Effects of a Sphingolipid Synthesis Inhibitor on Membrane Transport through the Secretory Pathway†

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ABSTRACT: We investigated the effects of an inhibitor of sphingolipid biosynthesis, 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), on cells in culture. Two Golgi-associated enzymes were affected by incubation of cells with PDMP. The synthesis of glucosylceramide was inhibited at low concentrations of PDMP (2.5–10 μM), and in the presence of higher concentrations (≥25 μM), synthesis of sphingomyelin was also reduced. Transport of vesicular stomatitis virus G protein through the Golgi complex was progressively retarded by increasing concentrations of PDMP. In the presence of 75 μM PDMP, the half-times of VSV-G protein arrival at the cis, medial, and trans Golgi and the cell surface were increased 1.5-, 2.1-, 2.4-, and 2.8-fold, respectively, compared to control values. Transport of fluorescent sphingolipids, synthesized de novo at the Golgi complex from fluorescent ceramide precursors, to the cell surface was retarded by ~20% in the presence of 50 μM PDMP and by ~50% in the presence of 100 μM PDMP. Control experiments demonstrated that PDMP had minimal effects on cell morphology and physiology (including microtubule and endoplasmic reticulum structure, mitochondrial function, and endocytosis). Although incubation of cells with relatively high concentrations of PDMP was required to see the effects on protein and sphingolipid transport, use of a fluorescent analogue of PDMP demonstrated that most cell-associated PDMP was sequestered in lysosomes, while the concentration at the Golgi complex, the site of the target synthetic enzymes, was relatively low. Taken together, these results suggest that transport of proteins and sphingolipids through the secretory pathway may be coupled to sphingolipid synthesis.

Determination of the molecular mechanisms underlying the transport of both proteins and lipids through the secretory pathway is currently the focus of much attention. Transport of proteins is likely to be mediated by fission of small vesicles from donor membranes, followed by fusion of these vesicles with the appropriate target membranes (Balch, 1989; Rothman & Orci, 1990). Cellular sphingolipids are also thought to be transported to the cell surface by a similar, if not identical, mechanism (Lipsky & Pagano, 1985; van Echten & Sandhoff, 1989; Wieland et al., 1987; Bennet et al., 1988; Kobayashi & Pagano, 1989; Helms et al., 1990; Karrenbauer et al., 1990; Schwarzmann & Sandhoff, 1990; Wattenberg, 1990; Puoti et al., 1991).

A common precursor for sphingolipid synthesis is ceramide (Cer)[1] (Hakomori, 1983; Kanfer, 1983; Sweeley, 1985). Recently, it was shown that the Golgi apparatus is the major site of sphingomyelin (SM) synthesis (Futerman et al., 1990; Jeckel et al., 1990). Glucosylceramide (GlcCer), a precursor of higher-order glycosphingolipids (GSLs), is synthesized at the Golgi apparatus and in a light, smooth vesicle fraction that may be a pre-Golgi compartment (Coste et al., 1985, 1986; Futerman & Pagano, 1991; Trinchera et al., 1991). Since newly synthesized proteins en route to the cell surface must first travel through the compartments containing the sphingolipid biosynthetic enzymes, and since sphingolipids comprise a significant fraction of cellular membrane lipids (Hakomori, 1983; Kanfer, 1983; Sweeley, 1985), these findings raise the intriguing possibility that transport of proteins through the secretory pathway and sphingolipid biosynthesis may be coupled processes. In the present study, we provide indirect evidence for a possible link between these processes using 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), a potent inhibitor of GlcCer synthase (Vunnam & Radin, 1980; Inokuchi & Radin, 1987).

Materials and Methods

Chemicals and Radiochemicals. 1-Phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP) was synthesized as previously described (Vunnam & Radin, 1980; Inokuchi & Radin, 1987). The D,L-threo forms were separated from the D,L-erythro forms of PDMP by crystallization (Inokuchi & Radin, 1987). PDMP throughout the text refers to the D,L-threo mixture. PDMP stocks (10 mM in 2-propanol) were

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[1] Abbreviations: BHK, baby hamster kidney; BSA, bovine serum albumin; C_5-DMB-Cer, *N*-[5-(5,7-dimethyldipyrromethaneboron difluoride)-1-pentanoyl]-D-*erythro*-sphingosine; C_6-NBD-Cer, *N*-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ε-aminohexanoyl]-D-*erythro*-sphingosine; Cer, ceramide (*N*-acylsphingosine); CHO, Chinese hamster ovary; DASMPI, 2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide; endo H, β-endo-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GlcCer, glucosylceramide; GSL, glycosphingolipid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [^{14}C]hexanoyl-Cer, *N*-([1-^{14}C]hexanoyl)-D-*erythro*-sphingosine; HMEM, 10 mM HEPES buffered Eagle's MEM, pH 7.4, without indicator and with 1.3 mM $CaCl_2$ and 0.8 mM $MgSO_4$; HSF, human skin fibroblast; MEM, minimal essential medium; NHS, *N*-hydroxysuccinimidyl; PBS, phosphate-buffered saline; PDMP, D,L-*threo*-1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol; SDS, sodium dodecyl sulfate; SM, sphingomyelin; TLC, thin-layer chromatography; VSV-G protein, vesicular stomatitis virus glycoprotein.

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stored at −20 °C. A fluorescent analogue of PDMP in which the decanoic acid moiety was replaced with the fluorescent fatty acid 5-(5,7-dimethyldipyrromethaneboron difluoride)-1-pentanoic acid (C_5-DMB) was synthesized as follows. Dried PDMP was dissolved in 0.8 M KOH in methanol, sealed under nitrogen, and incubated for 18 h at 100 °C. The reaction was then cooled, and the resulting compound, 1-phenyl-2-amino-3-morpholino-1-propanol, was partitioned into ethyl ether. The compound was then dried and N-acylated (Schwarzmann & Sandhoff, 1987) using the *N*-hydroxysuccinimidyl (NHS) ester (obtained from Molecular Probes, Inc., Eugene, OR) of the C_5-DMB-fatty acid. The resulting product was purified by preparative TLC using $CHCl_3/CH_3OH$/glacial acetic acid (9/1/1 v/v/v) as the developing solvent and had an R_f of ~0.35. Stock solutions of PDMP were diluted into the appropriate buffer so that the 2-propanol concentration was constant and did not exceed 2% in any experiment. This level of 2-propanol had no effect on any of the cells used in these studies.

N-[*N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-ε-aminohexanoyl]-D-*erythro*-sphingosine (C_6-NBD-Cer) and *N*-[5-(5,7-dimethyldipyrromethaneboron difluoride)-1-pentanoyl]-D-*erythro*-sphingosine (C_5-DMB-Cer) (Pagano et al., 1991) were obtained from Molecular Probes, Inc. (Eugene, OR). *N*-([1-^{14}C]Hexanoyl)-D-*erythro*-sphingosine ([^{14}C]hexanoyl-Cer) was synthesized as described (Futerman et al., 1990; Futerman & Pagano, 1992). The fluorescent and radioactive lipid analogues were stored in $CHCl_3/CH_3OH$ (19/1 v/v) at −70 °C.

Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; >1000 Ci/mmol) and β-endo-*N*-acetylglucosaminidase H (endo H) were from ICN (Costa Mesa, CA). Pansorbin was from Calbiochem (La Jolla, CA). Sulfo-NHS-biotin and streptavidin-conjugated agarose beads were from Pierce (Rockford, IL).

N-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) was from Research Organics (Cleveland, OH). Sulforhodamine–dextran (70 kDa) was from Molecular Probes, Inc. Bovine serum albumin (BSA) (defatted for making lipid/BSA complexes and grade V for use as a protein standard), horseradish peroxidase (type II), *o*-dianisidine dihydrochloride (horseradish peroxidase substrate), and 2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (DASMPI) were from Sigma Chemical Co. (St. Louis, MO). Rhodamine 3B was from Eastman (Rochester, NY). Triton X-100 (ultrapure, 10% stock) was from Pierce. H_2O_2 was from Mallinckrodt, Inc. (Paris, KY). All organic solvents were from Burdick and Jackson (Muskegon, MI). All other chemicals, unless otherwise noted, were from Sigma.

Cells and Cell Culture. Chinese hamster ovary cells (CHO-K1; CCL-61) and baby hamster kidney cells (BHK; CCL-10) were obtained from the American Type Culture Collection (Rockville, MD). Human skin fibroblasts (HSF; GM 5659B) were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository (Camden, NJ). CHO-15B (Lec1) cells were obtained from Dr. Pamela Stanley (Albert Einstein School of Medicine) (Gottlieb et al., 1975). All cells were maintained as previously described (Koval & Pagano, 1989, 1990; Pagano et al., 1989). Tissue culture dishes and flasks were obtained from Falcon (Falcon Division, Becton, Dickinson, and Co., Lincoln Park, NJ), Corning (Corning, NY), or NUNC (Thousand Oaks, CA).

Metabolism and Transport of Short-Chain Ceramides in Cultured Cells. For analysis of lipid metabolism, cells were grown for at least 2 days on 60-mm tissue culture dishes. The confluent monolayers were washed three times with cold Eagle's minimal essential medium (without phenol red) buffered with 10 mM HEPES, pH 7.4 (HMEM), and then incubated with 1 mL of either C_6-NBD-Cer (5 μM), C_5-DMB-Cer (5 μM), or [^{14}C]hexanoyl-Cer (2 μM) in 1:1 molar complexes with defatted BSA in HMEM (Pagano et al., 1989; Futerman et al., 1990) for 30 min in the cold (10 °C for CHO-K1 cells, CHO-15B cells, and CHO cells infected with VSV; 2 °C for HSF and BHK cells). The monolayers were then washed with HMEM to remove unincorporated lipid and incubated at 37 °C in 1 mL of HMEM containing 0–100 μM PDMP for 0–60 min. The monolayers were washed, scraped into 0.8 mL of HMEM, and extracted by the method of Bligh and Dyer (1959), using 0.9% NaCl and 10 mM HCl in the aqueous phase. Under these conditions, >95% of the short-chain sphingolipid analogues were extracted from cells. For analysis of metabolism in HSF cells, the cells were treated in the same manner as described above, except that all buffers contained 0.5 mM each choline chloride, ethanolamine, serine, and *myo*-inositol. Lipid extracts were analyzed by TLC on silica gel 60 plates (Merck, Elmsford, NY) using $CHCl_3/CH_3OH$/15 mM $CaCl_2$ (60/35/8 v/v/v) as the mobile phase. Quantification of radioactive lipids was performed by scraping the radioactive spots and counting as described (Futerman et al., 1990). Quantification of fluorescent lipids was performed by scraping the fluorescent spots from the silica, extracting by the Bligh and Dyer method (as above), and comparing the relative fluorescence to standards as described (Lipsky & Pagano, 1983).

To document the effect of PDMP on sphingolipid transport, the cells (grown on glass cover slips; see below) were labeled with C_5-DMB-Cer, warmed to 37 °C for 60 min in the absence of PDMP to allow for the metabolism of fluorescent Cer to products, washed with HMEM containing 0 or 75 μM PDMP, then incubated for an additional 60 min at 37 °C in the absence or presence of PDMP, observed by fluorescence microscopy, and photographed (described below). To determine the amount of fluorescent lipid transported to the plasma membrane, confluent monolayers of CHO-K1 cells on 100-mm dishes were labeled with C_6-NBD-Cer (2.5 nmol/dish) and warmed to 37 °C for 60 min in the absence of PDMP. To remove fluorescent lipid that had been transported to the plasma membrane during this initial warming period, the cells were incubated three times (10 min each) at 10 °C with dioleoylphosphatidylcholine large unilamellar vesicles prepared by extrusion (Hope et al., 1985) (400 μM in HMEM, "back-exchange" medium; Kobayashi & Pagano, 1989). The cells were then washed three times with HMEM containing 0, 50, or 100 μM PDMP and incubated at 37 °C in HMEM containing PDMP for an additional 60 min. The amount of transport of fluorescent lipids from the Golgi apparatus to the plasma membrane was then measured as previously described (Kobayashi & Pagano, 1989), by treating half of the dishes to a second round of back-exchange and comparing the values obtained to those from mock back-exchanged samples. Calculations were performed as described (Koval & Pagano, 1989) except fluorescence was normalized to cellular protein (Lowry et al., 1951) rather than DNA.

Analysis of VSV-G Protein Transport in Cultured Cells. For analysis of vesicular stomatitis virus glycoprotein (VSV-G) transport, CHO-K1 cells were plated at 5×10^4 cells per well in four-well culture dishes 1 day prior to the experiment. Cells were infected with vesicular stomatitis virus (VSV; San Juan strain, Indiana serotype) at a multiplicity of infection of 20 viral particles per cell by adsorbing the virus to cells in a small

volume of serum-free medium for 30 min at 37 °C, followed by addition of α-MEM containing 5% fetal calf serum with further incubation for an additional 3.5 h at 37 °C. Infected cells were incubated in serum-free, methionine-free Dulbecco's MEM supplemented with nonessential amino acids for 10 min; then the cells were incubated in the same medium containing 100 μCi/mL Tran^{35}S-label for 5 min at 37 °C (100 μL/well). Labeling medium was replaced with chase medium (HMEM containing a 3-fold excess of unlabeled methionine) with 0–100 μM PDMP, and incubation at 37 °C was continued for up to 2 h. At the end of each chase time, cells were washed in ice-cold phosphate-buffered saline (PBS; Dulbecco's formulation, obtained from GIBCO BRL, Gaithersburg, MD) and lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 50 mM NaCl. Debris was removed by centrifugation for 1 min in a microfuge (14000*g*), and samples were immunoprecipitated with 5 μL of a polyclonal anti-VSV serum followed by 40 μL of Pansorbin as described (Swift & Machamer, 1991). Pellets were resuspended in 20 μL of 50 mM Tris-HCl, pH 6.8, containing 1% sodium dodecyl sulfate (SDS). After heating to 100 °C for 3 min, half of the sample was mixed with 10 μL of 0.15 M sodium citrate, pH 5.5, and the other half in the same buffer containing 0.1 milliunit of endo H. After incubation at 37 °C for 16 h, samples were separated by electrophoresis in 10% polyacrylamide gels; then the gels were subjected to fluorography. The rates of acquisition of resistance to endo H and acquisition of sialic acid residues (resolved from the nonsialylated VSV-G protein by a marked decrease in R_f) were determined by densitometry.

For determining sialylation half-times (as in Table II), samples were prepared as above, except that endo H digestion was not performed. After the appropriate chase times, cells were washed in ice-cold PBS and lysed directly in 35 μL of electrophoresis sample buffer containing 5% 2-mercaptoethanol. After being heated to 100 °C for 3 min, the samples were separated by electrophoresis as above, and the percentage of VSV-G protein that was sialylated was determined by densitometry.

The rates of VSV-G protein delivery to the cis Golgi were determined by following oligosaccharide trimming in CHO-15B cells (Beckers et al., 1987). The cells were plated, infected, and labeled as described above. After the appropriate chase times, cells were washed and lysed directly in sample buffer as described above. After electrophoresis, the percentage of trimmed VSV-G protein was determined by densitometry.

The rates of VSV-G protein delivery to the cell surface were determined by a biotinylation assay. Cells infected and labeled as above were chased for up to 3 h. The chase medium was saved for determining the amount of VSV-G protein in virions (see below). Cells were rinsed in ice-cold PBS and incubated for 15 min at 4 °C in 0.2 mL of PBS containing 0.5 mg/mL sulfo-NHS-biotin, followed by another 15 min in fresh biotin/PBS. Cells were washed four times in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) with 10 mM glycine to inactivate the unreacted biotin and then lysed in 200 μL of 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 50 mM NaCl. After removal of debris by centrifugation in a microfuge for 30 s, lysates were incubated with 50 μL of streptavidin-conjugated agarose beads for 30 min at 4 °C. After several washes, the bound material was eluted from the streptavidin beads into sample buffer and loaded directly. Supernatants from the streptavidin–agarose bead precipitation (representing intracellular VSV-G protein not accessible to biotinylation) were precipitated in 10% trichloroacetic acid and washed one time with −20 °C acetone prior to resuspension in sample buffer. The chase medium was also precipitated with 10% trichloroacetic acid using 1% Triton X-100 as carrier. After electrophoresis, the resulting fluorograms were scanned, and the total VSV-G protein reaching the cell surface (biotinylated plus the amount in virions) was compared to the amount of nonbiotinylated (internal) VSV-G protein. To ensure the cells were not permeable during the biotinylation reaction, the amounts of intracellular VSV proteins (N, NS, and M) biotinylated under these conditions were also determined.

Fluorescence Microscopy. Cells were grown on glass cover slips in 35-mm culture dishes at subconfluent densities. Fluorescence microscopy was performed with a model IM-35 inverted microscope equipped with epifluorescence optics using a 100× planapo lens (1.3 n.a.) (Carl Zeiss, Inc., Thornwood, NY). Different filter combinations were used to view (a) fluorescein, NBD, and DASMPI, (b) rhodamine, and (c) DMB fluorescence (Pagano et al., 1991; also see Figure 4). Photography was performed as described (Pagano et al., 1989). Labeling of cells with C_6-NBD-Cer and C_5-DMB-Cer was performed as described above for metabolism and transport studies. Labeling of cells with sulforhodamine–dextran was performed as previously described (Koval & Pagano, 1989, 1990) but in the presence 0–100 μM PDMP. Mitochondria were stained with rhodamine 3B (Lipsky & Pagano, 1983) or DASMPI (Bereiter-Hahn, 1976) as described. The endoplasmic reticulum (ER) and microtubules were stained in fixed, permeabilized cells with anti-immunoglobulin binding protein (Bole et al., 1986) and anti-tubulin (Murphy et al., 1986) antibodies, respectively.

Miscellaneous Procedures. Protein concentrations were determined by the method of Bradford (1976) or Lowry et al. (1951) using BSA as a standard.

The effect of PDMP on protein synthesis was determined as described above for incubation of infected cells with Tran^{35}S-label, except the cells were uninfected and labeling was performed for 30 min rather than 5 min. The samples were precipitated with an equal volume of 20% trichloroacetic acid. Precipitates were washed with acetone (−20 °C), dried, resuspended in 20 μL of buffer containing 50 mM Tris-HCl, pH 6.8, and 1% SDS and heated to 100 °C for 3 min. The samples were then transferred to scintillation vials and counted.

The effect of PDMP on endocytosis was determined by monitoring uptake of horseradish peroxidase in the presence of 0–100 μM PDMP (Steinman et al., 1974).

Results

Effects of PDMP on Glucosylceramide and Sphingomyelin Synthesis in Cultured Cells. We first analyzed the effects of the GlcCer synthase inhibitor, PDMP, on the metabolism of short-chain fluorescent and radioactive Cer analogues. These analogues are useful for the study of lipid transport and metabolism (Pagano, 1990) and are metabolized to the corresponding derivatives of GlcCer and SM in vivo and in vitro (Lipsky & Pagano, 1983; Pagano et al., 1991; Futerman et al., 1990; Futerman & Pagano, 1991).

CHO-K1 cells were incubated with [^{14}C]hexanoyl-Cer at 10 °C for 30 min to allow transfer of the short-chain lipid into cells. Unincorporated lipid was removed by washing the monolayer several times with buffer containing PDMP (0–100 μM). The cultures were then warmed to 37 °C for 30 min in the presence of the same concentration of PDMP with which the cells were washed. The radioactive lipids were extracted from the cells and separated by TLC, and the amount of radioactivity comigrating with the substrate, [^{14}C]hexanoyl-

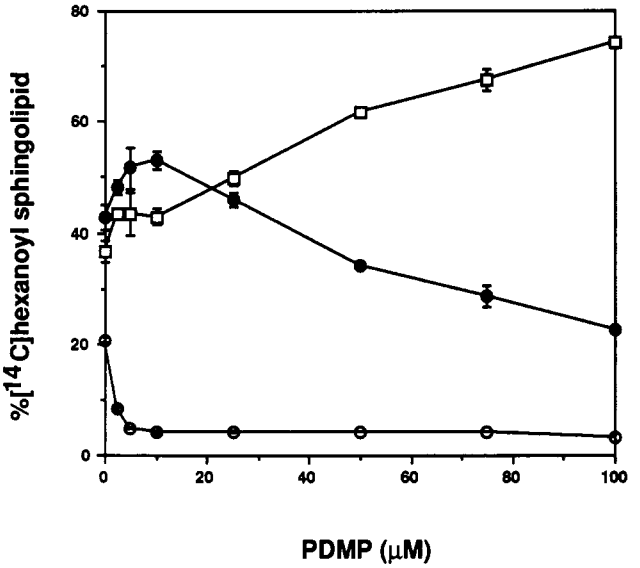

FIGURE 1: Metabolism of [^{14}C]hexanoyl-Cer by CHO-K1 cells in the presence of increasing amounts of PDMP. Monolayer cultures were labeled with [^{14}C]hexanoyl-Cer as described. The cells were then washed three times with HMEM containing 0–100 μM PDMP and incubated in 1 mL of HMEM containing 0–100 μM PDMP for 30 min at 37 °C. The radioactive lipids were extracted and analyzed by TLC. The data are presented as percent of total radioactivity recovered as the substrate, [^{14}C]hexanoyl-Cer (□), and its metabolites, [^{14}C]hexanoyl-GlcCer (○) and [^{14}C]hexanoyl-SM (●). Each point was determined in triplicate. The error bars show the standard deviation.

Table I: Effect of PDMP on Transport of VSV-G Protein through the ER/Golgi Complex[a]

transport to	half-time (min) control	+PDMP	fold change
cis Golgi[b]	11	16	1.5
medial Golgi[c]	13	27	2.1
trans Golgi[d]	18	44	2.4
PM[e]	26	72	2.8

[a] Half-times of transit of VSV-G protein through compartments of the ER/Golgi complex in the absence or presence of 75 μM PDMP were determined (see footnotes *b–e*). The data for transit through the medial Golgi and trans Golgi were obtained from Figure 2. The fold change was determined by dividing the value obtained for the half-time in the presence of PDMP by the half-time in the absence of PDMP. [b] Measured by oligosaccharide trimming in CHO-15B cells. [c] Measured by acquisition of endo H resistance. [d] Measured by acquisition of sialic acid residues. [e] Measured by accessibility to biotinylation plus amount in released virions.

Table II: Effect of Increasing Concentrations of PDMP on the Sialylation of VSV-G Protein[a]

PDMP (μM)	half-time (min)
0	16
10	18
25	23
50	27
75	44
100	53

[a] The rate of sialylation of VSV-G protein in the presence of increasing amounts of PDMP was determined as described under Materials and Methods.

Cer, and the products, [^{14}C]hexanoyl-GlcCer and [^{14}C]hexanoyl-SM, was determined (Figure 1). Under these conditions, [^{14}C]hexanoyl-GlcCer and [^{14}C]hexanoyl-SM were the only products of [^{14}C]hexanoyl-Cer metabolism detected. With low concentrations of PDMP (2.5–10 μM), decreased synthesis of radioactive GlcCer and increased synthesis of radioactive SM were seen. However, with increasing concentrations of PDMP (up to 100 μM), there was a concomitant decrease in the amount of [^{14}C]hexanoyl-SM synthesis. [Note that inhibition of GlcCer synthesis under these conditions was not complete. A small amount of conversion to products occurred during the initial incubation of cells with the radioactive Cer analogue at 10 °C in the absence of PDMP. When PDMP was included during this step, no GlcCer was detected (data not shown).] Results similar to those shown in Figure 1 were obtained with two fluorescent Cer analogues, C_6-NBD-Cer and C_5-DMB-Cer, and in other cell types, including CHO-15B, HSF, and BHK cells, and in CHO-K1 cells infected with VSV (data not shown).

The effects of PDMP on metabolism of the short-chain Cer analogues were also studied as a function of time. These experiments were similar to those in Figure 1, except that the cells were incubated with a constant amount of PDMP and were warmed to 37 °C for 0–60 min. Within the first 2.5 min of warming, at a low concentration of PDMP (10 μM), there was a substantial reduction in the amount of GlcCer formed and an increase in the amount of SM formed relative to control values, while at a high concentration of PDMP (50 μM), there was not only a decrease in the amount of GlcCer formed but also a decrease in the amount of SM formed (data not shown). Therefore, the effects of PDMP on sphingolipid metabolism appeared to be immediate.

Effects of PDMP on VSV-G Protein Transport. We next explored the effect of PDMP on transport of proteins through the Golgi apparatus using VSV-G protein since the modifications of its glycan structure and the intracellular sites where these modifications occur are well documented (Dunphy & Rothman, 1985; Rothman & Orci, 1990). To determine the effect of PDMP on this process, CHO-K1 cells were infected with VSV for 4 h. The viral proteins were pulse-labeled with Tran^{35}S-label for 5 min, and VSV-G protein was chased through the Golgi apparatus in the presence or absence of PDMP for up to 2 h. The labeled VSV-G protein was then analyzed by gel electrophoresis.

In the presence of 75 μM PDMP, the half-times for transit of VSV-G protein through a number of compartments were increased relative to controls (Figure 2; Table I). The half-times for arrival at the medial and trans Golgi compartments and at the cell surface were increased 2.1-, 2.4-, and 2.8-fold, respectively, relative to control values (Table I). These results suggest that transit through both early (the sites of GlcCer and SM synthases) and late Golgi compartments was altered in cells treated with PDMP. The decreased rate of transport through the Golgi complex was manifested as increased half-times for each step measured as well as longer lag times before processing began. Finally, note that the effect of PDMP on protein transport was immediate (see Figure 2, panels B and C), as was seen for lipid synthesis.

We also tested the effect of PDMP on arrival of VSV-G protein at cis Golgi compartments by analyzing trimming of glycans on VSV-G protein from VSV-infected CHO-15B cells where this step can be readily examined (Beckers et al., 1987) (Table I). This step was not as strongly affected by the presence of PDMP as was transit through the Golgi apparatus and transport to the plasma membrane. Since this step was the least affected by PDMP, while each subsequent step was increasingly more affected by PDMP, it appeared that the effects of PDMP on Golgi apparatus functions were cumulative. We also found that the rate of sialylation of VSV-G protein was sensitive to increasing concentrations of PDMP (Table II), as was found for inhibition of GlcCer and SM synthases (Figure 1).

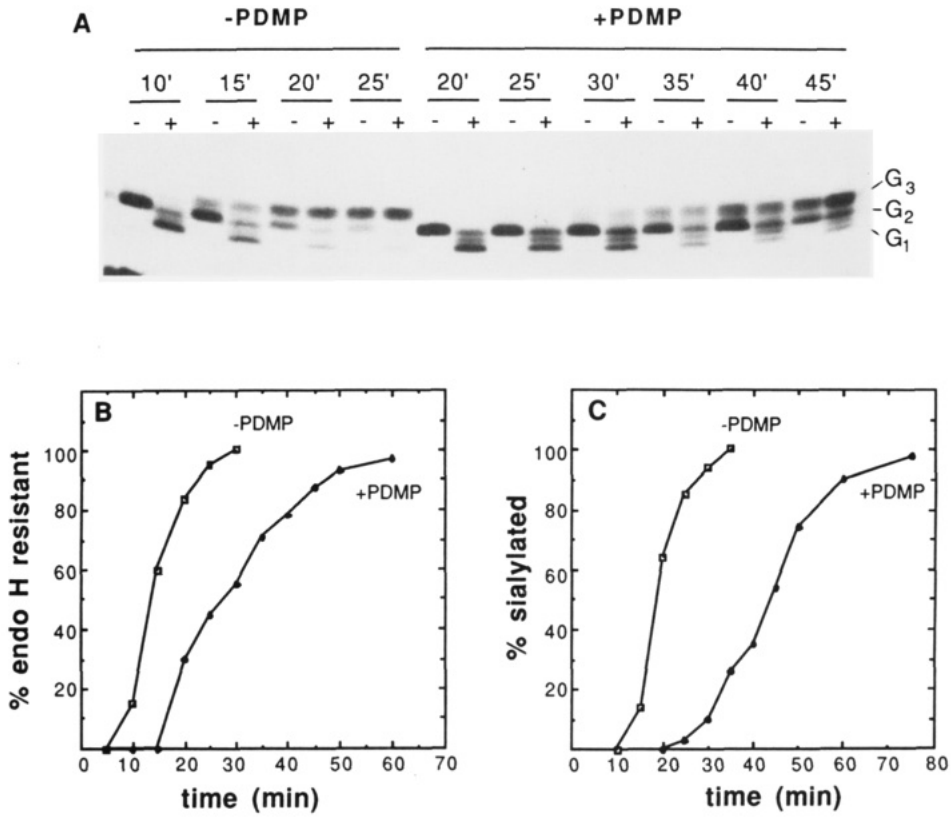

FIGURE 2: PDMP slows the rate of transport of VSV-G protein through the Golgi apparatus. CHO-K1 cells were plated, infected with VSV, and labeled as described under Materials and Methods. The cells were then chased with or without 75 μM PDMP for up to 2 h. VSV proteins were concentrated by immunoprecipitation, incubated with or without endo H, subjected to electrophoresis, fluorographed, and analyzed by densitometry as described. Panel A shows the fluorogram of the gel from one experiment. G_3 denotes sialylated VSV-G protein; G_2, nonsialylated, endo H resistant VSV-G protein; and G_1, endo H sensitive VSV-G protein. The rates of acquisition of endo H resistance (panel B) and the rates of acquisition of sialic acid residues (panel C) were determined from two experiments.

To determine if the effects of PDMP on VSV-G protein transport were reversible, labeled VSV-infected cells were incubated at 37 °C with medium containing PDMP, washed briefly with serum-containing medium to remove PDMP, and then incubated further at 37 °C in the absence of PDMP. As determined by the acquisition of sialic acid residues, the effects of PDMP on VSV-G protein transport were fully reversible (data not shown). These results are consistent with those of Inokuchi and co-workers, who demonstrated that other effects of PDMP on cells in culture are reversed upon removal of the drug (Inokuchi et al., 1989, 1990). (Experiments to determine the effect of PDMP removal on metabolism of the short-chain lipids were not attempted, since the conditions used to remove PDMP would also remove the short-chain Cer analogues from cells.) Finally, the effects of PDMP on cells appeared to be distinct from those observed with the drug brefeldin A (Lippincott-Schwartz et al., 1989) since we found that a VSV-G protein mutant that is retained in the ER (Δ1473; Rose & Bergmann, 1983) did not acquire endo H resistance in the presence of PDMP (data not shown), suggesting that resident Golgi enzymes were not redistributed to the ER in the presence of PDMP.

Effects of PDMP on Sphingolipid Transport. Sphingolipid transport from the Golgi apparatus to the plasma membrane was assessed in two ways. First, the effect of PDMP on transport of fluorescent Cer metabolites to the plasma membrane was monitored by fluorescence microscopy of HSF cells labeled with C_5-DMB-Cer in the manner described above for the metabolic experiments (see Figure 1). C_5-DMB-Cer and its metabolites, when concentrated in membranes, show a red spectral shift which can be visualized by fluorescence microscopy (Pagano et al., 1991). Cells containing C_5-DMB-Cer were first warmed to 37 °C for 60 min in the absence of PDMP. Under these conditions, ≥80% of the label is metabolized to products (mostly SM, as this analogue appears to be a poor substrate for GlcCer synthase; Pagano et al., 1991). After 60 min of warming, no red fluorescence was detected at the plasma membrane (data not shown), suggesting that any fluorescent lipid present there was not sufficiently concentrated to be detected. The cells were subsequently warmed for an additional 60 min in the presence of 0 or 75 μM PDMP and photographed (Figure 3). Cells that had been incubated with 75 μM PDMP (panel B) had much less label at the cell surface than control cells (panel A). Cells incubated in the same way, but with C_6-NBD-Cer, showed a similar inhibition of transport of fluorescent sphingolipids to the cell surface in the presence of PDMP (data not shown). Results similar to those obtained in Figure 3 were also obtained with CHO-K1 cells (data not shown).

Second, to quantify the amount of cell-associated sphingolipids transported to the cell surface in the presence of PDMP, CHO-K1 cells were labeled with C_6-NBD-Cer as described above. C_6-NBD-SM and -GlcCer, unlike their C_5-DMB counterparts (Pagano et al., 1991), can be removed from the cell surface by providing an exogenous acceptor for these lipids ("back-exchange" medium; see Materials and Methods). By measurement of the amount of cell-associated fluorescent GlcCer and SM before and after back-exchange, the amount of transport to the cell surface can be calculated (Lipsky & Pagano, 1985). Cells containing C_6-NBD-Cer were warmed to 37 °C for 60 min in the absence of PDMP. Under these conditions, ≥90% of the fluorescence was converted to C_6-NBD-GlcCer and -SM. During this initial warming period, some transport to the plasma membrane occurred (data not

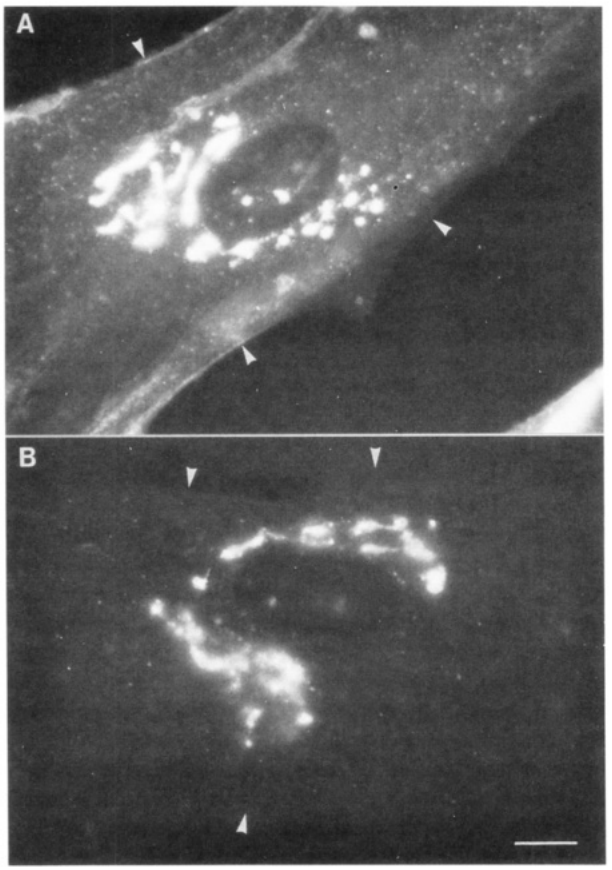

FIGURE 3: PDMP inhibits transport of fluorescent sphingolipids to the plasma membrane. HSF cells were labeled with 5 μM C_5-DMB-Cer for 30 min at 2 °C. The cells were washed and then incubated for 60 min at 37 °C to permit metabolism of the ceramide analogue in the absence of PDMP. The cells were then incubated for an additional 60 min at 37 °C in the presence of 0 or 75 μM PDMP. Samples were excited with 450–490-nm light and observed at ≥590 nm (red wavelengths) as described. PDMP concentrations during the second hour at 37 °C were 0 μM (panel A) or 75 μM (panel B). Note the difference in intensity of fluorescence at the plasma membrane in panel A versus panel B (arrows denote the cell perimeter). The bright reticular fluorescence in the perinuclear region corresponds to the Golgi apparatus [see Pagano et al., (1991)]. The small dots of fluorescence in the cell periphery may be transport vesicles carrying material to or from the plasma membrane. Bar is 20 μm.

shown), so all cells were subjected to a preliminary incubation with back-exchange medium. The cells were subsequently warmed to 37 °C for 60 min in the presence of 0, 50, or 100 μM PDMP and then treated with or without back-exchange medium (Table III). Transport of both fluorescent SM and GlcCer to the plasma membrane was inhibited by ~20% in the presence of 50 μM PDMP and by ~50% in the presence of 100 μM PDMP, consistent with the qualitative observations shown in Figure 3.

Effects of PDMP on Cell Morphology and Physiology. Control experiments were performed under conditions similar to or more extreme than conditions used to measure protein and sphingolipid transport to examine the effects of PDMP on cell morphology and physiology. First, we explored the appearance and function of subcellular organelles. The organization of the ER and microtubules was not affected by incubation for 2 h with 100 μM PDMP as assessed by immunofluorescence microscopy with antibodies directed against immunoglobulin binding protein and tubulin, respectively, in CHO-K1 cells. Lysosomal uptake of a fluorescent fluid-phase marker (sulforhodamine–dextran; Koval & Pagano, 1989, 1990) in CHO-K1 or HSF cells was unaffected by incubation

Table III: PDMP Inhibits Transport of C_6-NBD-SM and C_6-NBD-GlcCer to the Cell Surface[a]

PDMP (μM)	C_6-NBD-GlcCer	C_6-NBD-SM
0	60 ± 5	62 ± 5
50	51 ± 16	49 ± 9
100	26 ± 14	36 ± 7

[a] CHO-K1 cells on 100-mm culture dishes were incubated with C_6-NBD-Cer (1.25 μM; 2.5 nmol/dish) at 10 °C for 30 min as described under Materials and Methods. The cells were washed and incubated in HMEM for 60 min at 37 °C in the absence of PDMP. To remove fluorescent lipids at the surface, cells were incubated three times (10 min each) with back-exchange medium as described under Materials and Methods. The cells were then incubated for 60 min at 37 °C in the presence of 0, 50, or 100 μM PDMP. The amount of C_6-NBD-GlcCer and C_6-NBD-SM transported to the surface during the second incubation at 37 °C was determined by treating the cells with or without back-exchange medium as described under Materials and Methods. The data, determined in triplicate, are the percentages of fluorescent lipids (±standard deviation) available for back-exchange relative to mock back-exchanged samples.

with 100 μM PDMP as determined by fluorescence microscopy. The effect of PDMP on endocytosis in CHO-K1 cells was also measured by determining the amount of the fluid-phase marker horseradish peroxidase internalized during a 30-min incubation in the presence or absence of 100 μM PDMP (Steinman et al., 1974). The value obtained for uptake of horseradish peroxidase in the presence of PDMP was 90% of the control value. Mitochondrial structure and function in both CHO-K1 and HSF cells incubated for 30 min with 100 μM PDMP were analyzed using two different potential-sensitive fluorophores, rhodamine 3B (Johnson et al., 1981) and DASMPI (Bereiter-Hahn, 1976). There was little effect on uptake of either dye by mitochondria, although the mitochondria in cells treated with PDMP were more rounded than the mitochondria in control cells.

The appearance of the Golgi apparatus in HSF cells in the presence of PDMP as detected by fluorescence microscopy was slightly altered. The Golgi stacks labeled with fluorescent Cer were more swollen in PDMP-treated cells than in control cells [see Figure 3; compare panel A (control) and panel B (+PDMP)]. After long-term incubations with PDMP (≥4 h), the Golgi apparatus was increasingly fragmented compared to control cells (data not shown). Similar results were seen in CHO-K1 cells (data not shown). Thus, long-term inhibition of sphingolipid synthesis may cause altered morphology of this organelle.

Protein synthesis in the presence of PDMP was also analyzed. Cells were labeled with serum-free Dulbecco's MEM (methionine-free, 50 μCi/mL Tran^{35}S-label) for 30 min in the presence of 0–100 μM PDMP. Incorporation of label into protein was determined by precipitation of cell lysates with 10% trichloroacetic acid. PDMP inhibited protein synthesis in a concentration-dependent manner: at 50 μM PDMP, incorporation of label into protein was 50–60% of control values; at 100 μM, 30–40% of control values. When the labeled proteins were analyzed by gel electrophoresis, the qualitative pattern seen was unchanged, suggesting that the effect of PDMP on protein synthesis was a general one. [Shayman et al. (1991) have shown that PDMP inhibits DNA synthesis in a time- and concentration-dependent manner.] Given the immediate effects of PDMP on lipid synthesis and protein transport, it seemed unlikely that inhibition of protein synthesis was responsible for the changes seen in protein and lipid transport. To rule out this possibility, pulse-labeled cells were incubated for up to 1 h with cycloheximide at concentrations sufficient to completely block protein synthesis (100 μg/mL). This treatment did not affect the rate of VSV-G

protein transport. We also found that the extent of sphingolipid transport was not affected by this concentration of cycloheximide (data not shown).

PDMP is a lipophilic amine, and such compounds have been shown to have effects on lysosome structure and function (Roff et al., 1991). We determined that the effects on protein and sphingolipid transport were specific to PDMP and not a general property of lipophilic amines. Cells were incubated for 16–19 h with U18666A (Liscum & Faust, 1989), stearylamine, sphinganine, imipramine, or PDMP. All of these compounds were added to the cell medium from concentrated stocks in 2-propanol with the exception of imipramine which was added from a stock prepared in water. All of these compounds induced large membranous structures presumably as a result of their accumulation in lysosomes (Roff et al., 1991). However, none of these compounds, other than PDMP, inhibited transport of the fluorescent sphingolipids from the Golgi apparatus to the plasma membrane (as in Figure 3). In addition, inclusion of up to 50 μM stearylamine in place of PDMP in the chase medium did not affect transport of VSV-G protein. Finally, 50 μM stearylamine did not inhibit either GlcCer synthase or SM synthase activities (data not shown).

Intracellular Distribution of a Fluorescent Analogue of PDMP. We synthesized a fluorescent analogue of PDMP bearing the C_5-DMB-fatty acid moiety and found that it inhibited the synthesis of GlcCer from [^{14}C]hexanoyl-Cer in cultured cells at low concentrations (data not shown), similar to the parent compound. When HSF cells were incubated with fluorescent PDMP (10 μM) for 30 min at 37 °C and washed, two different patterns of intracellular fluorescence were seen, depending upon the spectral window chosen (Figure 4). When fluorescence at ≥520 nm was observed (green + red wavelengths; panel A), the Golgi apparatus, as well as other intracellular membranes, appeared green, while numerous large vesicle-like structures scattered throughout the cytoplasm appeared reddish orange. When the same specimen was viewed at ≥590 nm (red wavelengths; panel B), only the vesicle-like structures were visibly fluorescent. On the basis of previous studies with this fluorophore (Pagano et al., 1991), these results suggest that the fluorescent analogue of PDMP was present at low concentrations in the Golgi apparatus and other intracellular membranes (green fluorescence), while high concentrations were present in the vesicle-like structures (red fluorescence). When experiments were conducted with 10 μM fluorescent PDMP plus 40 or 90 μM nonfluorescent parent compound (to give 50 or 100 μM PDMP total), similar results were obtained although the overall fluorescence of the cells was dimmer (both green and red fluorescence), indicating that PDMP and its fluorescent analogue behave similarly with respect to binding to cell membranes. The vesicular structures labeled with this analogue were readily detected by phase microscopy (data not shown). In addition, other experiments have shown that when cells were labeled with the lysosomal marker sulforhodamine–dextran (Koval & Pagano, 1990) and a second fluorescent analogue of PDMP (containing an NBD-fatty acyl group in place of the C_5-DMB-fatty acyl group), the two probes showed extensive colocalization (Rosenwald and Pagano, manuscript in preparation). These results suggest that although retardation of lipid and protein transport required incubation of cells with high concentrations of PDMP, most of the cell-associated PDMP was sequestered in lysosomes, while its concentration at the Golgi complex, where GlcCer and SM synthesis occurs, was relatively low. Thus, the effects of PDMP on the function of this organelle apparently occurred at much lower concentrations than suggested by the concentration of PDMP in the medium.

Discussion

Retardation of Protein and Lipid Transport Is Correlated with Inhibition of Sphingolipid Synthesis. In the present study we examined the effects of PDMP on sphingolipid synthesis and on protein and sphingolipid transport in cultured cells. These three processes were increasingly hindered by incubation of cells with increasing concentrations of PDMP. It has been previously found that long-term incubation of cells with PDMP induces a number of changes in cells, including depletion of total cellular glycosphingolipids (discussed more fully below). In contrast to these results, we found that PDMP immediately retarded lipid synthesis and protein transport and therefore cannot represent effects caused by changes in the mass of cellular sphingolipids. Note also that the effect of PDMP on protein transport was quickly reversed upon removal of the drug. The effects of PDMP could not be attributed to generalized effects of lipophilic amines on cells, nor to its inhibition of protein synthesis. Thus, the effects of PDMP on sphingolipid synthesis and sphingolipid and protein transport appeared to be closely correlated.

Slowing of transport was most strongly correlated to inhibition of SM synthesis, since significant retardation of transport only occurred at high concentrations of PDMP. We cannot rule out the possibility that slowing of transport was due to inhibition of both SM and GlcCer synthesis. However, it does not appear that retardation of transport is due to the inhibition of GlcCer synthesis alone, since at low concentrations of PDMP (≤10 μM), little effect on transport was seen, despite nearly complete inhibition of GlcCer synthesis. On the basis of these results, we propose that there may be a linkage between sphingolipid synthesis at the Golgi complex and vesicular transport processes. Our results also corroborate the hypothesis that sphingolipids and proteins travel through the secretory pathway by an identical mechanism, since both were adversely affected by increasing concentrations of PDMP.

Potential Mechanisms of Action of PDMP. Previous work from our laboratory and others has shown that SM synthase activity is found in cis and medial compartments of the Golgi apparatus (Futerman et al., 1990; Jeckel et al., 1990). In contrast, GlcCer synthase appears to be more widely distributed, with activity present in the cis Golgi, as well as in a light vesicle fraction which may correspond to the intermediate (retrieval) compartment or cis Golgi network (Coste et al., 1985, 1986; Futerman & Pagano, 1991; Trinchera et al., 1991). In addition, previous studies have shown that when cells are cultured in low concentrations of PDMP for longer periods of time than in the studies described here, total GSL content decreases, while the amounts of Cer, SM, and *N*,*N*-dimethylsphingosine increase (Okada et al., 1988; Inokuchi et al., 1989, 1990; Felding-Habermann et al., 1990; Igarashi et al., 1990; Shayman et al., 1990, 1991; Goldkorn et al., 1991; Uemura et al., 1991). Given these observations, the addition of PDMP to cells could potentially decrease protein and lipid transport through the secretory pathway in a number of ways. (*i*) *Alterations in membrane composition*: On the basis of the intracellular distributions of SM synthase and GlcCer synthase, the concentrations of sphingolipids should be decreased in Golgi membranes, in pre-Golgi membranes, and in Golgi-associated transport vesicles in the presence of PDMP. If critical concentrations of sphingolipids in general, or a particular sphingolipid, are directly required for vesicle fission or fusion reactions, addition of PDMP would have a significant effect on vesicular transport. (*ii*) *Reduced rate of membrane formation*: Since synthesis of lipids should lead to an increase

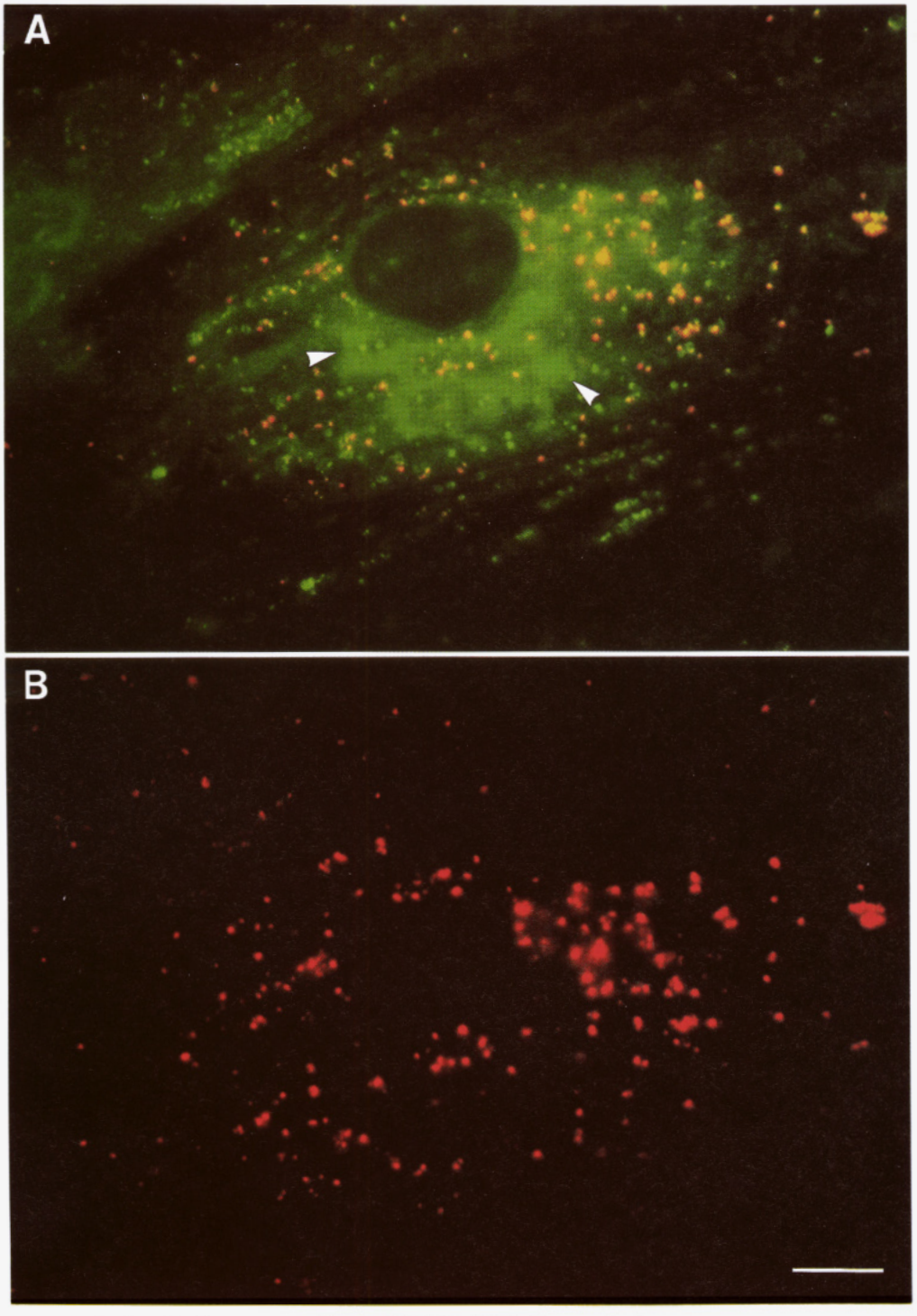

FIGURE 4: Intracellular distribution of a fluorescent analogue of PDMP. HSF cells were incubated with a C_5-DMB-labeled analogue of PDMP (10 μM) for 30 min at 37 °C. Samples were excited at 450–490 nm and observed at ≥520 nm (green + red wavelengths; panel A) or at ≥590 nm (red wavelengths; panel B). Red fluorescence results from regions of the specimen in which the fluorophore is highly concentrated, while green fluorescence indicates the probe is present at lower concentrations (Pagano et al., 1991). Note green fluorescence at the Golgi apparatus (arrows). Bar is 20 μm.

in membrane surface area, ongoing sphingolipid synthesis at the Golgi apparatus might promote vesicle formation and budding. If so, a reduction in sphingolipid synthesis by PDMP would lead to a decreased rate of membrane growth and a slowing of vesicle transport. In this regard, it has been observed that, in neuroblastoma cell lines, reduction of GSL mass by PDMP is correlated with inhibition of neurite outgrowth (Uemura et al., 1991). (*iii*) *Production of lipid second messengers*: Since incubation of cells with PDMP results in increased levels of *N*,*N*-dimethylsphingosine (Felding-Habermann et al., 1990; Igarashi et al., 1990) and this compound may be an inhibitor of protein kinase C in vivo (Hakomori, 1990), PDMP may exert its effect on vesicular transport by altering protein kinase C metabolism. Indeed, alterations in the amounts of GlcCer are correlated with changes in the amount of protein kinase C activity in cells (Shayman et al., 1991). Shayman et al. (1990) also found that long-term depletion of GSLs in cells by PDMP increased hormone-stimulated release of *myo*-inositol 1,4,5-trisphosphate. In addition, it should be noted that alterations in SM synthesis at the Golgi apparatus, as was found in this study, should affect the amount of diacylglycerol that is produced (Pagano, 1988),

which may alter protein kinase C activity at this organelle (Kikkawa et al., 1989).

Perspectives. Our results demonstrate that incubation of cells with a sphingolipid synthesis inhibitor can affect protein and lipid traffic through the secretory pathway, suggesting that vesicular transport along this pathway may be coupled to sphingolipid biosynthesis. In future studies, we hope to further evaluate this hypothesis using inhibitors which block other steps of sphingolipid biosynthesis (Sundaram & Lev, 1984; Medlock & Merrill, 1988; Wang et al., 1991).

In addition, PDMP may prove to be a valuable tool to evaluate the potential role of GSLs in sorting processes in polarized cells. It has been suggested that newly synthesized apical proteins associate with GSL domains at the trans Golgi network, by binding either directly to GSLs or indirectly through interaction with a transmembrane sorting protein (Simons & van Meer, 1988). Since growth of cells in PDMP leads to reduced levels of GSLs (Okada et al., 1988; Inokuchi et al., 1989, 1990; Felding-Habermann et al., 1990; Igarashi et al., 1990; Uemura, 1991), PDMP may disrupt sorting processes in polarized cells.

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Dissection of Helix Capping in T4 Lysozyme by Structural and Thermodynamic Analysis of Six Amino Acid Substitutions at Thr 59[†,‡]

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ABSTRACT: Threonine 59, a helix-capping residue at the amino terminus of the longest helix in T4 phage lysozyme, was substituted with valine, alanine, glycine, serine, asparagine, and aspartic acid. The valine, alanine, and glycine replacements were observed to be somewhat more destabilizing than serine, asparagine, and aspartic acid. The crystal structures of the different variants showed that changes in conformation occurred at the site of substitution, including Asp 61, which is nearby, as well as displacement of a solvent molecule that is hydrogen-bonded to the γ-oxygen of Thr 59 in wild-type lysozyme. Neither the structures nor the stabilities of the mutant proteins support the hypothesis of Serrano and Fersht (1989) that glycine and alanine are better helix-capping residues than valine because a smaller-sized residue allows better hydration at the end of the helix. In the aspartic acid and asparagine replacements the substituted side chains form hydrogen bonds with the end of the helix, as does threonine and serine at this position. In contrast, however, the Asp and Asn side chains also make unusually close contacts with carbon atoms in Asp 61. This suggests a structural basis for the heretofore puzzling observations that asparagine is more frequently observed as a helix-capping residue than threonine [Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648-1652] yet Thr → Asn replacements at N-cap positions in barnase were found to be destabilizing [Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 296-299]. Threonine and asparagine are shown to require different conformations of the backbone at the end of the α-helix in order to adopt optimal capping conformations. The energetic and structural consequences of substitutions at the N-cap position therefore depend not only on the nature of the substitution and the local environment in which it occurs but also on the backbone conformation that exists at the site of substitution.

A helix N-cap residue is perhaps best defined as the first residue in a helix whose α-carbon is within the cylinder defined by the helix (Richardson & Richardson, 1988). A survey of 215 helices in the structures of 45 different globular proteins (Richardson & Richardson, 1988) found that the most common residues at the N-cap position are Ser, Asn, Gly, Asp, and Thr, in order of decreasing frequency. In the evolution of proteins, some advantage evidently favors the choice of these residues although the reasons are not clear. Both kinetic (protein folding) and thermodynamic (protein stabilizing) roles for N-cap residues have been proposed (Presta & Rose, 1988; Richardson & Richardson, 1988).

The stability of helices in small synthetic peptides is known to be dependent on charged residues at the ends of those helices (Ihara et al., 1982; Shoemaker et al., 1985, 1987), presumably due to a favorable electrostatic interaction with the α-helix dipole (Blagdon & Goodman, 1975; Wada, 1976; Hol et al., 1978). Nicholson et al. (1988, 1991) have also shown that

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[‡] The coordinates for all T4 lysozyme structures discussed in this report have been deposited in the Protein Data Bank at the Brookhaven National Laboratory.

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