Sorting of an Internalized Plasma Membrane Lipid between Recycling and Degradative Pathways in Normal and Niemann-Pick, Type A Fibroblasts

Michael Koval and Richard E. Pagano
Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland 21210

Abstract. We examined the metabolism and intracellular transport of a fluorescent sphingomyelin analogue, N-(N-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl])-sphingosylphosphorylcholine (C₆-NBD-SM), in both normal and Niemann-Pick, type A (NP-A) human skin fibroblast monolayers. C₆-NBD-SM was integrated into the plasma membrane bilayer by transfer of C₆-NBD-SM monomers from liposomes to cells at 7°C. The cells were washed, and within 3 min of warming to 37°C, both normal and NP-A fibroblasts had internalized C₆-NBD-SM from the plasma membrane, resulting in a punctate pattern of intracellular fluorescence. Rates for C₆-NBD-SM internalization and transport from intracellular compartments to the plasma membrane (recycling) were similar for normal and NP-A cells.

With increasing time at 37°C, internalized C₆-NBD-SM accumulated in the lysosomes of NP-A fibroblasts, while normal fibroblasts showed increasing Golgi apparatus fluorescence with no observable lysosomal labeling. Since NP-A fibroblasts lack lysosomal (acid) sphingomyelinase (A-SMase), this result suggested that hydrolysis of C₆-NBD-SM prevented its accumulation in the lysosomes of normal fibroblasts during its transport along the degradative pathway.

We used the amount of C₆-NBD-SM hydrolysis by A-SMase in normal cells as a measure of C₆-NBD-SM transported from the cell surface to the lysosomes. After a lag period, C₆-NBD-SM was delivered to the lysosomes at a rate of ~8%/h. This rate was ~18-19 fold slower than the rate of C₆-NBD-SM recycling from intracellular compartments to the plasma membrane. Thus, small amounts of C₆-NBD-SM were transported along the degradative pathway, while most endocytosed C₆-NBD-SM was sorted for transport along the plasma membrane recycling pathway.

Receptors internalized from the plasma membrane are generally either recycled back to the plasma membrane or are transported along the "degradative" pathway to the lysosomes (reviewed in Goldstein et al., 1985; Wileman et al., 1985; Hubbard, 1989). An acidic intracellular compartment, known as sorting endosomes, 'early' endosomes, or CURL (compartment of uncoupling of receptor and ligand), has been implicated in the targeting of receptors to the appropriate destination (Geuze et al., 1983, 1987). One model for receptor sorting is that vesicles formed from tubular extensions of the sorting endosome are preferentially sorted to the plasma membrane. The remainder of the sorting endosome membrane, including most of the lumenal volume, moves to the lysosomal compartment (Rome, 1985; Linderman and Lauffenburger, 1988; Dunn et al., 1989).

One prediction of this model is that lipids internalized from the plasma membrane (Fig. 1, pathway I) should be transported through a sorting compartment to both the recycling pathway (Fig. 1, pathway II) and degradative pathway (Fig. 1, pathway III), provided that lipids are free to diffuse throughout the sorting endosome membrane. Further, if plasma membrane recycling is the major pathway for bulk flow of endocytosed membrane, one would expect that most of the endocytosed lipid would be returned to the plasma membrane.

Previously, we examined the transport of C₆-NBD-SM in CHO-K1 fibroblasts and found that the fluorescent lipid was recycled between intracellular compartments and the plasma membrane (Koval and Pagano, 1989). Both C₆-NBD-SM and Rh-transferrin, a protein that recycles, showed similar intracellular distributions upon internalization in CHO-K1 cells, suggesting that endocytosed C₆-NBD-SM is transported to sorting endosomes in these cells.

Although we measured C₆-NBD-SM recycling in CHO cells (i.e., Fig. 1, pathways I and II), C₆-NBD-SM did not label the lysosomes of these cells. One possibility is that C₆-NBD-SM recycling is dominant and the levels of C₆-
has been used to measure SM hydrolysis and turnover in NP-A fibroblasts only. After C\textsubscript{6}-NBD-SM hydrolysis, the fluorescent Cer produced can undergo transbilayer movement ("flip-flop") and subsequent transport to the Golgi apparatus (broken lines) where C\textsubscript{6}-NBD-Cer is metabolized to newly synthesized C\textsubscript{6}-NBD-SM and C\textsubscript{6}-NBD-GlcCer (see text).

NBD-SM transported along the degradative pathway (Fig. 1, pathway III) are too small to be detected. Alternatively, C\textsubscript{6}-NBD-SM might be transported to lysosomes but not retained there. For example, rapid retrograde transport of lipid from lysosomes to endosomes might occur. Alternatively, any fluorescent SM delivered to lysosomes might be rapidly hydrolyzed there to form C\textsubscript{6}-NBD-Cer. Since C\textsubscript{6}-NBD-Cer can undergo transbilayer movement (Pagano, 1989) and is capable of rapid and spontaneous monomeric diffusion (Pagano and Martin, 1988; Pagano, 1989), conversion of C\textsubscript{6}-NBD-SM to C\textsubscript{6}-NBD-Cer would permit rapid transport of C\textsubscript{6}-NBD-lipid from the lysosomes to other intracellular membranes (Fig. 1, broken lines). In particular, the Golgi apparatus has a high affinity for C\textsubscript{6}-NBD-Cer (Lipsky and Pagano, 1985b; Pagano and Martin, 1988; Pagano et al., 1989; Pagano, 1990). Consistent with this model, CHO-K1 cells labeled with C\textsubscript{6}-NBD-SM and then incubated at 37°C, hydrolyze increasing amounts of C\textsubscript{6}-NBD-SM with time and show concomitant increases in Golgi apparatus fluorescence (Koval and Pagano, 1989).

If hydrolysis of C\textsubscript{6}-NBD-SM is required for rapid depletion of C\textsubscript{6}-NBD-lipid from lysosomes, then elimination of A-SMase should result in accumulation of fluorescent SM in the lysosomes. Niemann-Pick, type A (NP-A) fibroblasts are deficient in A-SMase (Beaudet and Manschreck, 1982; Maziere et al., 1982). Differences in SM metabolism between normal and NP-A fibroblasts have previously been examined using exogenously added radiolabeled SM (Kudoh et al., 1983; Sutrina and Chen, 1984) and fluorescent (pyrene) SM (Levade and Gatt, 1987). Also, radiolabeling of endogenous SM, through the use of labeled metabolic precursors, has been used to measure SM hydrolysis and turnover in NP-A fibroblasts (Spence et al., 1983). Most studies on SM metabolism in NP-A fibroblasts focus on the long-term metabolism of SM (hours to days), and thus are difficult to interpret in the context of vesicular transport of SM between intracellular compartments, which occurs at rates of minutes to hours.

In this study, we examined normal and NP-A fibroblasts labeled at the plasma membrane with C\textsubscript{6}-NBD-SM and used fluorescence microscopy to demonstrate that the mutant fibroblasts accumulated endocytosed C\textsubscript{6}-NBD-SM in the lysosomes. This suggested that normal cells also transported C\textsubscript{6}-NBD-SM to the lysosomes. Since any C\textsubscript{6}-NBD-SM transported to the lysosomes in normal cells is likely to be rapidly hydrolyzed, the amount of C\textsubscript{6}-NBD-SM hydrolyzed by A-SMase should correspond to the amount of C\textsubscript{6}-NBD-SM transported along the degradative pathway. We were able to confirm this hypothesis, determine the amount of C\textsubscript{6}-NBD-SM transported to the lysosomes, and by comparison to the rate for C\textsubscript{6}-NBD-SM returned to the plasma membrane, determine the relative amount of C\textsubscript{6}-NBD-SM sorted along these two pathways.

### Glossary

- **A-SMase**: acid sphingomyelinas
- **C\textsubscript{6}-NBD**: N\textsubscript{6}-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)captorlyl
- **C\textsubscript{6}-NBD-Cer**: N\textsubscript{6}(C\textsubscript{6}-NBD)-sphingosine
- **C\textsubscript{6}-NBD-GlcCer**: N\textsubscript{6}(C\textsubscript{6}-NBD)-glucosylsphingosine
- **C\textsubscript{6}-NBD-SM**: N\textsubscript{6}(C\textsubscript{6}-NBD)-sphingosylphosphorylcholine
- **Cer**: ceramide
- **CHO-K1**: Chinese hamster ovary fibroblast cell line
- **DOPC**: dioleylophosphatidylcholine
- **GlcCer**: glucosylceramide
- **HCMP**: 10 mM Hepes-buffered calcium and magnesium-free Puck's saline, pH 7.4
- **hLAMP**: human lysosome-associated membrane protein
- **HMEM**: Hepes-buffered MEM
- **N-SMase**: neutral sphingomyelinas
- **NP-A**: Niemann-Pick disease, type A
- **SM**: sphingomyelina
- **SRh**: sulforhodamine
- **SUV**: small unilamellar vesicle

### Materials and Methods

#### Materials

- Dioleylophosphatidylincholine (DOPC) and dioleylophosphatidylethanolamin
- Avanti Polar Lipids, Inc. (Birmingham, AL). C\textsubscript{6}-NBD-fatty acid, Lucifer Yellow, sulforhodamine chloride, and sulforhodamine dextran, molecular mass 10 kDa (SRh-dextran) were purchased from Molecular Probes Inc. (Eugene, OR). Sodium cacodylate was from Electron Microscopy Sciences (Fort Washington, PA). All organic solvents were purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI). Fluorescein-conjugated secondary antibodies were from Organon Teknika-Cappel (Malvern, PA). Unless otherwise stated, all other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

#### Cell Culture

Monolayer cultures of normal (No. GM41) and Niemann-Pick, type A (No. GM112) fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ) and grown in DME (No. 430-2100; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) in a water-saturated atmosphere of 5% CO\textsubscript{2} in air. Cells were grown for 2-7 d on No. 1 thickness, 25-mm acid-washed glass coverslips to 20% confluency for microscopy, or on 100-mm plastic tissue culture dishes to 80% confluency for biochemical analysis. All cells were between passage numbers 12 and 20.
Lipid Synthesis and Analysis

9-erythro-C₆-NBD-SM was synthesized from C₆-NBD-fatty acid and sphingosylphosphorylcholine as described (Koval and Pagano, 1989). N-Sulfosorhodamine-conjugated dioleoylphosphatidylethanolamine (N-SRh-DOPE; Uster and Pagano, 1986) and N-(C₆-NBD)-9-erythro-sphingosine (C₆-NBD-Cer; Pagano and Martin, 1988) were synthesized and purified as described. Concentrations of lipid stock solutions were determined by phosphorus measurement (Rouser et al., 1966) or by reference to known concentrations of fluorescent standards.

Lipid Vesicles

Small unilamellar vesicles (SUV) consisting of either C₆-NBD-SM:DOPC or C₆-NBD-Cer:DOPC (2:3; mol/mol) were formed by ethanol injection (Kremer et al., 1977) as described (Koval and Pagano, 1989) and were typically diluted to a final working concentration of 2.5 μM total lipid in Hepes-buffered Eagle's MEM, pH 7.4 (HMEM), unless otherwise specified. DOPC vesicles for the back-exchange procedure (see below) were prepared as large unilamellar vesicles by extrusion (LUVET; Hope et al., 1985) and diluted to a final working concentration of 400 μM DOPC in HMEM, as described (Koval and Pagano, 1989).

Incubation of Lipid Vesicles with Cells

Monolayer cultures were cooled to 7°C for 5 min, washed twice with HMEM, and then incubated with vesicles containing fluorescent lipid, usually 25 μM C₆-NBD-SM:DOPC (2:3; mol/mol) SUV in HMEM at 7°C for 30 min. Incubations were stopped by washing the cells three times with cold HMEM. In most experiments, the cultures were subsequently warmed to 37°C by adding prewarmed HMEM to the cells and incubating at 37°C in a water bath.

To remove C₆-NBD-SM associated with the plasma membrane, the cells were back-exchanged (Sleight and Pagano, 1984; Struck and Pagano, 1980) at 7°C as described (Koval and Pagano, 1989). After back-exchange, cultures sometimes were further incubated at 37°C with either prewarmed HMEM alone or prewarmed HMEM containing LUVETs (back-exchange

Table I. Insertion of C₆-NBD-SM into the Plasma Membrane

| Cell type | C₆-NBD-SM/ cell DNA | Cell phospholipid/ cell DNA | C₆-NBD-SM/ cell phospholipid |
|-----------|---------------------|-----------------------------|-----------------------------|
| Normal    | 93.0 ± 12.3         | 3.70 ± 0.73                 | 25.1 ± 6.0                  |
| NP-A      | 95.3 ± 10.8         | 6.20 ± 0.72                 | 15.4 ± 2.5                  |

Both normal and NP-A fibroblasts were incubated with SUV containing C₆-NBD-SM for 30 min at 7°C, washed, and the amount of specific incorporation of C₆-NBD-SM was determined. Also, the total cellular phospholipid per microgram DNA was determined for both cell types. Values are the means of triplicate measurements ± SD.

Figure 2. Appearance of C₆-NBD-SM labeling and uptake in human fibroblasts. (a and c) Normal (a) or NP-A (c) human fibroblasts were labeled with C₆-NBD-SM/DOPC (2:3; mol/mol) SUV at 25 μM total lipid concentration for 30 min at 7°C, washed, and photographed. (b and d) Both normal (b) and NP-A (d) human fibroblasts were labeled as in a, washed, incubated for 10 min at 37°C in HMEM, treated with back-exchange medium at 7°C to remove C₆-NBD-SM remaining at the plasma membrane, washed, and photographed. Note the similarity between normal and NP-A fibroblasts treated under these conditions. Bar, 10 μm.
Figure 3. Transport of C₆-NBD-lipid to the Golgi apparatus in normal fibroblasts. Normal (a) and NP-A (b) human fibroblasts were labeled with SUV containing C₆-NBD-SM for 30 min at 7°C, washed, and incubated in HMEM for 60 min at 37°C. The cells were then treated with back-exchange medium at 7°C to remove C₆-NBD-SM remaining at the plasma membrane, washed, and photographed for C₆-NBD-lipid fluorescence. Although NP-A cells treated in this manner showed some perinuclear fluorescence (arrowheads), they did not show the same distinct reticular Golgi apparatus appearance exhibited by normal fibroblasts (a). Bar, 10 μm.
Figure 4. Distribution of lysosomes in human fibroblasts. Normal (a and b) or NP-A (c and d) human fibroblasts were incubated overnight in culture medium containing 0.6 μg SRh-dextran/ml, washed, incubated in HMEM for 2 h at 37°C, fixed, and photographed for SRh-dextran fluorescence (a and c). The cells were subsequently rendered permeable, treated with an antibody to a human lysosome-associated glycoprotein (hLAMP2), washed, labeled with fluorescein-conjugated goat anti-rabbit IgG, washed, and the field previously photographed for SRh fluorescence was rephotographed for fluorescein fluorescence (b and d). SRh-dextran and hLAMP were found to extensively colocalize. However, since this method required relocation of cells after indirect immunofluorescence labeling, subtle differences in fluorescein and SRh-fluorescence patterns are likely to arise from changes in the plane of focus. Bar, 10 μm.

To investigate metabolism of C6-NBD-Cer, cells were labeled for 30 min at 7°C with C6-NBD-Cer/DOPC (2:3; mol/mol) SUV at (a) concentrations of 3–50 μM total lipid, washed, and then incubated for 30 min at 37°C, or (b) at 25 μM total lipid, washed, and then incubated at 37°C for times ranging from 5 min to 2 h.

Analysis of Fluorescent Lipid Metabolism

Measurement of cell-associated C6-NBD-lipid species was determined as described (Koval and Pagano, 1989) by quantitative video imaging of lipid extracts (Bligh and Dyer, 1959) chromatographed on Silica Gel 60 thin-layer plates using CHCl3/CH3OH/28% NH4OH/H2O (72:48:2:9; vol/vol/vol/vol) as the developing solvent. DNA content from aliquots of cell samples was determined with Hoescht 33258 (Labarca and Paigen, 1980) using salmon sperm DNA as a standard.

The percentage of C6-NBD-SM removed by the back-exchange process [% (C6-NBD-SM)rem] was calculated using the equation:

\[
% \ (C_6\text{-NBD-SM})_{\text{rem}} = 1 - \frac{(C_6\text{-NBD-SM})_{\text{ax}}}{(C_6\text{-NBD-SM})_{\text{tot}}} \times 100, \quad (1)
\]

where the amount of C6-NBD-SM was determined as pmol fluorescent SM/μg DNA in both back-exchanged cells [(C6-NBD-SM)ax] and non-back-exchanged cells [(C6-NBD-SM)tot]. Back-exchange efficiency was determined by calculating the % (C6-NBD-SM)rem, expressed as a fraction, for cells labeled, maintained at 7°C, and treated with back-exchange medium at 7°C as described (Koval and Pagano, 1989). The amount of C6-NBD-lipid removed by back-exchange divided by the back-exchange efficiency (normal: 0.90; NP-A: 0.91) reflects the amount of fluorescent lipid located at the plasma membrane (Martin and Pagano, 1987; van Meer et al., 1987; Koval and Pagano, 1989).

The amount of newly-synthesized C6-NBD-SM in D-erythro-C6-NBD-SM labeled cells was calculated from the amount of cell-associated C6-NBD-GlcCer by:

\[
\text{(newly-synthesized C6-NBD-SM)} = R \times (C_6\text{-NBD-GlcCer}), \quad (2)
\]

where R was the ratio of C6-NBD-SM to C6-NBD-GlcCer synthesized by fibroblasts after incubation with C6-NBD-Cer [normal: \(R = 1.29 \pm 0.14\) (37°C; n = 18), 0.73 \pm 0.17 (19°C; n = 7); NP-A: \(R = 0.72 \pm 0.32\) (37°C; n = 19), 0.26 \pm 0.08 (19°C; n = 6)]. The amount of C6-NBD-SM that was not metabolized was determined by:

\[
\text{(nonmetabolized C6-NBD-SM)} = (\text{total C6-NBD-SM}) - (\text{newly synthesized C6-NBD-SM}) \quad (3)
\]

Values in Eqs. 2 and 3 were determined as pmol C6-NBD-SM/μg DNA.
In Vitro Sphingomyelinase Assay

Determination of sphingomyelinase activity in vitro was performed essentially as described by Chatterjee and Ghosh (1989). Briefly, monolayer cultures of cells were scraped into 5 ml H2O with a policeman in the presence of protease inhibitors (2 μg/ml antipain, 2 μg/ml leupeptin, 2 mM PMSF, 100 KIU/ml aprotinin). The culture dish was washed with an additional 5 ml H2O containing protease inhibitors, which was combined with the cell suspension. The cells were then placed in a nitrogen cavitation bomb (Parr Instrument Co., Moline, IL), which was maintained at 1,100 psi and 0°C for 20 min before disruption. The disrupted cells were then collected and centrifuged at 500 g for 5 min at 4°C and the resulting post nuclear supernatant was assayed for protein content using the Bradford reagent (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard. The pellet was discarded. 10 mM C6-NBD-SM/BSA complexes were prepared in a manner analogous to the preparation of C6-NBD-Cer/BSA complexes (Pagan and Martin, 1988). Each reaction mixture contained 400 μl cell homogenate, 400 μl of either 250 mM sodium acetate buffer (pH 4.0–5.6) or 250 mM tris-glycine buffer (pH 6.0–8.0), 10 μl C6-NBD-SM/BSA solution, and 8 μl of either 50 mM EDTA or 125 mM MgCl2. The reaction mixtures were incubated at 37°C for 1 h and then the lipids were extracted and analyzed as described (Koval and Pagano, 1989).

Microscopy and Lysosome Labeling Procedures

Specimens were observed using a Zeiss IM-35 microscope equipped with epi-fluorescence optics and filter combinations which eliminated cross-over between NBD (or fluorescein) and rhodamine fluorescence channels. Photomicrography and processing of photomicrographs were performed as described (Martin and Pagano, 1987). Lysosomes were labeled by incubating cells with 600 μg/ml SRh-dextran in DME for 12 h, followed by a chase of 2 h or more at 37°C with HMEM (Ferris et al., 1987). Alternatively, lysosomes were visualized by indirect immunofluorescence using an antibody to hLAMP-2, kindly provided by Drs. Y. Cha and J. T. August (Johns Hopkins School of Medicine) (Mane et al., 1989). For double-label experiments with hLAMP-2, cells were first labeled with SRh-dextran as described above. All remaining steps were performed at room temperature. The cells were fixed in 3% paraformaldehyde-0.2% glutaraldehyde as described (Koval and Pagano, 1989), washed, and then photographed using optics appropriate for SRh fluorescence. The samples were then treated with 0.2 M glycine in H2O for 5 min, followed by a 10-s treatment with 100% methanol at −20°C to render the cells permeable to antibodies. The cells were then washed with PBS containing 0.2% gelatin, and incubated with anti-hLAMP-2 at a 1:100 dilution in PBS-gelatin for 30 min. The cells were washed, incubated with fluorescein-conjugated goat anti-rabbit IgG at a 1:100 dilution in PBS-gelatin, and then observed using epi-fluorescence optics and filter combinations which eliminated cross-over between fluorescein and SRh fluorescence, with no observable lysosomal accumulation.

Results

C6-NBD-SM Labeling and Internalization

Normal and NP-A human fibroblasts incubated with 25 μM C6-NBD-SM/DOPC (2:3; mol/mol) SUV in HMEM for 30 min at 37°C showed equivalent amounts of labeling as a result of spontaneous transfer of fluorescent SM from SUV to the cells (Table I). In some experiments, SUV used for labeling also contained 2 mol % N-SRh-DOPE, a nonexchangeable fluorescent lipid marker (Slight and Pagano, 1984; Struck and Pagano, 1980; Struck et al., 1981) that provided a measure of SUV nonspecifically adsorbed to the cells. From these experiments we conclude that >96% of the cell-associated C6-NBD-SM fluorescence was due to insertion of C6-NBD-SM into the plasma membrane of both cell types. Both normal and NP-A fibroblasts labeled with fluorescent SM at 7°C showed bright plasma membrane fluorescence (Fig. 2, a and c). As long as the labeled cells were kept at 7°C, at least 90% of the cell-associated fluorescent lipid could be removed by back-exchange (normal: 90.2 ± 12.0% (n = 3); NP-A: 90.7 ± 10.4% (n = 3)). When cells labeled at 7°C were warmed to 37°C for 10 min, internalization of fluorescent lipid was observed. As shown in Fig. 2 b and d, both normal and NP-A fibroblasts treated under these conditions had a similar distribution of intracellular vesicles containing fluorescent lipid. With increasing incubation time at 37°C, C6-NBD-SM-labeled normal cells showed increasing Golgi apparatus fluorescence (Fig. 3 a), consistent with previous observations in CHO-K1 cells (Koval and Pagano, 1989). In contrast, NP-A cells showed some fluorescence in the region of the Golgi apparatus (Fig. 3 b), however the labeling pattern was less distinct.

Transport of C6-NBD-SM to Lysosomes in NP-A Fibroblasts

To examine the distribution of lysosomes in human fibroblasts, cells were incubated at 37°C overnight with culture medium containing SRh-dextran, a fluorescent fluid-phase marker, followed by incubation at 37°C in HMEM alone for at least 2 h (Ferris et al., 1987). As shown in Fig. 4, this resulted in SRh-dextran accumulation in the lysosomes, as indicated by a pattern of intracellular SRh fluorescence, which showed substantial overlap with the distribution of hLAMP-2, a human lysosome-associated membrane (glyco)protein (Mane et al., 1989), as visualized by indirect immunofluorescence.

We examined the intracellular distribution of C6-NBD-SM in NP-A and normal cells after vital staining of the lysosomes with SRh-dextran. As shown in Fig. 5, a and b, NP-A cells labeled with C6-NBD-SM at 7°C and then incubated for 30 min at 37°C did not show fluorescent SM localized to lysosomes labeled with SRh-dextran. However, when the 37°C incubation time was increased to 2 h, lysosomes, in addition to other endocytic compartments, were labeled with C6-NBD-SM (Fig. 5, c and d). In contrast, normal cells labeled with C6-NBD-SM and subsequently incubated for 2 h at 37°C did not accumulate any C6-NBD-SM in the lysosomes; rather, most of the intracellular C6-NBD-lipid was localized to the Golgi apparatus, similar to the cells shown in Fig. 3 a.

Normal and NP-A cells were labeled with C6-NBD-SM, incubated for 60 min at 37°C in HMEM, and then further incubated for 30 min at 37°C in back-exchange medium to remove C6-NBD-lipid from endosomes and the recycling pathway (Koval and Pagano, 1989). NP-A cells treated in this manner retained intracellular C6-NBD-SM with a punctate distribution, which colocalized with a subset of SRh-dextran labeled lysosomes (Fig. 6, a and b). However, equivalently treated normal cells showed almost exclusive Golgi apparatus fluorescence, with no observable lysosomal accumulation of C6-NBD-lipid (Fig. 6, c and d).

Fluorescent Sphingolipid Metabolism in Human Fibroblasts

Since NP-A cells are deficient in A-SMase (Beaudet and Manschreck, 1982; Maziere et al., 1982), differences in the intracellular distribution of C6-NBD-lipid between NP-A and normal fibroblasts were likely due to differences in the metabolism of C6-NBD-SM. As shown in Fig. 7, both normal and NP-A fibroblasts labeled with fluorescent SM at 7°C and then incubated at 37°C showed some hydrolysis of C6-NBD-SM to C6-NBD-Cer, as well as production of C6-NBD-GlcCer. To confirm that C6-NBD-SM hydrolysis by
NP-A fibroblasts was not due to low levels of A-SMase activity, homogenates were prepared from normal and NP-A fibroblasts and analyzed for in vitro SMase hydrolysis of fluorescent SM as a function of pH (Fig. 8). As expected, homogenates from normal cells had high levels of SMase activity at low pH. However, hydrolysis of C\textsubscript{6}-NBD-SM by NP-A cell homogenates was Mg\textsuperscript{2+}-dependent, with maximum activity at pH ~7.0 and no detectable SM hydrolysis at acidic pH. The pH profile of C\textsubscript{6}-NBD-SM hydrolysis by NP-A cell homogenates suggested that C\textsubscript{6}-NBD-SM hydrolyzed by intact NP-A cells was due to N-SMase activity.

To determine whether intact normal and NP-A fibroblasts contained similar amounts of N-SMase, we examined the metabolism of C\textsubscript{6}-NBD-SM in cells incubated at 19°C. It has been shown that incubation of cells at 19°C inhibits vesicular transport to the lysosomes (Dunn et al., 1980; Aulinskas et al., 1982). This was confirmed by examining cells containing lysosomes labeled with SRh-dextran, which were subsequently incubated for 2 h at 19°C in the presence of a fluid-phase marker, Lucifer Yellow. Neither normal nor NP-A cells transported Lucifer Yellow to the lysosomes during the 19°C incubation (Fig. 9, a–d). Further, normal or NP-A fibroblasts which were labeled with C\textsubscript{6}-NBD-SM and subsequently incubated at 19°C for 2 h did not accumulate fluorescent C\textsubscript{6}-NBD-SM in lysosomes (Fig. 9, e–h).

As shown in Table II, normal and NP-A cells incubated at 19°C hydrolyzed comparable amounts of C\textsubscript{6}-NBD-SM. Since this represents SMase activity in the absence of transport to the lysosomes, it seems likely that normal and NP-A cells have comparable amounts of nonlysosomal SMase activity (i.e., N-SMase). Thus, the difference between the amount of C\textsubscript{6}-NBD-SM hydrolyzed by intact normal human fibroblasts and by equivalently treated NP-A cells should reflect the amount of C\textsubscript{6}-NBD-SM hydrolyzed by A-SMase in normal human fibroblasts. As shown in Fig. 10, the calculated time course for A-SMase activity showed a ~20–30-min lag period, followed by increasing amounts of C\textsubscript{6}-NBD-SM hydrolysis. The lag period is likely to be related...
Comparison of C6-NBD-SM accumulation by normal and NP-A human fibroblasts. NP-A (a and b) or normal (c and d) human fibroblasts were labeled overnight at 37°C with SRh-dextran, washed, and then incubated for 2 h at 37°C in HMEM. The cells were then labeled with SUV containing C6-NBD-SM at 7°C for 30 min, washed, incubated for 60 min at 37°C in HMEM, followed by treatment with back-exchange medium at 7°C to remove fluorescent lipid at the plasma membrane. The cells were then further incubated in back-exchange medium at 37°C for 30 min to remove fluorescent lipid capable of recycling, washed, then photographed for either (a and c) C6-NBD-lipid or (b and d) SRh-dextran fluorescence. Note that NP-A fibroblasts (a and b) show accumulation in lysosomes, while normal cells (c and d) show prominent labeling of the Golgi apparatus (arrowheads) with no detectable lysosomal labeling. Bar, 10 μm.

The Journal of Cell Biology, Volume 111, 1990 436

Quantitation of C6-NBD-SM Endocytosis and Recycling

Fibroblasts were labeled with C6-NBD-SM at 7°C, incubated at 37°C for increasing amounts of time, and the internalization of C6-NBD-SM from the plasma membrane into intracellular compartments resistant to back-exchange was examined quantitatively. For normal fibroblasts, the amount of endocytosed, nonmetabolized C6-NBD-SM reached a plateau value of ~20% of the total cell-associated C6-NBD-lipid within 3 min, which was maintained for 2 h at 37°C (Fig. 11 a). In contrast, NP-A fibroblasts labeled with C6-NBD-SM and subsequently incubated at 37°C for 2 h accumulated more intracellular, nonmetabolized C6-NBD-SM than normal cells (normal: 19.3 ± 2.0% [n = 3]; NP-A: 35.8 ± 10.4% [n = 5]; Fig. 11 b). Differences between normal and NP-A cells in the amount of intracellular, nonhydrolyzed C6-NBD-SM appeared to reflect differences in C6-NBD-SM hydrolysis instead of differences in transport (see Discussion). Consistent with this explanation, the rate of internalization of nonmetabolized C6-NBD-SM from the plasma membrane was comparable for both normal and NP-A fibroblasts (Fig. 11 c). In particular, both cell types have the same rapid initial rate of uptake.
We also measured the rate of internalized fluorescent SM returned to the plasma membrane (recycling) in both cell types. Fibroblasts were labeled with C₆-NBD-SM at 7°C, washed, incubated at 37°C for 15 min, and treated with back-exchange medium at 7°C to remove fluorescent SM remaining at the plasma membrane. The cells were then further incubated at 37°C and the amount of C₆-NBD-SM returned from intracellular compartments to the plasma membrane was determined (Fig. 12). Both normal and NP-A cells showed comparable amounts of intracellular C₆-NBD-SM returned to the plasma membrane during further incubation at 37°C (Fig. 12 c). From this we estimate that the halftime of transport from intracellular compartments to the plasma membrane was ~5 min for both cell types, with an initial rate of 7.8 ± 1.6% C₆-NBD-SM recycled/min.

As seen in Fig. 12, after further incubation at 37°C in back-exchange medium for 1 h, NP-A fibroblasts retained more nonhydrolyzed C₆-NBD-SM in intracellular compartments (47.3 ± 19.8% [n = 3]) than did normal cells (17.6 ± 5.6% [n = 3]). This difference could be accounted for by the greater amount of C₆-NBD-SM hydrolyzed by normal fibroblasts (23.6 ± 4.9% [n = 3]). Also, the prominent Golgi apparatus labeling by normal cells observed in Fig. 6 c was consistent with the relatively high amounts of C₆-NBD-SM hydrolysis and accumulation of fluorescent SM in lysosomes (see Fig. 6 a).

Note that NP-A cells showed no hydrolysis of intracellular C₆-NBD-SM (Fig. 12 b), which was consistent with the localization of N-SMase to the cell surface. Thus, the amount of intracellular C₆-NBD-SM hydrolyzed by normal cells should directly reflect A-SMase activity, i.e., delivery to the

Figure 7. Metabolism of C₆-NBD-SM by normal and NP-A fibroblasts. Either normal (a) or NP-A (b) human fibroblasts were labeled with SUV containing C₆-NBD-SM for 30 min at 7°C, washed, and incubated for the indicated times at 37°C in HMEM. The cell-associated lipids were extracted, separated by TLC, and the fluorescent metabolites were measured and expressed as a percentage of total cell-associated C₆-NBD-lipid. (●) C₆-NBD-SM; (●) C₆-NBD-Cer; (▲) C₆-NBD-GlcCer. Data points are the means of triplicate measurements.

Figure 8. SMase activity of fibroblast homogenates. Either normal (a) or NP-A (b) cells were homogenized using a nitrogen cavitation bomb, and aliquots containing equal amounts of cell protein were adjusted to pH 4.5–8.0 in the presence of either Mg²⁺ or EDTA. C₆-NBD-SM was added to each aliquot and the samples were incubated at 37°C for 1 h. The lipids were then extracted, separated by TLC, and the amount of C₆-NBD-Cer formed in the presence of either Mg²⁺ (●) or EDTA (▲) was measured and expressed as pmol C₆-NBD-Cer formed/μg homogenate protein per minute. Note the difference in scale between a and b. Also, SMase hydrolysis in normal cells (a) is not Mg²⁺ dependent, suggesting that SM hydrolysis at pH 7.0–7.4 in normal cells is due to A-SMase activity that is sufficient to mask N-SMase activity in these homogenates, given the level of SMase activity found in NP-A cells (b).
lyosomes. Further, A-SMase activity was not detected until the cells had been incubated at 37°C for a total of ~20 min (15 min incubation for internalization followed by a 5-min second incubation), consistent with the lag observed in Fig. 10. We estimate that 0.42 ± 0.06% of the intracellular C6-NBD-SM/min was delivered to the lysosomes after this lag. From this, the rate of C6-NBD-SM returned to the plasma membrane was determined to be 18.6 ± 4.5-fold faster than the rate for C6-NBD-SM delivery to the lysosomes.

Discussion

In this study, we compared the transport and metabolism of C6-NBD-SM by normal human skin fibroblasts with NP-A fibroblasts. Both normal and NP-A cells incubated at 7°C with SUVs containing C6-NBD-SM incorporated equivalent amounts of fluorescent SM into the plasma membrane (Table I), and showed similar distributions of intracellular C6-NBD-lipid after short (10 min) incubations at 37°C (Fig. 2). In contrast to CHO-K1 fibroblasts, which accumulate intracellular vesicles containing endocytosed C6-NBD-lipid in the perinuclear region of the cell, human fibroblasts did not collect internalized C6-NBD-SM in a single region of the cell. Instead, vesicles containing internalized fluorescent lipid were distributed throughout the cell in both NP-A and human fibroblasts. Cell-specific differences in endosome distribution have also been observed by using both C6-NBD-phosphatidylcholine (Sleight and Abanto, 1989) and fluorescent conjugates of transferrin (Salzman and Maxfield, 1988) as markers for endocytosis.

Rates of endocytosis (Fig. 11 c) and recycling (Fig. 12 c) were comparable for both cell types. However, NP-A fibroblasts differed from normal cells in the amount of intracellular, nonmetabolized C6-NBD-SM accumulated during 37°C incubations. This observation can be interpreted in light of the accumulation of C6-NBD-SM in lysosomes by NP-A cells. For normal cells, the intracellular pool of nonmetabolized C6-NBD-SM is increased by endocytosis (Fig. 1, pathway I) and decreased by return of nonmetabolized C6-NBD-SM to the plasma membrane (pathway II) and by transport to the lysosomes (pathway III), followed by hydrolysis of C6-NBD-SM to C6-NBD-Cer by A-SMase (Fig. 1, broken lines). In contrast, NP-A fibroblasts do not hydrolyze C6-NBD-SM at the lysosomes. Therefore, delivery to the lysosomes redistributes, but does not decrease, the intracellular pool of nonmetabolized C6-NBD-SM. Thus, if rates of endocytosis, recycling, and delivery to the lysosomes are comparable for both cell types, then the amount of C6-NBD-SM internalized by NP-A fibroblasts should be equivalent to the sum of the amount of C6-NBD-SM hydrolyzed by A-SMase and the amount of nonmetabolized, intracellular C6-NBD-SM present in normal human fibroblasts. As shown in Table III, this appears to be the case.

Both normal and NP-A fibroblasts recycled plasma membrane lipid at rates similar to the rate of LDL receptor recycling in human fibroblasts, where receptors recycle between intracellular compartments and the plasma membrane once every 10 min (Brown and Goldstein, 1979; Goldstein et al., 1985). The rate for C6-NBD-SM recycling to the plasma membrane was ~18-19 times faster than the rate for C6-NBD-SM delivery to the lysosomes.

Table II. Hydrolysis of C6-NBD-SM at 37 and 19°C

| Cell type | 37°C | 19°C |
|-----------|------|------|
| Normal    | 51.4 ± 8.7 | 24.2 ± 4.7 |
| NP-A      | 19.1 ± 3.2 | 15.6 ± 6.3 |

Both normal and NP-A fibroblasts were labeled with SUV containing C6-NBD-SM at 7°C, washed, and incubated in HMEM at either 37 or 19°C for 4 h. The cell-associated lipids were then extracted, separated by TLC, and the total C6-NBD-SM hydrolyzed was calculated as described in the text. Values are the means ± SD of three measurements, except for NP-A cells at 19°C (six measurements).

Figure 9. Inhibition of transport to lysosomes in normal and NP-A cells incubated at 19°C. For all cases, cells were first labeled overnight at 37°C with SRh-dextran, washed, and then incubated for 2 h in HMEM at 37°C. (a-d) Normal (a and b) or NP-A (c and d) cells were subsequently incubated for 2 h at 19°C in HMEM containing the fluorescent fluid phase marker, Lucifer Yellow, washed, and then photographed for Lucifer Yellow (a and c) or SRh-dextran (b and d) fluorescence. Neither normal nor NP-A cells accumulated Lucifer Yellow in lysosomes (arrowheads) at 19°C. (e-h) Normal (e and f) or NP-A (g and h) cells were labeled with SUV containing C6-NBD-SM for 30 min at 7°C, washed, and incubated for 2 h at 19°C, followed by back-exchange at 7°C. The cells were then washed and photographed for NBD (e and g) and SRh-dextran (f and h) fluorescence. Note the lack of lysosomal labeling (arrowheads) by C6-NBD-SM in NP-A fibroblasts at 19°C, in contrast to the accumulation of C6-NBD-SM in lysosomes observed at 37°C (see Fig. 5, c and d). Bar, 10 μm.
Table III. Comparison of C6-NBD-SM Internalization for Normal and NP-A Cells

| Hours | Normal | NP-A |
|-------|--------|------|
|       | C6-NBD-SM | C6-NBD-SM | Sum | C6-NBD-SM |
| 1     | 22.7 ± 5.0 | 9.2 ± 2.4 | 31.9 ± 5.5 | 28.8 ± 18.8 |
| 2     | 19.3 ± 2.0 | 17.7 ± 3.7 | 37.0 ± 4.1 | 35.8 ± 10.4 |

Values for intracellular, nonmetabolized C6-NBD-SM for normal cells are from Fig. 11 a; values for C6-NBD-SM hydrolyzed by A-SMase are from Fig. 10; values for intracellular, nonmetabolized C6-NBD-SM for NP-A cells are from Fig. 11 b. Cells were labeled with SUV containing C6-NBD-SM at 7°C, followed by incubation for 1 or 2 h at 37°C. Sum is defined as the sum of intracellular, nonmetabolized C6-NBD-SM and C6-NBD-SM hydrolyzed by A-SMase for normal cells. See text for details.

It has been determined that 60–90% of the sorting endosome membrane of baby hamster kidney fibroblasts is organized into tubules, which are believed to be involved in plasma membrane recycling (Marsh et al., 1986; Griffiths et al., 1989). Although similar measurements have not been made on human skin fibroblasts, it appears that the sorting of endocytosed C6-NBD-SM reflects the relative amount of sorting endosome membrane transported along the recycling and degradative pathways, since C6-NBD-SM should be free to diffuse throughout the entire sorting endosome membrane. This is also consistent with the results of Dunn et al. on C6-NBD-SM delivery to lysosomes, after the lag period. We believe that this ratio reflects the relative amount of C6-NBD-SM sorted between the recycling and degradative pathways. Since there was a 20–30 min lag before detectable A-SMase hydrolysis, fluorescent SM destined for lysosomal hydrolysis was either sequestered into a nonrecycling portion of the sorting endosome or delivered to some other prelysosomal compartments that lack A-SM. If sorting of C6-NBD-SM to these compartments was faster than lipid movement along the degradative pathway, then the pool of nonmetabolized, intracellular C6-NBD-SM, shown in Fig. 11, should slowly increase during a 37°C incubation as a result of accumulation in prelysosomal compartments. However, after the rapid loading of endosomal and recycling compartments, levels of nonmetabolized, intracellular C6-NBD-SM remain constant in normal cells. Similar results were previously observed for CHO-K1 cells (Koval and Pagano, 1989). This suggests that C6-NBD-SM sorting between the plasma membrane recycling and degradative pathways is the rate limiting step in commitment of endocytosed C6-NBD-SM for transport to lysosomes.

Figure 11. Quantitative analysis of C6-NBD-SM internalization upon 37°C incubation. (a and b) Normal (a) or NP-A (b) human fibroblasts were labeled with SUV containing C6-NBD-SM for 30 min at 7°C, washed, incubated at 37°C in HMEM for the indicated amount of time, and then harvested either immediately, or, in parallel experiments, after treatment with back-exchange medium at 7°C to remove C6-NBD-SM remaining at the plasma membrane. The cell-associated lipids were extracted and analyzed by TLC as described in the text. The amount of back-exchangeable C6-NBD-SM was calculated using Eq. 1 (see Materials and Methods) and all values are expressed as a percentage of total cell-associated C6-NBD-lipid. The amounts of newly synthesized C6-NBD-SM and nonmetabolized C6-NBD-SM were calculated by Eqs. 2 and 3, respectively (see Materials and Methods). (c) Nonmetabolized C6-NBD-SM at the plasma membrane; (m) intracellular nonmetabolized C6-NBD-SM; (a) newly synthesized C6-NBD-SM at the plasma membrane; (u) intracellular newly synthesized C6-NBD-SM. Data points are the means of either three (a) or five (b) measurements and are expressed as percentages of the total cell-associated NBD lipid. (c) Comparison of the amount of nonmetabolized C6-NBD-SM remaining at the plasma membrane for normal (c) and NP-A cells (e) showing that uptake of C6-NBD-SM is similar for both cell types. Data points are the means ± SD.
Figure 12. Quantitation of C6-NBD-SM recycling to the plasma membrane. (a and b) Normal (a) or NP-A (b) human fibroblasts were labeled with SUV containing C6-NBD-SM at 7°C for 30 min, washed, incubated at 37°C for 15 min, and treated with back-exchange medium at 7°C to remove C6-NBD-SM remaining at the plasma membrane. The cells were then further incubated for the indicated amount of time at 37°C in either HMEM or back-exchange medium, washed, and the cell-associated lipids were extracted and analyzed by TLC (see Materials and Methods). The amount of nonmetabolized C6-NBD-SM was calculated using Eqs. 1-3, while the amount of C6-NBD-SM hydrolyzed was calculated as described in the legend of Fig. 11. (c) Nonmetabolized C6-NBD-SM returned to the plasma membrane; (D) intracellular non-

metabolized C6-NBD-SM; (E) total cell-associated C6-NBD-SM hydrolyzed. Data points are the means of triplicate measurements expressed as percentages of the total cell-associated lipid. (c) Comparison of the amounts of intracellular nonmetabolized C6-NBD-SM returned to the plasma membrane by normal (C) and NP-A cells (D) showing that recycling of C6-NBD-SM is similar for both cell types. Data points are the means ± SD of triplicate measurements expressed as percentages of the total initial cell-associated intracellular C6-NBD-SM.
selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of 125I-asialofetuin by the perfused rat liver. J. Biol. Chem. 255:5971-5978.

Ferris, A. L., J. C. Brown, R. D. Park, and B. Storrie. 1987. Chinese hamster ovary cell lysosomes rapidly exchange contents. J. Cell Biol. 105:2703-2712.

Geuze, H. J., J. W. Slot, G. J. A. M. Strous, H. F. Lodish, and A. L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. Cell. 32:277-287.

Geuze, H. J., J. W. Slot, and A. L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. J. Cell Biol. 104:1715-1723.

Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Rome. 1983. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1:1-39.

Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J. Cell Biol. 109:2703-2720.

Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochem. Biophys. Acta. 812:53-65.

Hubbard, A. L. 1989. Endocytosis. Curr. Op. Cell Biol. 1:675-683.

Koval, M., and R. E. Pagano. 1989. Lipid recycling between the plasma membrane and intracellular compartments: transport and metabolism of fluorescent sphingomyelin analogues in cultured fibroblasts. J. Cell Biol. 108:2169-2181.

Kremer, J. M. H., M. W. J. v. d. Esker, C. Pathmamanoharan, and P. H. Wiersema. 1977. Vesicles of variable diameter prepared by a modified injection method. Biochemistry. 16:3932-3935.

Kudoh, T., M. A. Velkoff, and D. A. Wenger. 1983. Uptake and metabolism of radioactively labeled sphingomyelin in cultured skin fibroblasts from control and patients with Niemann-Pick disease and other lysosomal storage diseases. Biochim. Biophys. Acta. 793:169-179.

Linderman, J. L., and D. A. Lauffenhurger. 1988. Analysis of intracellular receptor/ligand sorting in endosomes. J. Theor. Biol. 132:203-245.

Lippeicot-Swartz, J., and D. M. Fambrough. 1987. Cycling of the integral membrane protein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. Cell. 49:669-685.

Lipsky, N. G., and R. E. Pagano. 1983. Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue. Proc. Natl. Acad. Sci. USA. 80:2608-2612.

Lipsky, N. G., and R. E. Pagano. 1985a. Intracellular translocation of fluorescent sphingolipids in cultured fibroblasts: endogenously synthesized sphingomyelin and glucosylcerebroside analogues pass through the Golgi apparatus en route to the plasma membrane. J. Cell Biol. 100:27-34.

Lipsky, N. G., and R. E. Pagano. 1985b. A vital stain for the Golgi apparatus. Science (Wash. DC). 228:745-747.

Mane, S. M., L. Marzelia, D. P. Bainton, V. K. Holt, Y. Cha, J. E. K. Hildreth, and J. T. August. 1989. Purification and characterization of human lysosomal membrane glycoproteins. Arch. Biochem. Biophys. 268:360-378.

Marsh, M., G. Griffiths, G. E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. Proc. Natl. Acad. Sci. USA. 83:2899-2903.

Martin, O. C., and R. E. Pagano. 1987. Transbilayer movement of fluorescent analogs of phosphatidylycerine and phosphatidylcholine at the plasma membrane of cultured cells. J. Biol. Chem. 262:5890-5898.

Maziere, J. C., C. Maziere, L. Morn, J. D. Routier, and J. Polonovski. 1982. In situ degradation of sphingomyelin by cultured fibroblasts and fibroblasts from patients with Niemann-Pick disease type A and C. Biochem. Biophys. Res. Commun. 108:1101-1106.

Pagano, R. E. 1989. A fluorescent derivative of ceramide: physical properties and use in studying the Golgi apparatus of animal cells. Methods Cell Biol. 29:75-85.

Pagano, R. E. 1990. The Golgi apparatus: insights from lipid biochemistry. Biochem. Soc. Trans. 18:361-366.

Pagano, R. E., and O. C. Martin. 1988. A series of fluorescent N-(acyl)-sphingosines: synthesis, physical properties, and studies in cultured cells. Biochemistry. 27:4439-4445.

Pagano, R. E., M. A. Sepanski, and O. C. Martin. 1989. Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy. J. Cell Biol. 109:2067-2079.

Rome, H. L. 1985. Curling receptors. Trends Biochem. Sci. 10:151.

Rouser, B., A. N. Siakotos, and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorous analysis of spots. Lipids. 1:35-86.

Salzman, N. H., and F. R. Maxfield. 1988. Intracellular fusion of sequentially formed endocytic compartments. J. Cell Biol. 106:1083-1091.

Sleight, R. G., and M. N. Abanto. 1989. Differences in intracellular transport of a fluorescent phosphatidylcholine analog in established cell lines. J. Cell Sci. 63:363-374.

Sleight, R. G., and R. E. Pagano. 1984. Transport of a fluorescent phosphatidylcholine analog from the plasma membrane to the Golgi apparatus. J. Cell Biol. 99:742-751.

Spence, M. W., J. T. R. Clarke, and H. W. Cook. 1983. Pathways of sphingomyelin metabolism in cultured fibroblasts from normal and sphingomyelin lipidosis subjects. J. Biol. Chem. 258:8595-8600.

Struck, D. K., and R. E. Pagano. 1983. Insertion of fluorescent phospholipids into the plasma membrane of a mammalian cell. J. Biol. Chem. 258:5404-5410.

Struck, D. K., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. Biochemistry. 20:4093-4099.

Sutrina, S. L., and W. W. Chen. 1984. Lysosomal involvement in cellular turnover of plasma membrane sphingomyelin. Biochim. Biophys. Acta. 793:169-179.

Uster, P. S., and R. E. Pagano. 1986. Resonance energy transfer microscopy: observations of membrane-bound fluorescent probes in model membranes and in living cells. J. Cell Biol. 103:1221-1234.

van Meer, G., E. H. K. Stelzer, W. Wijnaendts-van-Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. J. Cell Biol. 105:1623-1635.

Wileman, T. C., Harding, and P. Stahl. 1985. Receptor-mediated endocytosis. Biochem. J. 232:1-14.