature, the fluorescent ceramide transfers as soluble monomers from DF-BSA to the plasma membrane bilayer of the fixed cells. At the plasma membrane, the fluorescent lipid could undergo transbilayer movement to the cytoplasmic leaflet of the plasma membrane, where it could spontaneously transfer by monomer diffusion to all intracellular membranes. These processes would account for the initial labeling pattern seen in fixed cells (Fig. 2 *a*). After this labeling, the cell-associated fluorescent ceramide could be back-exchanged from the cells with DF-BSA by reversing the processes described above. We speculate that because of its interaction with Golgi components, the off-rate for C_6-NBD-Cer at the Golgi apparatus relative to other membranes is reduced, and the fluorescent ceramide present there is more resistant to back-exchange. This model is consistent with the data in Fig. 3 in which a much larger fraction of NBD fluorescence was removed from the cells during the initial back-exchanges, resulting in prominent labeling of the Golgi apparatus (Fig. 2 *b*).

Several pieces of evidence suggest that the trapping of C_6-NBD-Cer at the Golgi apparatus of fixed cells was due to its interaction with Golgi lipids. First, accumulation of C_6-NBD-Cer occurred in cells fixed in a variety of ways, but this accumulation was inhibited when fixation protocols that extracted or modified cellular lipids were used (Table I). Thus, glutaraldehyde-fixed cells rendered permeable by brief treatment with detergents and then washed extensively did not exhibit postfixation staining of the Golgi apparatus by C_6-NBD-Cer, although such treatments have no obvious effect on Golgi morphology seen at the level of the light microscope with fluorescent antibodies (Lipsky and Pagano, 1985*b*). Second, quantitation of the amount of C_6-NBD-Cer present after back-exchange at 24°C indicated that 1–4.2 × 10^8 molecules of the fluorescent lipid were present at the labeled Golgi cisternae of each cell, and electron microscopic localization studies demonstrated that the majority of this lipid was restricted to the *trans*-Golgi stacks. If C_6-NBD-Cer labeling of the Golgi apparatus was due to its binding to a resident Golgi protein(s), the required number of copies of this protein(s) would be extraordinarily high. Finally, Filipin, which forms complexes with cellular cholesterol, labeled the Golgi apparatus of fixed cells and inhibited accumulation of C_6-NBD-Cer at the Golgi apparatus. We conclude that C_6-NBD-Cer most likely accumulates at the *trans*-Golgi apparatus by interaction with endogenous Golgi lipids and speculate that the lipid composition of the *trans*-Golgi apparatus must be unique since the fluorescent lipid did not accumulate in the *cis*- or *medial*-Golgi stacks under these conditions. Recently, Simons and van Meer (1988) have suggested that microdomains of glycosphingolipids, perhaps formed through interlipid hydrogen bonding, might be involved in lipid and protein sorting at the *trans*-Golgi network. If such domains exist, C_6-NBD-Cer might readily integrate into them because of its structural similarity to endogenous sphingolipids. Alternatively, C_6-NBD-Cer might accumulate within the *trans*-Golgi network as a result of interactions with endogenous lipoproteins present there.

Implications of Molecular Trapping of C_6-NBD-Cer at the Golgi Apparatus for In Vivo Studies

We have previously shown that Monensin blocks the appearance of C_6-NBD-sphingomyelin and -glucosylceramide at the plasma membrane, but does not inhibit their intracellular synthesis from C_6-NBD-Cer (Lipsky and Pagano, 1985*a*). Since sphingomyelin synthesis most likely takes place in the Golgi apparatus (for review see Pagano, 1988) and since Monensin is thought to block *medial*-to-*trans* movement of molecules through the Golgi complex (Griffiths et al., 1983; Quinn et al., 1983), synthesis of C_6-NBD-sphingomyelin from C_6-NBD-Cer must occur proximal to this block. (These points have been confirmed using a radioactive analogue of ceramide in isolated rat liver Golgi membranes and in Golgi subfractions [Futerman, A. H., B. Stieger, A. L. Hubbard, and R. E. Pagano, manuscript in preparation.]) In the present study, we have shown that C_6-NBD-Cer selectively partitions into the *trans*-Golgi stacks of fixed cells. If this also occurs in living cells, a paradox arises since C_6-NBD-Cer would be delivered distal to the (Golgi) compartments where it would be further metabolized. In future studies we hope to determine whether C_6-NBD-Cer selectively partitions into the *trans*-Golgi stacks of living cells, and, if so, to resolve this paradox.

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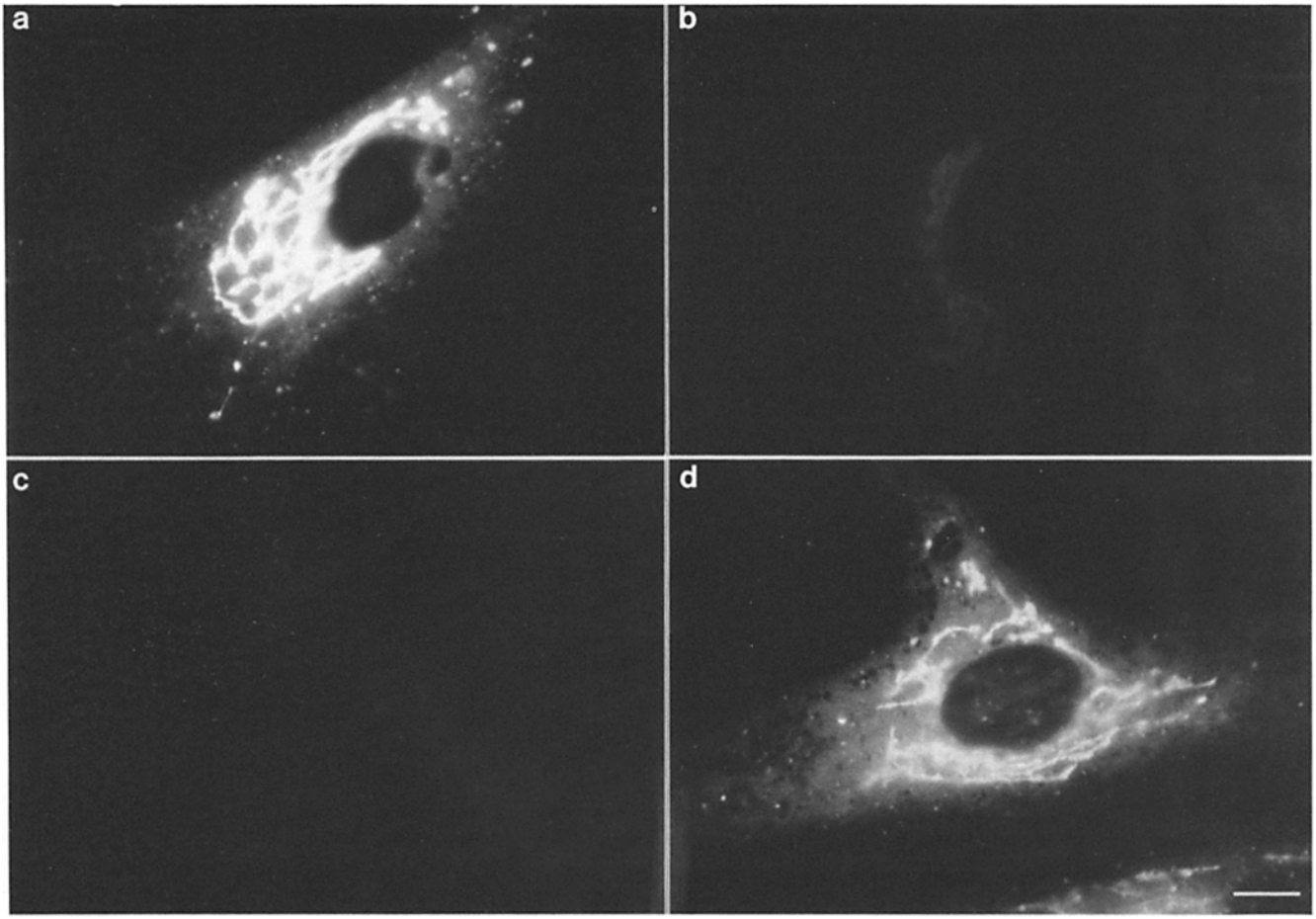

Figure 8. Labeling of the Golgi apparatus of fixed cells by fluorescent ceramide analogues is dependent on the stereochemistry of the fluorescent fatty acid and the nature of long-chain base. Human skin fibroblasts were fixed with glutaraldehyde, washed, and incubated for 30 min at 2°C with 5 nmol/ml of the indicated fluorescent *N*-(acyl)-sphingosine–DF-BSA complexes. The cells were then washed and back-exchanged with 3.4 mg DF-BSA/ml HCMF (four times; 30 min each at room temperature) before washing in HCMF and photography. (*a*) *N*-(ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine; (*b*) *N*-(ε-NBD-aminohexanoyl)-3-keto-sphingosine; (*c*) *N*-(L-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine; (*d*) *N*-(D-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine. All micrographs were exposed and printed under identical conditions. Bar, 10 μm.

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