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A Series of Fluorescent *N*-Acylsphingosines: Synthesis, Physical Properties, and Studies in Cultured Cells†

Richard E. Pagano* and Ona C. Martin
Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, Maryland 21210-3301

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ABSTRACT: We have previously shown that when cultured fibroblasts are briefly incubated at 2 °C with a fluorescent (NBD) analogue of ceramide, *N*-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ε-aminohexanoyl]-D-*erythro*-sphingosine, fluorescent labeling of the mitochondria, endoplasmic reticulum, and nuclear envelope occurs. During further incubation at 37 °C, the Golgi apparatus and later the plasma membrane become intensely fluorescent. Concomitantly, the fluorescent ceramide is metabolized to fluorescent analogues of sphingomyelin and glucosylceramide [Lipsky, N. G., & Pagano, R. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2608–2612]. In the present study we synthesized fluorescent *N*-acylsphingosine analogues using various long-chain bases (D-*erythro*-sphingosine, L-*erythro*-sphingosine, D-*threo*-sphingosine, L-*threo*-sphingosine, D-*erythro*-dihydrosphingosine, L-*threo*-dihydrosphingosine, phytosphingosine, and 3-ketosphingosine) and fluorescent fatty acids (ε-NBD-aminohexanoic acid; D- or L-α-OH-ε-NBD-aminohexanoic acid; D- or L-α-NBD-aminohexanoic acid). Using previously described resonance energy transfer assays, we examined the rates of spontaneous transfer of these compounds between liposomes and their ability to undergo transbilayer movement. The fluorescent *N*-acylsphingosine analogues had half-times for spontaneous transfer of 0.3–4.0 min at 25 °C, and all were capable of transbilayer movement in lipid vesicles. The metabolism and intracellular distribution of analogues in cultured fibroblasts were also studied. While most of the fluorescent *N*-acylsphingosines were significantly metabolized to the corresponding sphingomyelin analogues, metabolism to glucosylceramide was strongly dependent on the long-chain base and the stereochemistry of the fluorescent fatty acid moiety. When cells were incubated with the various *N*-acylsphingosine analogues under appropriate conditions, labeling of the Golgi apparatus was seen in all cases except for *N*-(ε-NBD-aminohexanoyl)-3-ketosphingosine.

We have developed a series of fluorescent (NBD)[1] lipid derivatives which are useful in studying the synthesis, molecular sorting, and intracellular transport of lipids in animal cells [reviewed in 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 and their metabolites within *living* cells by fluorescence microscopy. One lipid, a fluorescent analogue of ceramide, is particularly interesting because it prominently labels the Golgi apparatus of cells (Lipsky & Pagano, 1983, 1985a,b) and provides a means for studying the traffic of sphingolipids through this organelle (Lipsky & Pagano, 1983, 1985a; van Meer et al., 1987). When cells are treated with this lipid at low temperature, washed, and warmed to 37 °C, the Golgi apparatus and later the plasma membrane become intensely fluorescent. During this redistribution of intracellular fluorescence, the fluorescent ceramide is metabolized to fluorescent SM and GlcCer. Consistent with the increasing fluorescence at the plasma membrane over time, increasing amounts of each fluorescent metabolite can be removed from the cell surface by backexchange to nonfluorescent acceptor liposomes. Furthermore, the ionophore monensin, which inhibits the transport of newly synthesized glycoproteins to the cell surface, also inhibits the delivery of these fluorescent lipid metabolites to the cell surface and causes accumulation of fluorescence in the region of the Golgi apparatus. These results suggest that both the fluorescent SM and GlcCer analogues are synthesized intracellularly from the fluorescent ceramide precursor and then translocated through the Golgi apparatus

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* Address correspondence to this author.

[1] Abbreviations: BSA, bovine serum albumin; C, chloroform; DOPC, dioleoylphosphatidylcholine; GlcCer, glucosylceramide; HCMF, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Puck's saline without calcium or magnesium; HMEM, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Eagle's minimal essential medium, pH 7.4, without indicator; HPLC, high-pressure liquid chromatography; M, methanol; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NHS, *N*-hydroxysuccinimidyl; RET, resonance energy transfer; SM, sphingomyelin; TLC, thin-layer chromatography.

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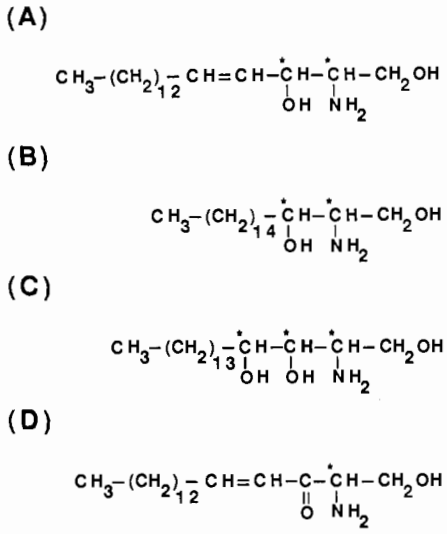

FIGURE 1: Structures of long-chain bases used in the synthesis of fluorescent *N*-acylsphingosine derivatives: (A) sphingosine; (B) dihydrosphingosine; (C) phytosphingosine; (D) 3-ketosphingosine. Asterisks indicate chiral carbon atoms.

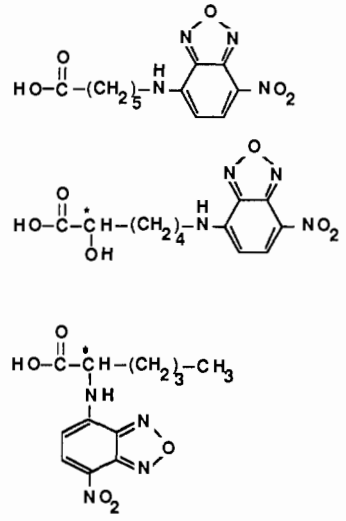

FIGURE 2: Structures of NBD fatty acids used in the synthesis of fluorescent *N*-acyl-D-*erythro*-sphingosines: (A) ϵ-NBD-aminohexanoic acid; (B) L- or D-α-OH-ϵ-NBD-aminohexanoic acid; (C) L- or D-α-NBD-aminohexanoic acid. Asterisks indicate chiral carbon atoms.

to the plasma membrane (Lipsky & Pagano, 1983, 1985a).

These previous studies utilized a fluorescent ceramide analogue synthesized from the naturally occurring long-chain base D-*erythro*-sphingosine,[2] and ϵ-NBD-aminohexanoic acid. In the present paper we synthesized other *N*-acylsphingosine analogues by varying the chemistry of both the long-chain base and the fluorescent (NBD) fatty acid. Our goals were (i) to learn if subtle changes in the chemistry of the fluorescent *N*-acylsphingosines would affect its physical properties in liposomes, (ii) to define which constituents of the molecule were important in accumulation of the lipid and its metabolites in the Golgi apparatus of cells, and (iii) to attempt to affect the metabolism of the fluorescent *N*-acylsphingosine analogue and learn whether this altered metabolism would lead to an altered intracellular distribution of the lipid.

Materials and Methods

Long-Chain Bases. Several long-chain bases (Figure 1) were used in the synthesis of fluorescent (NBD) *N*-acylsphingosines. The sources of these were as follows: (i) D-*erythro*-sphingosine, obtained from bovine brain SM, (ii) a mixture of D-*erythro* and L-*threo* isomers of dihydrosphingosine, and (iii) phytosphingosine (yeast) were purchased from Sigma Chemical Co. (St. Louis, MO); (iv) D-*erythro*-sphingosine, containing approximately 20–40% L-*threo*-sphingosine, was from Serdary Research Laboratories (London, Ontario, Canada); (v) 3-ketosphingosine was formed after N-acylation of D-*erythro*-sphingosine with ϵ-NBD-aminohexanoic acid (see below) according to the procedure of Kishimoto and Mitry (1974); (vi) DL-*erythro*-sphingosine and DL-*threo*-sphingosine were generous gifts of Dr. John Pike of the Upjohn Co. (Kalamazoo, MI).

The purity of each long-chain base was determined by TLC in C/M/2 N NH_4OH (40/10/1) on silica gel 60 TLC plates (Merck & Co., Inc., Rahway, NJ). The materials were detected by spraying the TLC plates with ninhydrin (Krebs et al., 1969), or anisaldehyde–sulfuric acid (Krebs et al., 1969). In the cases of (i), (ii), and (iv) above, the materials were used directly for the synthesis of the *N*-(ϵ-NBD-aminohexanoyl)-sphingosines or -dihydrosphingosines (see below), and the fluorescent diastereoisomers produced were purified by preparative reverse-phase HPLC (see below).

Separation of the four stereoisomers of sphingosine by use of the starting materials in (vi) was performed as follows. Step 1: DL-*erythro*-Sphingosine and DL-*threo*-sphingosine were purified by preparative TLC in C/M/2 N NH_4OH (40/10/1) to remove minor impurities. The purified sphingosines chromatographed as single spots on TLC in the above solvent (R_f 0.29 for DL-*erythro*- and 0.22 for DL-*threo*-sphingosines). Step 2: The purified sphingosines were N-acylated, as described below, with the NHS ester of the optically active fatty acid, (*R*)-(−)-*O*-acetylmandelic acid, to produce diastereoisomers of the DL-sphingosines. TLC of the reaction mixtures using C/M (9/1) as the developing solvent demonstrated that the N-acylated DL-*erythro*-sphingosines chromatographed as two well-resolved components. A similar result was obtained for the DL-*threo*-sphingosine reaction mixture. Each of the four N-acylated sphingosines was purified by preparative TLC using C/M (9/1). Step 3: Each purified N-acylated sphingosine was hydrolyzed to sphingosine and fatty acid as follows. The dried *N*-acylsphingosines were dissolved in 2 mL of 0.8 M KOH in methanol, sealed under nitrogen, and incubated for 18 h at 100 °C. The reactions were then cooled, and the resulting sphingosines were partitioned into ethyl ether as described (Kates, 1972). This procedure resulted in four sphingosine isomers, two of which had the same mobility in C/M/2 N NH_4OH (40/10/1) as authentic D-*erythro*-sphingosine and two of which had the same mobility as authentic L-*threo*-sphingosine. Each of these sphingosine isomers was then N-acylated with ϵ-NBD-aminohexanoic acid (see below).

Fluorescent Fatty Acids and Derivatives. The fluorescent (NBD) fatty acids shown in Figure 2 were obtained as follows. ϵ-NBD-aminohexanoic acid and its NHS ester were purchased from Molecular Probes (Eugene, OR). D-α-OH- and L-α-OH-ϵ-aminohexanoic acids, generous gifts of Dr. Jack Folk [see Schrode and Folk (1979)], were used to synthesize D-α-OH- or L-α-OH-ϵ-NBD-aminohexanoic acid by derivatization (Longmuir et al., 1985) with NBD-Cl. D- and L-norleucine were used to synthesize D- and L-α-NBD-aminohexanoic acids by derivatization with NBD-Cl.

Synthesis of N-Acylsphingosines. Fluorescent *N*-acylsphingosine derivatives were synthesized from the corresponding long-chain bases and fluorescent fatty acids by oxidation–reduction condensation with triphenylphosphine and

[2] In the *R*/*S* system, D-*erythro*, L-*erythro*, D-*threo*, and L-*threo* correspond respectively to 2*S*,3*R*, 2*R*,3*S*, 2*R*,3*R*, and 2*S*,3*S*.

2,2'-dipyridyl disulfide (Kishimoto, 1975) or by reaction of the long-chain base with the NHS ester of the fluorescent fatty acid (Schwarzmann & Sandhoff, 1987). The products were purified by preparative TLC using C/M/H_2O (65/25/4) or C/M/NH_4OH/H_2O (160/40/1/3) as the developing solvent. The use of reactions in which the long-chain base was omitted from the reaction mixture was necessary in some cases to identify the desired product. In several instances, the TLC-purified materials were subjected to further purification by reverse-phase HPLC (see below).

Mass spectral determinations of all samples were performed at the Middle Atlantic Mass Spectrometry Laboratory, using a KRATOS MS-50 instrument.

Synthesis of Other Fluorescent Lipids. Fluorescent (NBD) SM and GlcCer TLC standards were synthesized as described above (Schwarzmann & Sandhoff, 1987), by use of the NHS esters of each of the NBD fatty acids shown in Figure 2 and (i) a mixture of D-*erythro*- and L-*threo*-sphingosylphosphocholine or (ii) D-*erythro*-glucopsychosine.

HPLC Analysis of N-Acylsphingosines. HPLC analysis and purification of some of the fluorescent *N*-acylsphingosines was carried out on a Waters Model 510 HPLC equipped with a McPherson Model 749 spectrofluorometric detector and a Shimadzu C-R3A plotter/integrator (Martin & Pagano, 1986). The fluorescent lipids were separated on an analytical reverse-phase ODS-II column (Regis Chemical Co., Morton Grove, IL) with M/H_2O/H_3PO_4 (850/150/1.5 v/v/v) as the mobile phase. Samples were injected in 20 μL of the HPLC solvent and eluted at a flow rate of 1 mL/min. Chloroform, methanol, and water were added to the eluted materials to form a two-phase system, C/M/H_2O (1/1/0.9), and the chloroform phase was washed several times with theoretical upper phase. The fluorescent lipids were then dried under nitrogen and stored at −70 °C in C/M (9/1).

Assays for Spontaneous Transfer and Transbilayer Movement of Fluorescent N-Acylsphingosine Analogues. The half-time for spontaneous transfer of each fluorescent *N*-acylsphingosine between small unilamellar DOPC vesicles (Nichols & Pagano, 1982) and its ability to undergo transbilayer movement (Pagano & Longmuir, 1985; Pagano, 1988) were determined according to previously described RET assays.

Preparation of Fluorescent N-Acylsphingosine/BSA Complexes. Fluorescent *N*-acylsphingosine/BSA complexes were prepared by a modification of the procedure of Lipsky and Pagano (1985b). Briefly, an aliquot of a C/M stock solution of the desired NBD lipid (50 nmol) was dried, first under a stream of nitrogen and then in vacuo. The dried lipid was dissolved in 200 μL of ethanol and injected into 10 mL of HMEM containing 0.34 mg of defatted BSA (Sigma Chemical Co.)/mL while being vortex mixed. This solution was dialyzed overnight at 4 °C against 500 mL of HMEM, dispensed into plastic tubes, and stored frozen at −20 °C. The complexes therefore contained approximately a 5 μM concentration of both the fluorescent lipid and BSA.

Cells and Cell Culture. Monolayer cultures of Chinese hamster V79 lung fibroblasts (Ford & Yerganian, 1958) were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Monolayer cultures of BHK-21 (C-13) (American Type Culture Collection) fibroblasts were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 10% tryptose phosphate broth. Both cell types were grown in a water-saturated atmosphere of 5% CO_2 in air. Cells were grown on 60 mm diameter tissue culture dishes for biochemical studies and on 25 mm diameter (No. 1 thickness) glass cover slips for microscopy studies.

Incubation of Fluorescent Lipids with Cells and Lipid Analysis. Cells were washed in HMEM and incubated with the fluorescent *N*-acylsphingosine/BSA complex (5 nmol/mL) for 60 min at 2 °C, washed, and further incubated in HMEM for 0 or 60 min at 37 °C prior to lipid extraction or fluorescence microscopy. Cells were harvested for lipid extraction with a rubber policeman and 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 TLC using C/M/H_2O (65/25/4) or C/M/NH_4OH/H_2O (160/40/1/3) as the developing solvent. In some experiments, an alternate extraction procedure (Sonderfeld et al., 1985) for retention of gangliosides was used, but no additional fluorescent lipids were detected.

Other Procedures. Fluorescence microscopy was performed with a Zeiss IM-35 inverted microscope equipped with a Planapo 100× (1.3 n.a.) objective. Photomicrographs were taken with Kodak Tri-X film and processed at ASA 1600 with Diafine developer.

Concentrations of NBD lipid stock solutions were determined by measurements of relative fluorescence compared to a fluorescent standard containing a known amount of NBD lipid. All measurements were made in C/M (2/1) on an AMINCO-BOWMAN spectrophotofluorometer (λ_{ex} = 470 nm; λ_{em} = 530 nm).

Other Chemicals. Glucopsychosine and sphingosylphosphocholine (a mixture of the D-*erythro* and L-*threo* isomers)[3] were obtained from Sigma Chemical Co. (*R*)-(−)-*O*-Acetylmandelic acid and D- and L-norleucine were from Aldrich Chemical Co. Tissue culture media were from Gibco Laboratories (Grand Island, NY).

RESULTS

Synthesis and Purification of Fluorescent (NBD) Compounds. In the present study we synthesized two groups of fluorescent *N*-acylsphingosines. First, four different long-chain bases (Figure 1) and some of their stereoisomers were N-acylated with ϵ-NBD-aminohexanoic acid, and second, D-*erythro*-sphingosine was N-acylated with several different fluorescent (NBD) fatty acids (Figure 2). The compounds synthesized are shown in Table I with their R_f values in two TLC systems. Most of the compounds were N-acylated by each of two procedures (Kishimoto, 1975; Schwarzmann & Sandhoff, 1987), both of which yielded the same product. The expected molecular weights of all the fluorescent *N*-acylsphingosines were confirmed by mass spectral analysis.

We prepared the four stereoisomers of *N*-(ϵ-NBD-aminohexanoyl)sphingosine using DL-*erythro*-sphingosine and DL-*threo*-sphingosine as the starting materials. This was accomplished by derivatizing each of the DL-sphingosine mixtures with an optically active fatty acid to form the *N*-acylsphingosine diastereoisomers [see Shoyama et al. (1978)] which could then be separated and purified by TLC. Each of the *N*-acylsphingosines was then hydrolyzed to sphingosine and fatty acid, and the stereochemically pure sphingosines obtained from the hydrolysis were N-acylated with ϵ-NBD-aminohexanoic acid (see Materials and Methods). Two of the four possible stereoisomers of *N*-(ϵ-NBD-aminohexanoyl)dihydrosphingosine were synthesized with a commercial source of the long-chain base containing a mixture of the D-*erythro* and L-*threo* isomers. After N-acylation, the fluorescent product was first purified by preparative TLC and the diastereoisomers separated by reverse-phase HPLC (see below).

[3] M. Koval and R. E. Pagano, unpublished observations.

Table I: Properties of Fluorescent (NBD) *N*-Acylsphingosine Analogues

fluorescent (NBD) fatty acid	long-chain base	R_f[a]	R_f[b]	half-time for spontaneous transfer at 25 °C (min)[c]	trans-bilayer move-ment[d]	Golgi labeled[e]	metabolized to SM	metabolized to GlcCer
ε-NBD-aminohexanoic acid	D-*erythro*-sphingosine	0.60	0.80	0.47 ± 0.01	+	+	+	+
ε-NBD-aminohexanoic acid	L-*erythro*-sphingosine	0.60	0.80	0.42 ± 0.03	+	+	+	+
ε-NBD-aminohexanoic acid	D-*threo*-sphingosine	0.60	0.80	0.50 ± 0.05	+	+	+	–
ε-NBD-aminohexanoic acid	L-*threo*-sphingosine	0.60	0.80	0.54 ± 0.03	+	+	++	–
ε-NBD-aminohexanoic acid	D-*erythro*-dihydrosphingosine	0.60	0.80	1.42 ± 0.06	+	+	+	–
ε-NBD-aminohexanoic acid	L-*threo*-dihydrosphingosine	0.60	0.80	1.54 ± 0.01	+	+	+	–
ε-NBD-aminohexanoic acid	phytosphingosine	0.46	0.71	0.29 ± 0.03	+	+	+	+
ε-NBD-aminohexanoic acid	3-ketosphingosine	0.73	0.86	0.83 ± 0.03	+	–	–	–
L-α-OH-ε-NBD-aminohexanoic acid	D-*erythro*-sphingosine	0.54	0.75	0.52 ± 0.03	+	+	+	tr[f]
D-α-OH-ε-NBD-aminohexanoic acid	D-*erythro*-sphingosine	0.48	0.71	0.60 ± 0.00	+	+	+	+
L-α-NBD-aminohexanoic acid	D-*erythro*-sphingosine	0.67	0.83	4.01 ± 0.13	+	wk[g]	tr	–
D-α-NBD-aminohexanoic acid	D-*erythro*-sphingosine	0.70	0.83	1.91 ± 0.14	+	+	+	–

[a] R_f in C/M/NH_4OH/H_2O (160/40/1/3 v/v/v/v). [b] R_f in C/M/H_2O (65/25/4 v/v/v). [c] Small unilamellar donor vesicles containing NBD lipid/*N*-Rh-PE/DOPC (1/1/98) and small unilamellar acceptor vesicles containing DOPC alone were formed in HCMF by ethanol injection (Kremer et al., 1977). The vesicles were diluted to 500 nmol of total lipid/mL; 100 μL of donor vesicles was added to 1.3 mL of HCMF and allowed to equilibrate in the fluorometer at 25 °C. The fluorescence (λ_{ex} = 470 nm; λ_{em} = 530 nm) was continuously monitored before and after the addition of 100 μL of acceptor vesicles, and the half-time for the change in fluorescence was then determined. Values represent the mean ± SD of three determinations. See Nichols and Pagano (1982). [d] Small unilamellar donor vesicles containing NBD lipid/*N*-Rh-PE/DOPC (1/1/98) were prepared by ethanol injection, and the RET between NBD and rhodamine was measured. This measurement was then repeated after the addition of increasing amounts of nonfluorescent acceptor vesicles (up to a 15-fold excess). Addition of an excess of acceptor vesicles removes the NBD lipid from the *outer* leaflet only (no transbilayer movement) or *both* leaflets (transbilayer movement) of the donor vesicle bilayer and reduces the RET signal accordingly [see Pagano and Longmuir (1985) and Pagano (1988)]. [e] BHK cells and V79 fibroblasts were incubated for 60 min at 2 °C with the indicated fluorescent lipid/BSA complex. The cells were then washed and further incubated for 60 min at 37 °C in HMEM. The cells were then extracted and analyzed by TLC, or identically treated cultures were observed under the fluorescence microscope. No differences in the metabolic profile or intracellular distribution of fluorescence were seen in the two cell types. Labeling of the Golgi apparatus was based on previous observations of the staining of this organelle in these and other cell types [see Lipsky and Pagano (1985b)]. [f] tr = trace amount seen on TLC. [g] wk = weak labeling.

A reverse-phase HPLC method readily separated the D-*erythro* and L-*threo* isomers of *N*-(ε-NBD-aminohexanoyl)-sphingosine and -dihydrosphingosine (Figure 3) but not enantiomeric mixtures of these compounds. Thus, when purified D-*erythro*-sphingosine or DL-*erythro*-sphingosine was N-acylated with ε-NBD-aminohexanoic acid, purified by preparative TLC, and analyzed, the product eluted as a single peak corresponding to position 1 of the chromatogram in Figure 3, while a single peak at position 2 was seen when purified L-*threo*-sphingosine or DL-*threo*-sphingosine was used. When D-*erythro*-sphingosine obtained from natural sources was used without further purification, two fluorescent *N*-acylsphingosines could be separated from one another by HPLC. These were a major peak corresponding to the D-*erythro* analogue and a minor peak corresponding to the L-*threo* analogue. (The relative amounts of the two depended on the commercial source of sphingosine.) Similar results were obtained with the dihydrosphingosine derivatives except that the D-*erythro* and L-*threo* isomers eluted respectively at positions 3 and 4 of the chromatogram.

Spontaneous Transfer of Fluorescent N-Acylsphingosine Derivatives between Liposomes and Their Transbilayer Movement in Liposomes. The fluorescent (NBD) *N*-acylsphingosines exhibited rapid spontaneous transfer between liposomes, with half-times of 0.29–4.0 min at 25 °C (Table I). These rapid rates of transfer are consistent with previous observations using lipids derivatized with a short-chain NBD-labeled fatty acid (Nichols & Pagano, 1982). When an excess of nonfluorescent acceptor vesicles was added to donor vesicles containing the fluorescent (NBD) *N*-acylsphingosines, all of the NBD lipid transferred into the acceptor vesicles, indicating transbilayer movement had occurred (Pagano & Longmuir, 1985; Pagano, 1988) (Table I). In contrast, when such studies were performed with the corresponding NBD derivative of GlcCer or SM, no transbilayer movement was detected (data not shown).

Metabolism and Intracellular Distribution of N-(ε-NBD-aminohexanoyl)sphingosines in Cells. Cultured fibroblasts

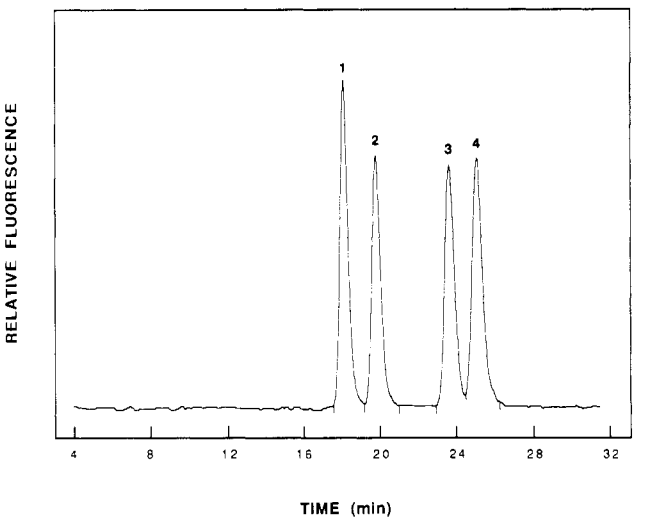

FIGURE 3: HPLC chromatogram of a mixture of *N*-(ε-NBD-aminohexanoyl)sphingosine derivatives. The peaks correspond to N-acylated D-*erythro*-sphingosine (1), L-*threo*-sphingosine (2), D-*erythro*-dihydrosphingosine (3), and L-*threo*-dihydrosphingosine (4).

were incubated with the ε-NBD-aminohexanoic acid labeled *N*-acylsphingosine/BSA complexes for 60 min at 2 °C, washed, and further incubated for 60 min at 37 °C. The cell lipids were then extracted and the extracts analyzed by TLC. All the *N*-(ε-NBD-aminohexanoyl)sphingosines were significantly metabolized to the corresponding fluorescent SM except for the 3-ketosphingosine analogue. The L-*threo*-sphingosine analogue produced significantly more fluorescent SM than the other analogues studied, but this effect was not quantified. Metabolism to fluorescent GlcCer was seen only with *N*-(ε-NBD-aminohexanoyl)-*erythro*-sphingosine (D or L isomer) and with the phytosphingosine derivative (Table I). The thin-layer chromatogram in Figure 4 documents the results for three of the lipids, fluorescent *N*-acyl-D-*erythro*-sphingosine, *N*-acyl-L-*threo*-sphingosine, and *N*-acyl-3-ketosphingosine analogues in BHK cells. (Identical profiles of the fluorescent metabolites

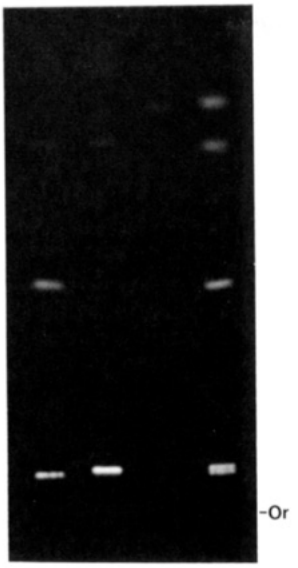

FIGURE 4: Metabolism of *N*-(ϵ-aminohexanoyl)sphingosine analogues in BHK cells. Cells were incubated with the indicated fluorescent lipid for 60 min at 2 °C, washed, and further incubated for 60 min at 37 °C in HMEM prior to lipid extraction. The lipid extracts were chromatographed in $C/M/H_2O$ (65/25/4), and the developed chromatogram was photographed under UV light. Incubations were with *N*-(ϵ-aminohexanoyl)-D-*erythro*-sphingosine (lane 1), L-*threo*-sphingosine (lane 2), and 3-ketosphingosine (lane 3). Fluorescent lipid standards: ϵ-NBD-aminohexanoic acid labeled 3-keto-sphingosine, D-*erythro*-sphingosine, GlcCer, L-*threo*-SM, and D-*erythro*-SM (lane 4, top to bottom).

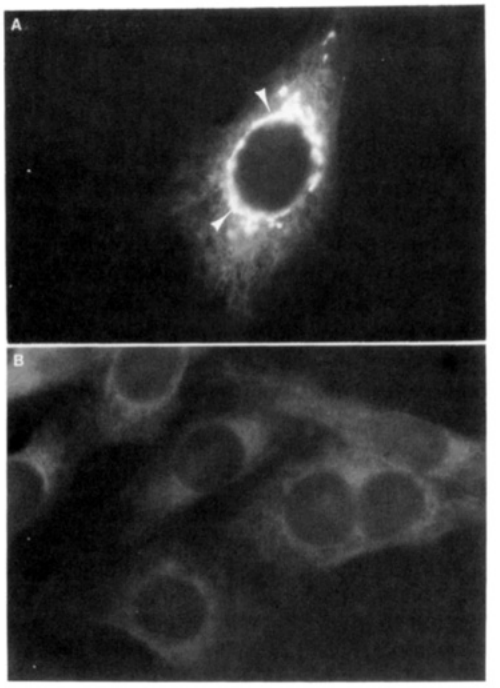

FIGURE 5: Fluorescence micrographs of BHK fibroblasts treated with two fluorescent *N*-(ϵ-NBD-aminohexanoyl)sphingosines: (A) *N*-(ϵ-NBD-aminohexanoyl)-D-*erythro*-sphingosine; (B) *N*-(ϵ-NBD-aminohexanoyl)-3-ketosphingosine. Prominent perinuclear labeling at arrows is the Golgi apparatus [see Lipsky and Pagano (1985b)].

were obtained with either BHK cells or Chinese hamster V79 lung fibroblasts.) The absence of higher order fluorescent glycolipids in the cell lipid extracts was not an artifact of the extraction procedure since an alternate method (Sonderfeld et al., 1985) which retains gangliosides gave identical results with those shown in Table I.

The intracellular distribution of fluorescence of each of the fluorescent *N*-(ϵ-NBD-aminohexanoyl)sphingosines and their metabolites in BHK and V79 cells was studied by fluorescence microscopy. When cells were incubated with the various NBD lipids for 60 min at 2 °C, washed, and further incubated for 60 min at 37 °C, labeling of the Golgi apparatus was seen in all cases except when the 3-ketosphingosine analogue was used (Table I; Figure 5). In control experiments, no *rhodamine* fluorescence could be detected when cells were incubated with *N*-(ϵ-NBD-aminohexanoyl)-D-*erythro*-sphingosine/rhodamine-labeled BSA for 60 min at 2 °C, suggesting that most of the NBD lipid became cell associated by lipid transfer from BSA at 2 °C rather than by adsorption of the NBD lipid/BSA complex.

Metabolism and Intracellular Distribution of N-Acyl-D-erythro-sphingosines Containing Different Fluorescent (NBD) Fatty Acids. Table I also summarizes the metabolism and intracellular distribution of fluorescent D-*erythro*-sphingosines which were N-acylated with the NBD-labeled fatty acids shown in Figure 2. In agreement with previous studies (Lipsky & Pagano, 1983, 1985a), when ϵ-NBD-aminohexanoic acid was used, pronounced labeling of the Golgi apparatus was seen, and metabolism to fluorescent SM and GlcCer occurred. When the α-OH-ϵ-NBD-aminohexanoic acids were used, both the L and D stereoisomers resulted in labeling of the Golgi apparatus, and both were metabolized to fluorescent SM. However, only the D-α-OH analogue was metabolized to GlcCer. When the α-NBD-aminohexanoic acids were used, labeling of the Golgi apparatus, although detectable with both stereoisomers, was much weaker with the L isomer. No metabolism to GlcCer was seen for either stereoisomer, and only a trace amount of SM synthesis was detected with the L isomer.

DISCUSSION

Physical Properties of Fluorescent N-Acylsphingosines. All of the fluorescent *N*-acylsphingosines were capable of transbilayer movement in liposomes, and all rapidly transferred between liposomes. However, the half-time for spontaneous transfer was dependent on both the long-chain base and the fluorescent fatty acid constituents of the molecules (Table I). Among the ϵ-NBD-aminohexanoic acid labeled compounds, the *N*-acylphytosphingosine derivative had the shortest half-time, whereas the *N*-acyldihydrosphingosine and *N*-acyl-3-ketosphingosine derivatives had the longest half-times. These differences might reflect the relative polarities of these compounds. For example, addition of a hydroxyl group in the case of the phytosphingosine derivative would be expected to increase its polarity relative to that of the sphingosine derivative, whereas substitution of a keto group for a hydroxyl group in the case of the 3-ketosphingosine analogue would be expected to decrease the polarity of the fluorescent derivative. A surprising result was obtained when the half-times for spontaneous transfer of *N*-acyl-D-*erythro*-sphingosines containing *different* fluorescent fatty acids (Figure 2) were studied. As seen in Table I, the half-times for spontaneous transfer of the D and L isomers of *N*-(α-NBD-aminohexanoyl)sphingosine were significantly longer than those of the other derivatives synthesized. The reason for this is unknown, but one possibility is that the hexanoic acid side chain can better intercalate into the membrane bilayer when the NBD fluorophore is close to the polar portion of the *N*-acylsphingosine than when it is at the end of the fatty acid chain. The recent study of Chattopadhyay and London (1987) is consistent with this interpretation. They presented evidence to suggest that the NBD group is sufficiently polar to cause it to "loop back" to the

polar/hydrocarbon interface when attached to the ends of flexible acyl chains of phosphatidylcholine.

Metabolism and Intracellular Distribution of the Fluorescent N-Acylsphingosines in Cultured Cells. In the present study we synthesized 12 fluorescent *N*-acylsphingosines for use in cellular studies. Several conclusions can be drawn from this work. (*i*) *Most of the fluorescent N-acylsphingosines were significantly metabolized to fluorescent SM.* The two exceptions were *N*-(L-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine and *N*-(ε-NBD-aminohexanoyl)-3-ketosphingosine, where little or no fluorescent SM was formed (Table I; Figure 4). Consistent with previous studies demonstrating that the nonnatural L-*threo* isomer of ceramide is a better substrate in vitro than the naturally occurring D-*erythro* isomer [see Kanfer (1983)], qualitatively more fluorescent SM was formed when the *N*-(ε-NBD-aminohexanoyl)-L-*threo*-sphingosine was incubated with intact cells (Figure 4). (*ii*) *Metabolism of the fluorescent N-acylsphingosines to fluorescent GlcCer was strongly dependent on the nature of the long-chain base and on the stereochemistry of the fluorescent fatty acid moiety.* Of the eight long-chain bases which were N-acylated with ε-NBD-aminohexanoic acid (Table I), only the phytosphingosine and D- or L-*erythro*-sphingosine analogues were metabolized to the corresponding fluorescent GlcCer. The formation of fluorescent GlcCer from the L-*erythro* compound was somewhat surprising given the earlier studies of Stoffel and co-workers in which the metabolism of the four possible stereoisomers of *dihydro*sphingosine was studied in rat liver (Stoffel & Bister, 1973). In that study only the D-*erythro* isomer was metabolized to cerebrosides. When *N*-(D- or L-α-OH-ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine was used, only the D isomer was metabolized to the corresponding fluorescent GlcCer. This is consistent with the occurrence of D- (but not L-) α-OH fatty acids as constituents of glycosphingolipids in nature (Tatsumi et al., 1974). (*iii*) *Labeling of the Golgi apparatus in cells was insensitive to the nature of the long-chain base and the fluorescent fatty acid moiety of the N-acylsphingosines.* All of the compounds in Table I labeled this organelle except for *N*-(ε-NBD-aminohexanoyl)-3-ketosphingosine and *N*-(L-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine. The molecular basis for this labeling is not known. One possibility is that labeling results from binding of the fluorescent *N*-acylsphingosines to specific proteins which might be involved in the metabolism or transport of (glyco)sphingolipids. Alternatively, the fluorescent *N*-acylsphingosines may interact strongly with the lipids of the Golgi membranes, possibly by hydrogen-bond formation. We are presently exploring various experimental strategies for distinguishing between these possibilities.

Perspectives. The fluorescent *N*-acylsphingosines described in this paper provide a basis for designing other *N*-acylsphingosine derivatives which might be useful in future studies of (glyco)sphingolipid metabolism, sorting, and transport. In particular, an *N*-acylsphingosine analogue which accumulates in the Golgi apparatus and which bears a radioactive and photoactivatable fatty acid could be used to selectively label proteins in the Golgi apparatus which are involved in these processes. Although a large number of membrane proteins would probably be labeled in such an experiment, the use of different long-chain bases as outlined here could modify the metabolism and intracellular distribution of the labeled *N*-acylsphingosine and aid in identifying the proteins of interest.

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Registry No. ε-NBD-aminohexanoic acid, 88235-25-0; L-α-OH-ε-NBD-aminohexanoic acid, 114301-90-5; D-α-OH-ε-NBD-aminohexanoic acid, 114301-91-6; L-α-OH-ε-NBD-aminohexanoic acid, 114301-92-7; D-α-NBD-aminohexanoic acid, 114301-93-8; D-*erythro*-sphingosine, 123-78-4; L-*erythro*-sphingosine, 6036-75-5; D-*threo*-sphingosine, 6036-85-7; L-*threo*-sphingosine, 25695-95-8; D-*erythro*-dihydrosphingosine, 764-22-7; L-*threo*-dihydrosphingosine, 15639-50-6; phytosphingosine, 554-62-1; 3-ketosphingosine, 19767-43-2; *N*-(ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine, 94885-02-6; *N*-(ε-NBD-aminohexanoyl)-L-*erythro*-sphingosine, 114301-94-9; *N*-(ε-NBD-aminohexanoyl)-D-*threo*-sphingosine, 114301-95-0; *N*-(ε-NBD-aminohexanoyl)-L-*threo*-sphingosine, 114301-96-1; *N*-(ε-NBD-aminohexanoyl)-D-*erythro*-dihydrosphingosine, 114301-97-2; *N*-(ε-NBD-aminohexanoyl)-L-*threo*-dihydrosphingosine, 114301-98-3; *N*-(ε-NBD-aminohexanoyl)phytosphingosine, 114301-99-4; *N*-(ε-NBD-aminohexanoyl)-3-ketosphingosine, 114302-00-0; *N*-(L-α-OH-ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine, 114302-01-1; *N*-(D-α-OH-ε-NBD-aminohexanoyl)-D-*erythro*-sphingosine, 114376-28-2; *N*-(L-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine, 114302-02-2; *N*-(D-α-NBD-aminohexanoyl)-D-*erythro*-sphingosine, 114376-29-3.

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Effects of Amino Acid Replacements in Yeast Iso-1 Cytochrome *c* on Heme Accessibility and Intracomplex Electron Transfer in Complexes with Cytochrome *c* Peroxidase†

James T. Hazzard,‡ George McLendon,§ Michael A. Cusanovich,‡ Goutam Das,∥ Fred Sherman,∥ and Gordon Tollin*,‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, Department of Chemistry, University of Rochester, Rochester, New York 14627, and Departments of Biochemistry and Biophysics, University of Rochester Medical School, Rochester, New York 14642

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ABSTRACT: The kinetics of reduction of wild type and several site-specific mutants of yeast iso-1 cytochrome *c* (Arg-13 → Ile, Gln-16 → Ser, Gln-16 → Lys, Lys-27 → Gln, Lys-72 → Asp), both free and in 1:1 complexes with yeast cytochrome *c* peroxidase, by free flavin semiquinones have been studied. Intramolecular one-electron transfer from the ferrous cytochromes *c* to the H_2O_2-oxidized peroxidase at both low (8 mM) and high (275 mM) ionic strengths was also studied. The accessibility of the cytochrome *c* heme within the electrostatically stabilized complex and the rate constants for intramolecular electron transfer at both low and high ionic strength are highly dependent on the specific amino acids present at the protein–protein interface. Importantly, replacement by uncharged amino acids of Arg or Lys residues thought to be important in orientation and/or stabilization of the electron-transfer complex resulted in increased rates of electron transfer. In all cases, an increase in ionic strengths from 8 to 275 mM also produced increased intramolecular electron-transfer rate constants. The results suggest that the electrostatically stabilized 1:1 complex is not optimized for electron transfer and that by neutralization of key positively charged residues, or by an increase in the ionic strength thereby masking the ionic interactions, the two proteins can orient themselves to allow the formation of a more efficient electron-transfer complex.

Both biological energy transduction and much of intermediary metabolism ultimately depend on the control of simple one electron transfer reactions between proteins. Therefore, understanding the factors that govern the rate and specificity of protein to protein electron transfer is a matter of intense current research (Cusanovich et al., 1987a; Marcus & Sutin, 1985; McLendon et al., 1985; Scott et al., 1985).

One particularly useful paradigm for such studies is provided by the reduction of peroxide-oxidized cytochrome *c* peroxidase [commonly referred to as either ferryl peroxidase, compound I, "ES", or CcP(IV,R$^{\bullet+}$)] by ferrous cytochrome *c* [cyt*c*(II)].[1] This reaction is assumed to be involved in peroxide detoxification in yeast, utilizing cyt *c*(II) and CcP(IV,R$^{\bullet+}$) as natural redox partners. These two proteins are particularly well suited for detailed studies of protein to protein electron transfer for several reasons:

(1) The crystal structure of each protein is known at high resolution for various oxidation states (Poulos et al., 1980; Edwards et al., 1987; Swanson et al., 1976; Takano & Dickerson, 1981; Louie et al., 1988).

(2) Cyt *c* and CcP form a thermodynamically well characterized complex (Nicholls & Mochan, 1971; Mochan & Nicholls, 1971; Erman & Vitello, 1980), and a detailed hypothetical model has been proposed for the relative orientations of cyt *c* and CcP within this electron-transfer complex (Poulos & Kraut, 1980; Koppenol & Margoliash, 1982; Poulos & Finzel, 1984) although recent efforts to cocrystallize the two proteins have led to ambiguous results (Poulos et al., 1987). A number of specific ionic interactions within the complex have been suggested by chemical modification and/or covalent cross-linking measurements (Kang et al., 1977, 1978; Waldmeyer & Bosshard, 1985).

(3) Photochemical techniques have been developed which permit detailed single-turnover kinetic measurements of the reaction cyt *c*(II)·CcP(IV,R$^{\bullet+}$) → cyt *c*(III)·CcP(III,R$^{\bullet+}$) (Hazzard et al., 1987, 1988a,b).

(4) Numerous amino acid replacements of yeast cyt *c* (Hampsey et al., 1986; Pielak et al., 1985; Liang et al., 1987) as well as several site-specific mutants of CcP (Goodin et al., 1986; Fishel et al., 1987; Cusanovich et al., 1988; Mauro et

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*Author to whom correspondence should be addressed.
‡University of Arizona.
§University of Rochester.
∥University of Rochester Medical School.

[1] Abbreviations: cyt *c*(II) and cyt *c*(III), ferrous and ferric species of cytochrome *c*; CcP(III) and CcP(IV,R$^{\bullet+}$), ferric and H_2O_2-oxidized species of cytochrome *c* peroxidase; LfH$^{\bullet}$ and LfH^{-}, semiquinone and fully reduced lumiflavin species; 5-DRf and 5-DRfH$^{\bullet}$, oxidized and semiquinone species of 5-deazariboflavin; EDTA, ethylenediaminetetraacetic acid; Ser-16 etc., altered iso-1 cytochromes *c* with Ser-16 etc. replacement (see Table I).

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