In situ assay of acid sphingomyelinase and ceramidase based on LDL-mediated lysosomal targeting of ceramide-labeled sphingomyelin

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Abstract The activity of lysosomal sphingolipid hydrolases is usually estimated in vitro from complex assays on cell lysates under artificial conditions including the presence of detergents and substrate analogs. However, the measure of their effective activity in situ (i.e., in living cells) is necessary to understand the normal intracellular sphingolipid turnover. Moreover, their determination in cells from patients with genetic enzyme deficiencies represents a key parameter of the pathophysiology of sphingolipid storage disorders. In this report, we have developed a procedure for estimating the effective activity of lysosomal sphingomyelinase and ceramidase in situ. This procedure is based on the selective targeting to lysosomes of a natural substrate under physiological conditions of substrate influx. Epstein-Barr virus-transformed human lymphoid cells and human skin fibroblasts were incubated with purified human low density lipoproteins (LDL) containing [^{3}H]ceramide-labeled sphingomyelin. Data demonstrate that this substrate is internalized through the apolipoprotein B/E receptor pathway and targeted to lysosomes. Lysosomal localization of the incorporated substrate was evidenced by ultrastructural autoradiography and subcellular fractionation as well as by metabolic studies in mutant cells. Short-term pulse-chase experiments with LDL-associated [^{3}H]ceramide-labeled sphingomyelin allowed us to determine the effective activity of lysosomal sphingomyelinase and ceramidase in normal cells. Initial velocities of sphingomyelin and ceramide degradation were, respectively, estimated at 0.66 and 1.14 nmol·h^{-1}·mg cell protein^{-1} in lymphoid cells, and 5.4 and 3 nmol·h^{-1}·mg cell protein^{-1} in skin fibroblasts. The advantages and applications of these in situ studies are discussed—**Levade, T., M. Leruth, D. Graber, A. Moisand, S. Vermeersch, R. Salvayre, and P. J. Courtoy.** In situ assay of acid sphingomyelinase and ceramidase based on LDL-mediated lysosomal targeting of ceramide-labeled sphingomyelin. *J. Lipid Res.* 1996. **37:** 2525–2538.

Supplementary key words Niemann-Pick disease • Farber disease • I-cell disease

Sphingolipids are important constituents of eukaryotic cell membranes and are implicated in a variety of cell functions (for reviews, see refs. 1–6). Their degradation mainly takes place in the lysosomes through the sequential action of various sphingolipid-specific acid hydrolases (1, 7). Inherited functional defects of individual lysosomal hydrolases result in sphingolipidoses, a group of diseases characterized by intralysosomal accumulation of the corresponding sphingolipid substrates (7, 8). These deficiencies may be caused by an alteration of the enzyme itself or by a defect in a sphingolipid-activator protein that is required for efficient activity of the corresponding sphingolipid hydrolase in vivo (for reviews, see refs. 9 and 10).

Most human sphingolipidoses occur in various clinical forms that differ by the age of onset and the severity of symptoms (from the first months of life to late adulthood, e.g., in gangliosidoses, Krabbe disease, or metachromatic leukodystrophy), or by the occurrence of neurologic involvement (e.g., in Niemann-Pick or Gaucher diseases; 7, 8). The explanation in molecular terms for different phenotypes of the same enzyme deficiency remains elusive, yet is necessary for a correct understanding of their pathophysiology. The generally accepted hypothesis considers that different gene alterations lead to unequal loss of activity of the affected enzyme. While molecular biology techniques have recently permitted the identification of a number of mutations for several lipid hydrolases (8, 11), the cru-

Abbreviations: SPM, sphingomyelin; NPD, Niemann-Pick disease; LCL, lymphoid cell lines; FH, familial hypercholesterolemia; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; TLC, thin-layer chromatography; apo, apolipoprotein.

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cial problem of determining effective residual activities in intact cells has remained unsolved.

The activity of lysosomal sphingolipid hydrolases is usually determined in vitro by incubating lysates with the appropriate lipid substrates (or substrate analogs) in the presence of detergents or activator proteins (12). Under these artificial conditions, the different clinical forms of the same lipidosis cannot be accurately distinguished. Efforts have then been made to measure the activity of lysosomal enzymes in intact, living fibroblasts by loading tests with exogenous substrates (see Discussion). However, although these in situ experiments have indicated some differences among various forms of lipidoses, their interpretation remains difficult. Indeed, despite the fact that the natural lipidic substrates are not water-soluble, they were simply added to the cell culture medium. Accordingly, no information was provided about the physico-chemical state of the substrate, its mode of entry into cells, its subcellular compartmentalization and the enzyme involved in its degradation. In addition, the initial rates of substrate degradation were not determined, leading to incorrect estimations of residual activities in mutant cells.

In view of the existence of non-lysosomal sphingolipid hydrolases, we consider that a lysosomal enzyme activity is best measured when the substrate is specifically targeted to the lysosomes. Therefore, this study aimed at developing a procedure to determine the effective activity of two lysosomal sphingolipid hydrolases, sphingomyelinase and ceramidase, i.e., enzymes responsible for the degradation of sphingomyelin (SPM) and ceramide, two lipids that are important components of cell membranes and plasma lipoproteins and may participate in intracellular signaling (3, 13).

MATERIALS AND METHODS

Chemicals and radiolabeled lipids

[^{3}H]ceramide-labeled sphingomyelin ([^{3}H]ceramide-SPM, 400 mCi/mmol) was obtained from CEA (Gif-sur-Yvette, France) by catalytic tritiation of bovine brain SPM (Sigma, St. Quentin Fallavier, France); the sphingolipid was purified by preparative TLC, using chloroform–methanol–water 100:42:6 (by vol) as developing solvent. The purity of the radioactive SPM was further checked by analytical TLC and corresponded to 97–99%. Acidic hydrolysis showed that the SPM was radiolabeled on both the sphingoid base and fatty acid moieties, in which the radiolabel represented 45 and 55%, respectively. Radiolabeled ceramide was obtained by hydrolysis of [^{3}H]ceramide-SPM using *B. cereus* sphingomyelinase (Sigma). [^{3}H]AMP (25.5 Ci/mmol) was supplied from DuPont NEN (Courtaboeuf, France). 4-Methyl-umbelliferyl substrates, *p*-iodonitrotetrazolium and chloroquine were from Sigma; silica gel 60 TLC plates (No. 5721) were from Merck (Darmstadt, Germany). All solvents and other reagents obtained from Merck or SDS (Peypin, France) were of analytical grade.

Nycodenz, RPMI 1640 medium, penicillin, streptomycin, L-glutamine, trypsin-EDTA, and fetal calf serum were from Gibco BRL (Cergy-Pontoise, France). The serum substitute, Ultroser HY, was from IBF (Villeneuve-la-Garenne, France).

Human cell lines

Long-term lymphoid cell lines (LCL) were established by Epstein-Barr virus-transformation of peripheral blood B lymphocytes (14, 15) from normal individuals (males or females) or from homozygous patients affected with NPD Type A (lines ElG, MS-325, Tre), with Farber disease (line GM 5748), or with LDL receptor-negative form of familial hypercholesterolemia (FH; lines GM 1459, GM 1767). Human skin fibroblasts were derived from normal individuals (males or females) and from homozygous patients affected with NPD Type A (line GM 370), Farber disease (line GM 5752), or I-cell disease (Mucolipidosis Type II; line 406). The MS-325 cell line was kindly provided by Prof. R.J. Desnick (Division of Medical Genetics, The Mount Sinai Hospital, New York) and the line 406 was obtained from Dr. M.T. Zabot (Laboratoire de Biochimie, Hôpital Debrousse, Lyon, France). The GM 5748 and GM 5752 cell lines were derived from the same patient with Farber disease; both leukocytes and fibroblasts of this patient showed undetectable acid ceramidase activity (16). The GM cell lines were from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Identical culture conditions were used for lymphoid cells and fibroblasts. The cells were routinely grown in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium containing L-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (10%) as previously reported (15, 17). Cultured skin fibroblasts were studied between the 8th and 18th passages and used just after reaching confluency.

Preparation of LDL-associated [^{3}H]ceramide-SPM

[^{3}H]ceramide-SPM-labeled LDL were prepared as follows. An ethanolic solution of tritiated SPM (about 400 10^6 dpm) was added to 10 ml of filtered pooled, freshly collected human sera, gently mixed, and incubated overnight at 37°C. The subsequently labeled serum was adjusted to a density of 1.185 g/ml with solid KBr. Thirty ml of 0.9% NaCl adjusted to a density of 1.025

g/ml with KBr was transferred into a 40-ml centrifugation tube and the serum mixture was layered under the saline to fill the tube (18). Centrifugation was performed in a Beckman vertical VTi 50 rotor at 4°C and 140 000 *g* for 6 h in a Beckman L8-70 ultracentrifuge. In our first series of experiments, 10 fractions of 4 ml were collected from the tube bottom and analyzed for density, radioactivity, and apolipoprotein content. In subsequent experiments, only the fraction containing the LDL (the LDL band was clearly seen in the upper third of the tube) was removed by puncturing the tube at the lowest level of the band and aspiration through a syringe.

In other experiments, LDL and lipoprotein-deficient serum (LPDS) were isolated from human sera by sequential ultracentrifugation at densities of 1.063 and 1.21 g/ml, respectively. [^{3}H]ceramide-SPM was incubated with LPDS alone or a mixture of LPDS and LDL, and the mixtures were analyzed by discontinuous density gradient ultracentrifugation as described above for whole serum.

The [^{3}H]ceramide-SPM-labeled LDL preparation was extensively dialyzed at 4°C against saline (150 mM NaCl, 0.3 mM EDTA, pH 7.4), filtered through a 0.2-μm pore diameter membrane filter, and stored at 4°C until use.

Electrophoretic migration of lipoprotein fractions was studied on agarose gels (Hydragel-Lipo Sebia, Issyles-Moulineaux, France). Apolipoprotein (apo) B and A-I contents were determined by nephelometry (Behring, Marburg, Germany).

Incubation of intact cells with radiolabeled SPM

Before the experiments were initiated, the cells were grown for 2–3 days in RPMI 1640 medium containing L-glutamine, antibiotics, and 2% Ultroser HY, a serum substitute devoid of lipoproteins. Then, cells were incubated at 37°C for the indicated periods with medium containing 2% Ultroser HY and LDL-associated [^{3}H]ceramide-SPM (unless otherwise stated, the final concentration of LDL in the incubation medium averaged 50 μg apoB/ml). For lymphoid cells, uptake was terminated by low-speed sedimentation, and pelleted cells were washed thrice with PBS containing bovine serum albumin (2 mg/ml) and then twice with PBS alone (15). Skin fibroblasts were similarly washed in the dish, then harvested using a rubber policeman. In "pulse-chase" experiments, after incubation with LDL-associated [^{3}H]ceramide-SPM, cells were briefly washed twice at 37°C in RPMI medium containing 10% fetal calf serum. Fresh medium supplemented with 10% fetal calf serum was added to the cells and incubation continued for the indicated times. At the end of chase period, cells were washed as mentioned above. The cell pellets were stored at −20°C.

Lipid extraction and analyses

Cell pellets were suspended in 0.6 ml distilled water and sonicated for 3 × 15 sec (Soniprep MSE sonicator). After an aliquot was taken for protein determination (19) and another for estimating the total cell-associated radioactivity by liquid scintillation counting (Packard Tricarb 4530 spectrometer), the remainder was extracted with 2.5 ml of chloroform–methanol 2:1 (by vol.), vortex-mixed, and centrifuged at 1000 *g* for 15 min (20). The lower phase of the Folch extract was evaporated under nitrogen and the lipids were resolved by analytical TLC developed in chloroform–methanol–water 100:42:6 (by vol.) up to 2/3 of the plate and then in chloroform–methanol–acetic acid 94:1:5 (by vol.) or in hexane–diethylether–formic acid 55:45:1 (by vol.). The distribution of the radioactivity on the plate was analyzed using a Berthold LB 2832 radiochromatoscan. Unlabeled and radioactive lipid standards were used to identify the various [^{3}H]ceramide-SPM metabolic products.

Electron microscopic autoradiography

After a 24-h incubation with [^{3}H]ceramide-SPM in the presence of 10% fetal calf serum or 2% Ultroser HY, lymphoid cells were sedimented and washed twice in RPMI 1640 medium. Cells were resuspended in medium and fixed by increasing concentrations of glutaraldehyde (up to 1.75% final) in 100 mM sodium cacodylate, 2 mM $MgCl_2$, for 1 h at room temperature. Then, cells were centrifuged at 4000 *g* for 10 min in BEEM conical capsules and further fixed by 2.5% glutaraldehyde in 100 mM cacodylate, 2 mM $MgCl_2$, for 3 h at room temperature. Samples were then rinsed 3 times with 100 mM cacodylate and 2 mM $MgCl_2$, and postfixed by 2% OsO_4 in water for 3 h at 4°C. After dehydration in ethanol followed by epoxy-1,2-propane, samples were embedded in Epon for 48 h. Sections of 150–250 nm thickness were collected on copper grids, covered with a 25% monogranular Ilford L4 emulsion (applied with a wire loop), and left for 6 months at 4°C. Sections were developed using Kodak D19b, fixed, stained with 5% uranyl acetate followed by lead citrate, and observed with a Philips EM301 electron microscope.

Subcellular fractionation studies

After a 24-h incubation with LDL-associated [^{3}H]ceramide-SPM, lymphoid cells were washed as described and resuspended in 6 ml of ice-cold homogenization buffer (0.25 M sucrose, 2 mM HEPES, 1 mM $MgCl_2$, pH 7.4). Cells were homogenized at 4°C by repeated (usually 20) passages through a Balch's ball-bearing device with an inside diameter of 8.02 mm using a ball of 8.006 mm in diameter (21). Cellular debris and nuclei (Frac-

tion N) were sedimented by low-speed centrifugation (1200 *g* for 8 min) and resuspended in homogenization buffer. The postnuclear supernatant was centrifuged at 4°C and 22,000 *g*, for 60 min using a Beckman 50Ti rotor. The supernatant (Fraction S) was isolated and the pellet (designated Fraction MLP, containing postnuclear particles) was resuspended in 0.5 ml of homogenization buffer using a Dounce homogenizer. Postnuclear particles (0.3 ml) were then mixed with 0.85 ml of a Nycodenz–sucrose solution adjusted at the density of 1.33 g/ml, and layered under a continuous gradient of Nycodenz in homogenization buffer, prepared from two 5.5-ml solutions of 1.05 and 1.20 g/ml densities. After centrifugation at 4°C and 15,000 *g* for 2.5 h in a Kontron TST41.14 swinging rotor, 10–12 fractions were collected from the tube bottom using a peristaltic pump, and the density was determined.

These fractions as well as fractions N, S, and MLP were analyzed for volume, radioactivity, protein content (22), and marker enzymes: N-acetyl-β-hexosaminidase (23), 5′-nucleotidase (24), succinate-tetrazolium reductase (25), and α-glucosidase (26).

RESULTS

Effect of serum on lysosomal targeting of SPM

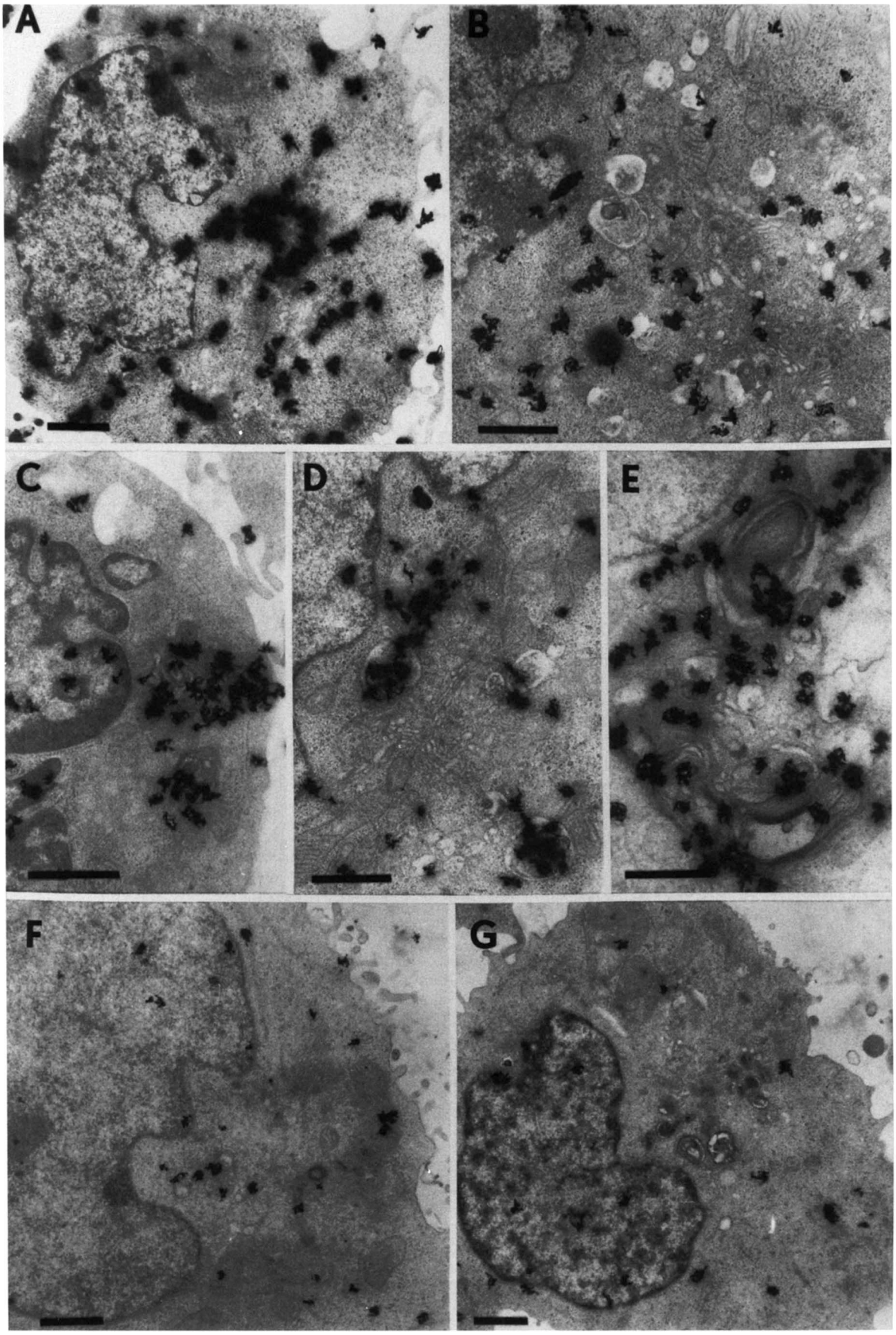
As previous studies showed association of fluorescently-labeled SPM to fetal calf serum lipoproteins (27), and as uptake of this SPM added to cultured cells occurs through the LDL receptor-mediated pathway (15, 17), initial experiments aimed at studying the effect of serum lipoproteins present in the cell culture medium on the targeting of [^{3}H]ceramide-SPM to lysosomes. Lymphoid cells from normal individuals and Niemann-Pick disease (NPD) Type A patients were incubated with [^{3}H]ceramide-SPM in the absence or presence of 10% fetal calf serum. While incubation in the absence of serum produced no significant difference in the rates of radiolabeled SPM degradation between normal and lysosomal sphingomyelinase-deficient cells (undegraded SPM representing 75–85% of the total cell-associated radioactivity in both cell lines after 48 h), a clear difference was seen after incubation in the presence of serum. Under the latter conditions, intact SPM represented 38.4 ± 12% (n = 9) of the total cell-associated radioactivity in control cells after 24 h, compared with 86.5 ± 4% (n = 5) in NPD type A cells. Similar data were obtained after a 12-h incubation with [^{3}H]ceramide-SPM followed by a 24-h chase (data not shown). These results are in good agreement with previous data using another radiolabeled SPM (15, 17) and suggest that in the presence of serum lipoproteins a major part of [^{3}H]ceramide-SPM is directed to, and degraded within, lysosomes.

Localization of [^{3}H]ceramide-SPM by ultrastructural autoradiography

To get further insight into the fate of [^{3}H]ceramide-SPM taken up by lymphoid cells in the absence or presence of serum, the subcellular localization of the radioactive lipid was investigated by ultrastructural autoradiography. Preliminary experiments indicated that >50% of the cell-associated radioactivity present after cell washes was recovered after all the fixation steps required for electron microscopy processing. As shown in **Fig. 1** (F and G), cells incubated without serum showed few autoradiographic grains. In the presence of fetal calf serum, normal cells contained many more grains, representing [^{3}H]ceramide-SPM or its metabolites (28, 29), mostly distributed in compartments other than lysosomes (Fig. 1A and B). In marked contrast, the distribution of [^{3}H]ceramide-SPM in NPD Type A cells incubated with serum was essentially lysosomal (Fig. 1C, D, E). Association with lysosomes was evident, as these cells exhibit enlarged residual bodies with characteristic concentrically laminated structures (the so-called "myelin-figures"; 30). In NPD Type A cells, the density of radioactive grains over lysosomes ranged from 10 to 19 per μm^2, while it averaged only one grain/μm^2 over the cytoplasm or the nucleus, a value considered as background radioactivity.

Although this ultrastructural autoradiographic analysis supported a major lysosomal localization of the [^{3}H]ceramide-SPM delivered to the NPD cells in the presence of serum, there was still a clearly detectable residual SPM turnover in severely lysosomal sphingomy-

Fig. 1. Ultrastructural localization of [^{3}H]ceramide-SPM offered to lymphoid cells in the presence or absence of fetal calf serum. Lymphoid cells from normal individuals (A, B, and F) and from patients with NPD Type A (C, D, E, and G) were incubated for 20 h with [^{3}H]ceramide–SPM (10^7 dpm/ml) in the presence of 10% fetal calf serum (A to E) or 2% Ultroser HY, a serum substitute devoid of lipoproteins (F and G). At the end of this incubation, cells were processed as described in Materials and Methods. When the [^{3}H]ceramide-SPM was offered to normal cells in the presence of serum, autoradiographic grains are distributed throughout the cell cytoplasm (A and B), consistent with the finding that more than 60% of the [^{3}H]ceramide-SPM taken up is degraded and converted into a variety of metabolic products that are reused for membranes (notice the low background of autoradiographic grains over nuclei). In contrast, in lysosomal sphingomyelinase-deficient, NPD Type A cells, where 85–90% of the radioactive SPM remains undergraded, most grains are concentrated in lysosomes (C to E), which display the myelin-like figures characteristic of the disease. Irrespective of their origin from normal (F) or NPD Type A patients (G), cells fed [^{3}H]ceramide-SPM in the absence of serum lipoproproteins do not concentrate grains over lysosomes. All bars indicate 1 μm.



elinase-deficient NPD Type A cells. This indicated that not all SPM was internalized via LDL and sequestered within lysosomes, and pointed to the participation of a non-lysosomal sphingomyelinase, a metabolic pathway that compounded the in situ assay of acid sphingomyelinase. As SPM can stably associate with LDL (15, 27), we purified this complex before incubation with cells, in order to target this substrate exclusively through the LDL receptor-mediated pathway for processing by the lysosomes of intact cells.

Association of [^{3}H]ceramide-SPM with LDL

The association of [^{3}H]ceramide-SPM with lipoproteins was investigated by incubating the radiolabeled SPM with human serum overnight at 37°C and by following the density distribution of the radioactivity in relation with the various lipoprotein fractions. As shown in **Fig. 2,** a major fraction of the radiolabeled SPM codistributed with apoB (and visually with the yellow LDL band), at an average density of 1.052 g/ml. A similar result was obtained when the lipoproteins were separated by agarose gel electrophoresis (**Fig. 3**). A minor proportion of [^{3}H]ceramide-SPM cofractionated with apoA/HDL (Fig. 2 and Fig. 3B).

When [^{3}H]ceramide-SPM was incubated in the presence of human LPDS, most of the radioactivity remained at the bottom of the gradient (Fig. 2B). However, when LPDS was supplemented with purified LDL during the incubation with radiolabeled SPM, the radioactivity peaked again at ~1.049 g/ml, a pattern similar to that of complete serum (Fig. 2B).

Analysis of the LDL band isolated after incubation of [^{3}H]ceramide-SPM with serum showed that this LDL preparation migrated on agarose gel as a single band with the typical mobility of LDL (Fig. 3A) and did not contain apoA (data not shown). In addition, more than 95% of the radioactive SPM present in this preparation was associated with LDL (Fig. 3C). Under our experimental conditions, the incorporation of [^{3}H]ceramide-SPM into LDL ranged from 4000 to 12000 dpm/μg apoB.

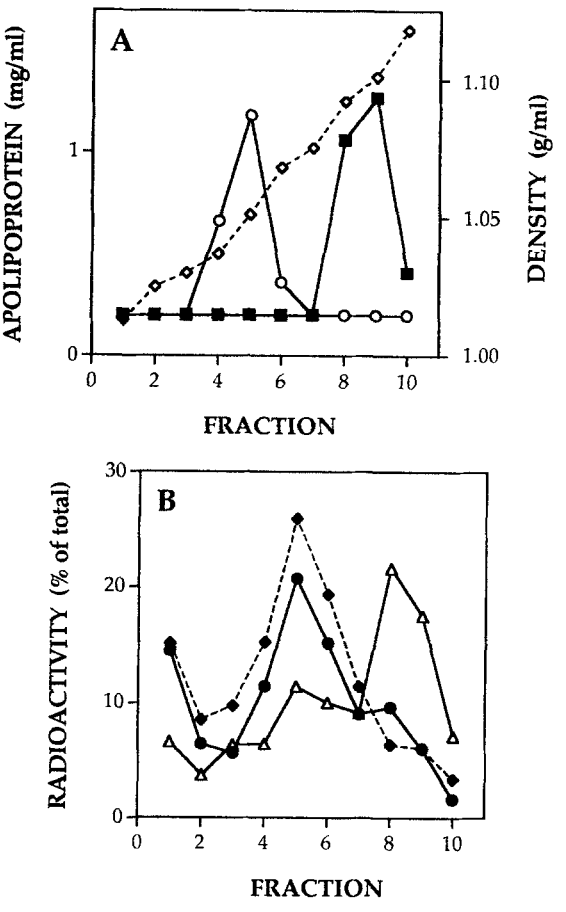


Fig. 2. Relation of [^{3}H]ceramide-SPM with serum lipoproteins after density gradient ultracentrifugation. A: 10 ml of complete human serum layered under 30 ml of saline was centrifuged as described in Materials and Methods. Ten fractions of 4 ml each were collected and analyzed for apoA (—■—) and apoB (—○—) content and density (–◊–). B: an ethanolic solution of [^{3}H]ceramide-SPM (about 75 × 10^6 dpm) was mixed with 10 ml of complete human serum (indicated SPM + SERUM, —●—), or with 10 ml of lipoprotein-deficient serum in the absence (SPM + LPDS, –△–) or presence (SPM + LPDS + LDL, –♦–) of purified LDL (1.2 mg apoB/ml). After overnight incubation at 37°C, the mixtures were centrifuged as in A, and the fractions were analyzed for radioactivity. The data are the means of 4 separate experiments.

Uptake of LDL-associated [^{3}H]ceramide-SPM

The fate of LDL-associated [^{3}H]ceramide-SPM was examined by studying first its mode of uptake by cultured cells derived from normal individuals and from patients with familial hypercholesterolemia (FH). As shown in **Fig. 4A,** total uptake of SPM by normal lymphoid cells was compatible with a saturable process, that was strongly competed for by unlabeled LDL. After subtracting nonspecific uptake (measured in the presence of an excess of LDL), saturation was found to occur at ~50 μg apoB/ml (~0.1 μmol/l). In contrast, a very low uptake of LDL-associated [^{3}H]ceramide-SPM was observed in cells from patients with FH, which lack LDL receptors (Fig. 4A). Similar results were obtained using cultured skin fibroblasts (data not shown). In addition, preincubation of cells for 30 min at 4°C or in the presence of metabolic poisons (such as 30 mM sodium fluoride, 20 mM sodium fluoride plus 2 mM potassium cyanide, or 5 mM sodium azide plus 50 mM deoxyglucose) markedly reduced (up to 90%) the uptake of LDL-associated [^{3}H]ceramide-SPM (data not shown). When studied as a function of incubation time, the total cell-associated radioactivity showed a non-linear increase (Fig. 4B). The above data strongly suggested that the radioactive SPM-labeled LDL behaved as native

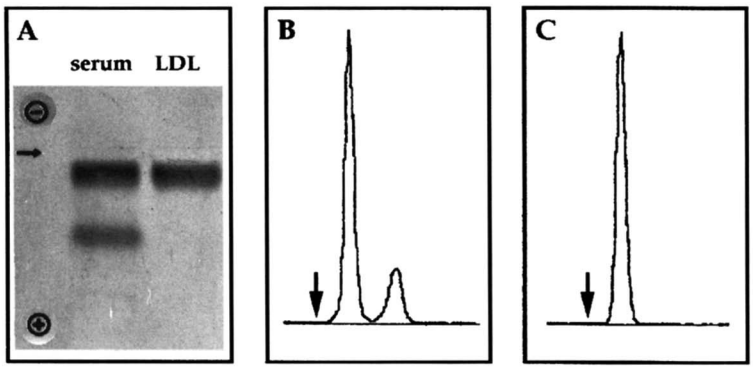


Fig. 3. Relation of [^{3}H]ceramide-SPM with serum lipoproteins after agarose gel electrophoresis. In A, aliquots of a mixture of human serum and [^{3}H]ceramide-SPM (serum), and of the LDL band isolated from the mixture SPM + serum by density gradient ultracentrifugation as described in Fig. 2 (LDL), were submitted to agarose gel electrophoresis and stained with Sudan Black. In B and C, radiochromatoscan of the gel bands from serum and LDL, respectively. The arrows indicate the origin of migration. In B, the major peak corresponds to LDL and the minor peak to HDL. This figure is representative of more than 5 separate experiments.

LDL, i.e., they were taken up by lymphoid cells and fibroblasts through the apoB/E receptor pathway.

Subcellular localization of internalized LDL-associated [^{3}H]ceramide-SPM in Niemann-Pick disease

The distribution within the cell of the internalized LDL-associated [^{3}H]ceramide-SPM was examined by subcellular fractionation studies on lymphoid cells derived from patients with NPD Type A. Cells were homogenized using Balch's ball-bearing device, resulting in ~90% cell disruption. Differential sedimentation analysis demonstrated that <10% of the [^{3}H]ceramide-SPM was found in the cell debris/nuclear fraction, while about 88% of the radioactivity present in the postnuclear supernatant was sedimentable (MLP fraction; T. Levade and P.J. Courtoy, unpublished results). These postnuclear particles were further analyzed by flotation in Nycodenz density gradients (**Fig. 5**). While most of the plasma membrane marker, 5′-nucleotidase activity was found in light fractions, the lysosomal marker, β-hexosaminidase activity peaked at a density of 1.17 g/ml. Similar results were obtained by studying the distribution of exogenously added radioactive cholesterol to label the plasma membrane, and of other lysosomal enzymes such as α-mannosidase or β-glucuronidase (data not shown). Markers for mitochondria (succinate reductase) and endoplasmic reticulum (neutral α-glucosidase) usually peaked at a density slightly higher than that of lysosomes. The distribution of [^{3}H]ceramide-

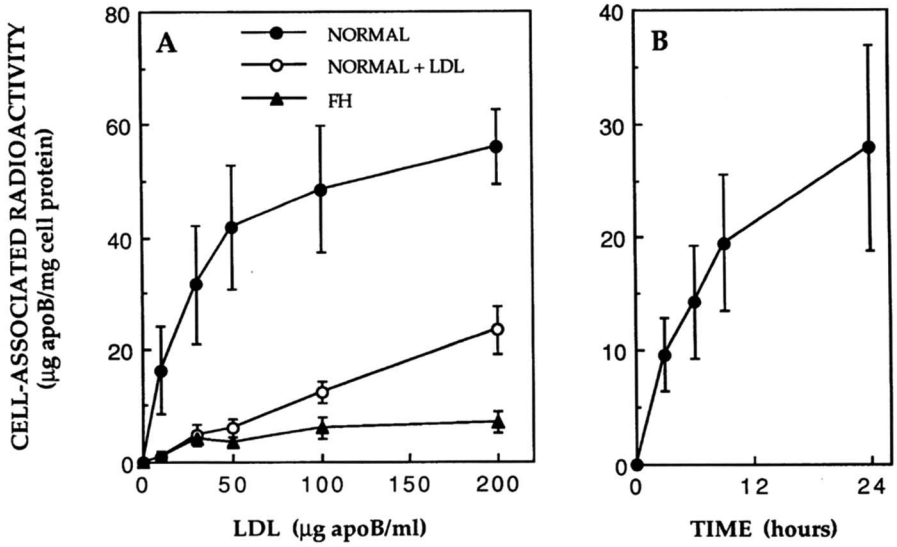


Fig. 4. Uptake of LDL-associated [^{3}H]ceramide-SPM by lymphoid cells from normal individuals and patients with familiar hypercholesterolemia. In A, lymphoid cells derived from normal subjects and patients with the LDL receptor-negative form of familial hypercholesterolemia (FH) were incubated for 24 h with the indicated concentrations of LDL-associated [^{3}H]ceramide-SPM, in the absence or presence (+LDL) of native LDL (600 μg apoB/ml). In B, normal cells were incubated with LDL-associated [^{3}H]ceramide-SPM (50 μg apoB/ml) for the indicated times. The cell-associated radioactivity was measured as described in Materials and Methods. The data are means ± SD of 3–6 separate experiments performed on different cell lines.

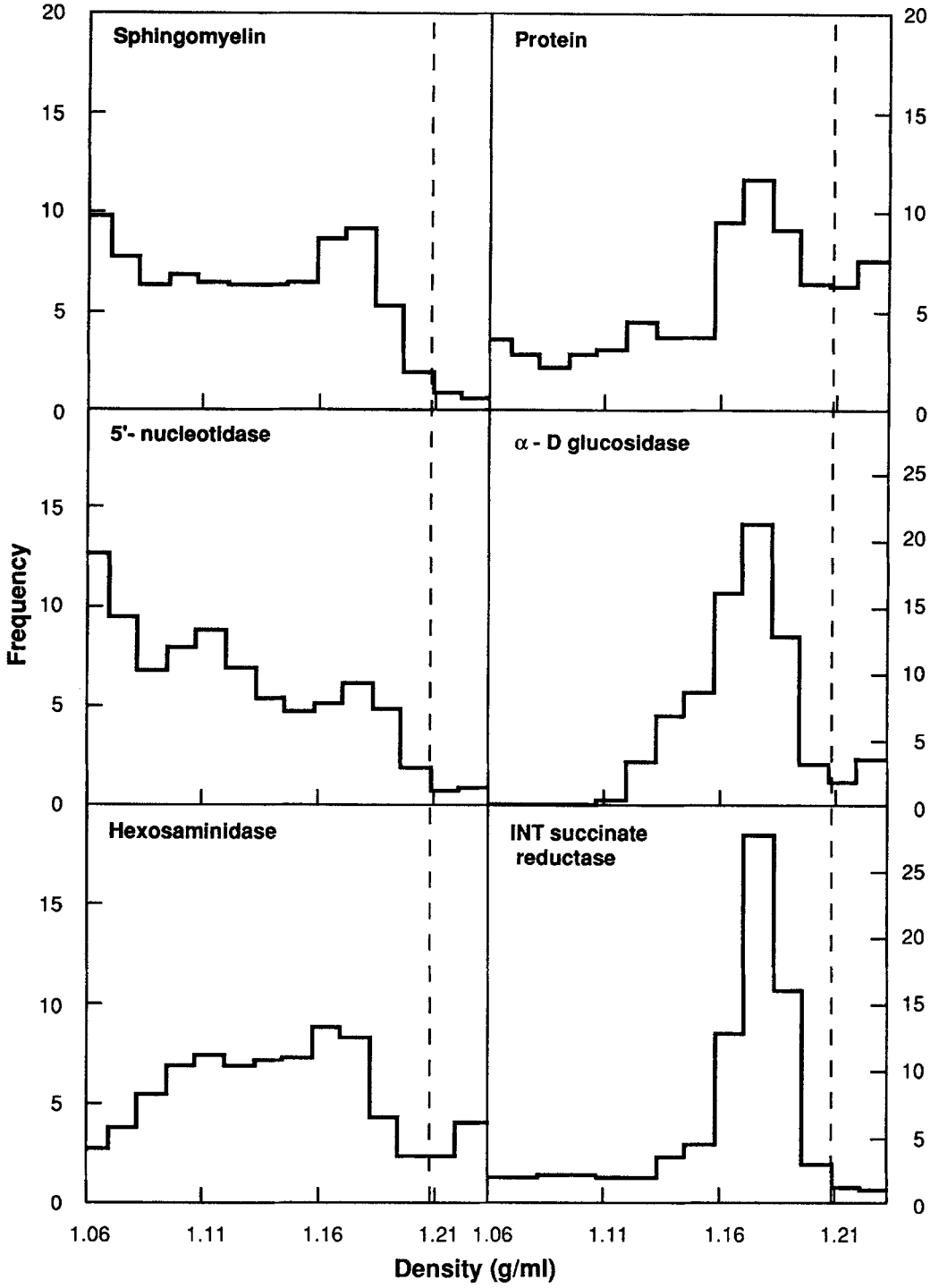


Fig. 5. Subcellular fractionation of lymphoid cells from a Niemann-Pick patient incubated with LDL-associated [^{3}H]ceramide-SPM. Lymphoid cells (about 50 × 10^6) derived from a patient with NPD Type A were incubated for 24 h with LDL-associated [^{3}H]ceramide-SPM (about 50 μg apoB/ml). Then, cells were washed, suspended in homogenization buffer, and homogenized as described in Materials and Methods. Postnuclear particles were prepared and layered under a continuous 1.05–1.20 g/ml Nycodenz density gradient. After centrifugation at 15,000 *g* for 150 min, twelve fractions were collected and analyzed as detailed in Materials and Methods. Density distributions are presented as frequency histograms, normalized according to Leighton et al. (65). Recoveries ranged between 64 and 128%, except for neutral α-D-glucosidase that is inhibited (70%) by Nycodenz in the range of concentrations where its activity was measured. The loading zone is indicated by a broken line. Distributions are averages from a representative experiment performed in duplicate; similar results were obtained in 4 other separate experiments.

SPM (which was undegraded in these cells) was consistently found to parallel that of the lysosomal markers. To exclude a relocation artifact during the fractionation procedure, postnuclear particles (MLP fraction) were isolated from normal lymphoid cells and incubated in vitro for 10 min with [^{3}H]ceramide-SPM labeled LDL prior to equilibration by flotation or sedimentation in the Nycodenz density gradients. In both cases, 95% of the radioactivity remained in the loading zone, suggesting that the density distribution of radioactivity in gradients from cells incubated with LDL-associated [^{3}H]ceramide-SPM accurately reflected the subcellular localization in the intact cells.

Degradation of LDL-associated [^{3}H]ceramide-SPM by normal cells

The metabolism of LDL-associated [^{3}H]ceramide-SPM in cells derived from control individuals was next studied by analyzing the distribution of the radiolabel in the various cellular lipids. TLC analyses of normal lymphoid cells and skin fibroblasts extracts showed that [^{3}H]ceramide-SPM was degraded in both cases to ceramide and that the two radioactive products of ceramide breakdown (i.e., sphingoid base and fatty acid) were reincorporated into sphingolipids (SPM being the major one), glycerophospholipids, and neutral lipids (29). The degradation of [^{3}H]ceramide-SPM was evident already after 3 h incubation, the production of radioactive ceramide metabolites being maximal about 48 h after the end of the incubation with LDL-associated [^{3}H]ceramide-SPM (28).

As previous evidence was obtained in support of a lysosomal targeting and processing of LDL-associated [^{3}H]ceramide-SPM, we examined the effect of chloroquine, a well-known inhibitor of lysosomal functions, on the degradation of [^{3}H]ceramide-SPM. After 24 h incubation with chloroquine, the cell viability (as assessed by the Trypan blue dye exclusion test and [^{3}H]thymidine incorporation) remained unaffected up to a concentration of 200 μM for lymphoid cells, and up to 50 μM for skin fibroblasts. **Figure 6** demonstrates that chloroquine (which did not affect SPM uptake) strongly inhibited the hydrolysis of LDL-associated [^{3}H]ceramide-SPM by normal lymphoid cells and particularly by fibroblasts in a dose-dependent manner.

In situ activities of lysosomal sphingomyelinase and ceramidase in normal cells

The kinetic parameters of the intracellular (intralysosomal) degradation of SPM and its direct metabolite, ceramide, were assessed by following the hydrolysis of [^{3}H]ceramide-SPM to [^{3}H]ceramide by intact normal cells that had been incubated with LDL-associated [^{3}H]ceramide-SPM for a relatively short pulse (3 h), chosen as a compromise between a limited interval of lysosomal loading and a sufficient uptake for adequate sensitivity. The data, presented in **Fig. 7,** showed that the two sphingolipids were rapidly degraded by lymphoid cells and skin fibroblasts. During the time intervals studied, most of the SPM present in normal cells corresponded to the internalized, undegraded substrate (de novo synthesized SPM appeared only after 6 h incubation and accounted for <10% of the total SPM metabolites). Based on the slope of the degradation curves, the half-time for in situ SPM degradation (i.e., the time required for degradation of 50% of the cell-associated SPM) averaged 6 h in normal lymphoid cells and 2.5 h in skin fibroblasts. For ceramide degradation, corresponding half-times were 1.3 and 2.7 h. Based on the mean values determined for cellular uptake of LDL-associated [^{3}H]ceramide-SPM, we concluded that the initial velocities of degradation of radiolabeled SPM and ceramide were comparable (22 and 38 pmol·h^{-1} · mg cell protein^{-1}, respectively, in lymphoid cells, and

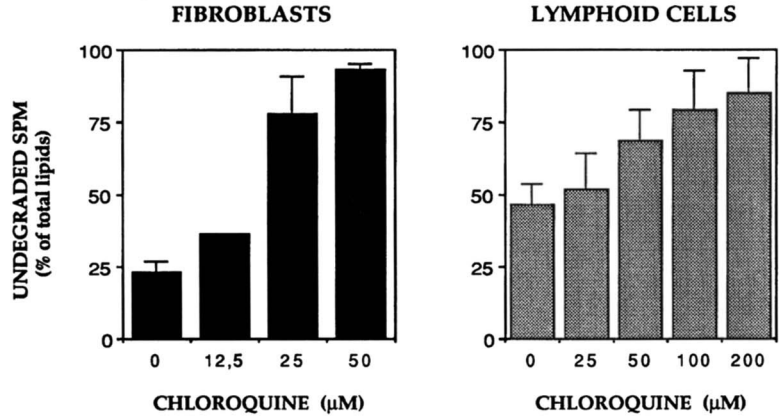


Fig. 6. Effect of chloroquine on the degradation of LDL-associated [^{3}H]ceramide-SPM by normal skin fibroblasts or lymphoid cells. Normal skin fibroblasts and lymphoid cells were incubated for 24 h with LDL-associated [^{3}H]ceramide-SPM (50 μg apoB/ml) in the presence of the indicated concentrations of chloroquine. At the end of the incubation, cells were washed and the lipids were extracted and analyzed as described in Materials and Methods. The data (means ± SD) are from 3–8 separate experiments.

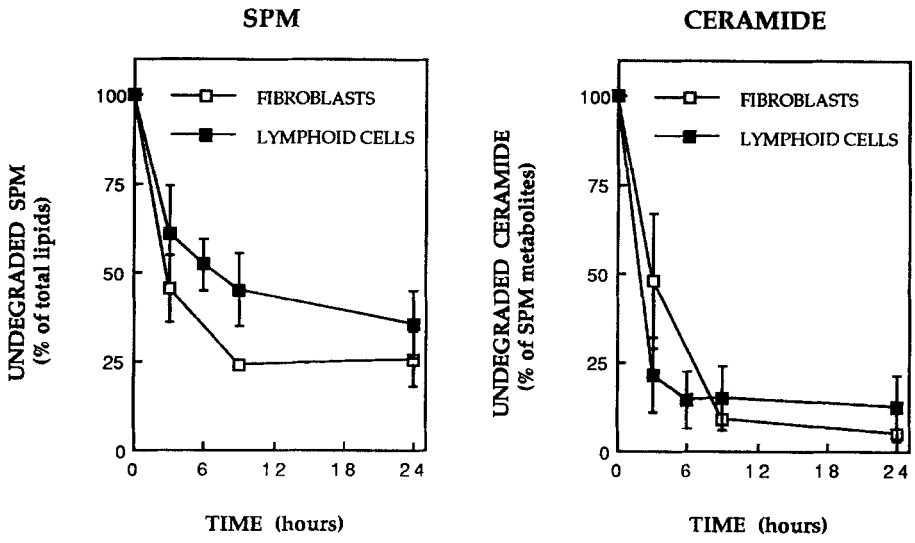


Fig. 7. Degradation of LDL-associated [^{3}H]ceramide-SPM and of the subsequently formed [^{3}H]ceramide by skin fibroblasts and lymphoid cells from normal individuals. At time 0, normal fibroblasts and lymphoid cells were incubated for 3 h with LDL-associated [^{3}H]ceramide-SPM (about 50 μg apoB/ml). Then, cells were washed and further incubated in fresh medium containing 10% fetal calf serum. At the indicated times, cells were washed and the lipids were extracted and analyzed as described in Materials and Methods. In A, the amounts of undegraded [^{3}H]ceramide-SPM are expressed as percentage of the total cell-associated radioactivity. In B, the levels of [^{3}H]ceramide are expressed as percentage of the total lipid products of SPM hydrolysis, i.e., by neglecting the SPM itself but considering the ceramide as the substrate. The data correspond to duplicates or means ± SD of triplicates for skin fibroblasts, and to the means ± SD of 5–12 separate experiments for lymphoid cells.

180 and 100 pmol · h^{-1}·mg cell $protein^{-1}$ in skin fibroblasts). Assuming that LDL particles contain 300 pmol SPM/μg apoB and a mean incorporation of radiolabeled SPM into LDL of 9000 dpm (that is only 10 pmol/μg apoB), the values for initial velocities of degradation should be multiplied by a factor of about 30.

Degradation of LDL-associated [^{3}H]ceramide-SPM in genetically defective cells

Degradation of LDL-associated [^{3}H]ceramide-SPM was next studied in cultured cells genetically defective in the activity of lysosomal sphingomyelinase (NPD Type A), ceramidase (Farber disease), or both (Mucolipidosis Type II). There was no difference in the SPM uptake levels between normal and defective cells (data not shown). As shown in **Table 1,** after 24 h incubation of skin fibroblasts or lymphoid cells derived from NPD Type A patients with LDL-associated [^{3}H]ceramide-SPM, undegraded SPM represented almost 100% of the total cell-associated radioactivity. An obvious, but less severe, accumulation of SPM was also observed in Mucolipidosis Type II fibroblasts. As for ceramide degradation, skin fibroblasts and lymphoid cells from a patient with Farber disease exhibited a marked accumulation of radiolabeled ceramide, which accounted for about 75% of the total metabolic products of [^{3}H]ceramide-

TABLE 1. Degradation of LDL-associated [^{3}H]ceramide-SPM and of the subsequently formed [^{3}H]ceramide by skin fibroblasts and lymphoid cells from normal subjects and from patients with defective lysosomal enzymes

Cell Type	Undegraded Lipid	
	SPM	Ceramide
	% of total lipids	*% of SPM metabolites*
Fibroblasts		
Normal	20.7 ± 4.0 (10)	11.1 ± 5.0 (10)
NPD Type A	100 (3)	nd
ML II	79.0; 80.0	,80.0; 82.0
Farber	16.2 ± 5.1 (3)	76.0 ± 12.0 (3)
Lymphoid cells		
Normal	34.3 ± 6.3 (4)	8.1 ± 4.5 (6)
NPD Type A	99.5 ± 1.0 (5)	nd
Farber	34.4 ± 6.1 (5)	73.6 ± 13.8 (5)

Skin fibroblasts and lymphoid cells from normal individuals and patients afflicted with Niemann-Pick disease (NPD) Type A, Farber disease, or Mucolipidosis Type II (ML II) were incubated for 24 h with LDL-associated [^{3}H]ceramide-SPM (about 50 μg apoB/ml). Then cells were washed and the lipids were extracted and analyzed as described in Materials and Methods. The levels of undegraded [^{3}H]ceramide-SPM are expressed as percentage of total radioactive lipids, and those of [^{3}H]ceramide are expressed as percentage of the total lipid products of SPM hydrolysis, i.e., by neglecting the SPM itself but considering the ceramide as the substrate. Data are individual values or means ± SD of the number of separate experiments indicated in parentheses; nd, not detectable.

SPM. A similar defect of ceramide hydrolysis was found in Mucolipidosis Type II cells.

DISCUSSION

Lysosomal sphingolipid hydrolases have been so far mostly characterized by in vitro studies on tissue or cell homogenates, but their effective activity in intact living cells has not been satisfactorily measured. There are several reports in the literature on the degradation by intact cells of various sphingolipids. Thus, the activity of the hydrolases acting on cerebroside-sulfate (31–35), sphingomyelin (36–41), G_{M1} and G_{M2} gangliosides (42–45), glucosylceramide (46–49), galactosylceramide (50, 51), lactosylceramide (51), and ceramide (52) has been tentatively determined in cultured fibroblasts in situ.

Such studies met with several difficulties, among which the hydrophobicity of the substrate represents a major obstacle. In order to circumvent this problem, some authors have used sphingolipid analogs that are more hydrophilic and more cell-permeable than their natural counterparts. These synthetic analogs include short-acyl chain ceramide derivatives (35, 49), as well as fluorescent (27, 33, 40, 41, 53, 54) or fluorogenic (47, 48, 55) compounds. To what extent these derivatizations could affect enzyme activity measurements is not totally clear.

The next problem in loading living cells with sphingolipids concerns the conditions for presenting the substrate. While in most studies the mode of entry was not investigated, various attempts were made to incorporate the sphingolipid into complexes that would enhance its cellular uptake and facilitate its transport into the lysosomes. For instance, Agmon et al. (41) and Kohen et al. (48) have proposed the use of liposomes coated with recombinant apolipoprotein E and Rousseau and Gatt (56) used lipids incorporated into viral envelopes. Mannosylated liposomes have also been described for delivery of glucosylceramide to cultured macrophages (57).

In addition, the intracellular trafficking of the incorporated natural sphingolipid has generally received little attention. However, the question of the subcellular compartmentalization of the lipid taken up by a living cell is of crucial importance as the enzyme activity to be measured is essentially restricted to the lysosomal compartment. The lysosomal localization of exogenously added sphingolipids has been documented in only a few cases, e.g., for ceramide (52), cerebroside sulfate (34, 35), G_{M2} ganglioside (42), and SPM (40). In some instances, the administered sphingolipid has been found both in plasma membrane-enriched and lysosomal fractions, e.g., for galactosylceramide (51), cerebroside sulfate (35), G_{M2} ganglioside (42), and fluorescent ceramide derivatives (4). Finally, other studies provided indirect evidence suggesting the involvement of non-lysosomal compartments in the degradation of the exogenously supplied sphingolipid (15, 58–60).

The last problem in previous sphingolipid loading studies concerns the measurement of the lysosomal activity itself. In many reports, the hydrolase activity was not measured by a kinetic assay but only after a single incubation end-point, by measuring substrate accumulation or metabolite production while the cells were continuously fed with the sphingolipid. To our knowledge, the only studies where the intracellular activity has been accurately determined have been carried out with non-natural, fluorescent substrate analogs (47, 54). Thus, the values reported may not accurately reflect the effective intracellular enzyme activities and the residual values in genetically defective cells.

In the present study, we have developed a system that addresses the various problems mentioned above, all of which are critical for a relevant and accurate measurement of the activity of lysosomal hydrolases. First, a natural substrate, radiolabeled SPM, was used rather than a fluorescent analog. Indeed, previous observations from our laboratory have shown that fluorescent and natural derivatives of SPM can exhibit different pathways for cellular uptake and degradation (15, 17).

Second, the present study was carried out on two different human cell types, cultured skin fibroblasts and lymphoid cell lines. These two experimental cell systems were chosen because: *i*) these cells express sufficient apoB/E receptors to avidly incorporate lipids through the LDL endocytic pathway (61); *ii*) they also express a non-lysosomal pathway for SPM, possibly mediated by a neutral, Mg^{2+}-dependent sphingomyelinase (15, 60); and *iii*) both fibroblasts and lymphoid cells from patients with inherited deficiencies of lysosomal sphingomyelinase or ceramidase are available. In these mutant cells, an incomplete lysosomal targeting of SPM will result in a detectable hydrolysis mediated by the non-lysosomal pathway; in contrast, if the substrate is completely targeted to lysosomes, no residual degradation will be observed in the defective cells (see Results).

Third, the physical state of the substrate delivered to the cells has been well defined: SPM associated to LDL particles has been cleared of liposomal SPM. Our data also demonstrate that the physical and biological properties of the [^{3}H]ceramide-SPM-labeled LDL were similar to their native counterparts. This mode of sphingolipid delivery to the cells offers several advantages: *i*) it is specific to the apoB/E receptor pathway; *ii*) it allows for a selective transport to the lysosomes; and *iii*) it results in a physiological influx of exogenous substrate

(the SPM being a normal constituent of lipoproteins). The latter notion is important with respect to the determination of the effective intracellular activity (35).

Fourth, evidence is provided that LDL indeed targets the associated [^{3}H]ceramide-SPM to the lysosomal compartment where degradation occurs. This conclusion is supported by: *i*) the subcellular localization studies, i.e., the ultrastructural autoradiography and subcellular fractionation studies, which both showed that SPM was located in lysosomes; *ii*) the inhibition by chloroquine of the hydrolysis of internalized SPM; and *iii*) the block of degradation of SPM or the released ceramide in cells with deficient activity of the corresponding lysosomal enzymes. The fact that LDL-targeted [^{3}H]ceramide-SPM is not hydrolyzed at all in NPD Type A cells suggests that no other sphingomyelinase than the lysosomal one contributes to its degradation. The accumulation of SPM in cells from NPD Type A patients or of ceramide in Farber cells also suggests that, in contrast to fluorescent derivatives (4, 53), these natural, radiolabeled lipids most likely do not get across the lysosomal membrane prior to degradation.

As a result, a major advantage of the LDL-loading procedure is the possibility to determine the effective activity in situ of two lysosomal hydrolases, acid sphingomyelinase and ceramidase. These activities have been defined by two kinetic parameters, the initial velocity and the half-time of degradation of SPM and ceramide. To our knowledge, this is the first report on the measurement of the intracellular activities of lysosomal sphingomyelinase and ceramidase in situ using a natural substrate. The present study also compares favorably with other reports on the intracellular activities of lysosomal sphingolipid hydrolases (47, 54), by the use of physiological substrates. Confrontation of the effective activities measured in intact cells with the in vitro activities determined on cell homogenates after disintegration and addition of detergents shows that the lysosomal sphingomyelinase activity in situ (as half-time of hydrolysis) is quite similar in skin fibroblasts and lymphoid cell lines while those found in vitro are very different (80–100 nmol $\cdot$ h^{-1} $\cdot$ mg protein^{-1} in fibroblasts versus 2–4 nmol $\cdot$ h^{-1} $\cdot$ mg^{-1} in lymphoid cells). As for lysosomal ceramidase, its in situ activity is about the same in the two cell types. Remarkably, while in vitro acid ceramidase activity in skin fibroblasts is about 100-fold lower than that of acid sphingomyelinase, their activities in situ are very similar. This indicates that lysosomal ceramidase is a relatively active enzyme, and may explain why ceramide, the end-product of the catabolism of all sphingolipids and an interesting second messenger, does not accumulate in normal cells. Differences between the in vitro and in situ activities of another lysosomal enzyme, glucosylceramidase, have already been reported (47), emphasizing the importance of the second type of assay. However, these differences might be related to the actual substrate concentrations in situ. It is also possible that high intracellular concentrations of SPM or ceramide (higher than those herein obtained) would result in apparent differences between acid sphingomyelinase and ceramidase activities.

The procedure for in situ determination of the activities of lysosomal sphingomyelinase and ceramidase has two other applications. The first one is the evaluation of the effective residual enzyme activities in cells from patients with lipidoses, an approach that showed an inverse correlation between the residual intracellular activity of sphingomyelinase or ceramidase and the severity of the various phenotypic forms of the ensuing lysosomal storage disorder, i.e., Niemann-Pick and Farber diseases (29, 62). We believe this procedure is a useful complement to the in vitro assays both for postnatal and prenatal (63) diagnosis of these enzyme deficiencies. The second interest of the in situ assay is to allow investigation of the role of natural activator proteins (saposins) in the lysosomal degradation of sphingolipids, as compared to the metabolism of endogenously-labeled sphingolipids (64). Such studies on the metabolism of SPM and ceramide in saposin-deficient cells are currently being performed.

The authors thank Mrs. A. Lebreton for her valuable technical assistance in subcellular fractionation studies, and Dr. M. Lagente and Mr. J.C. Thiers for their help in electrophoretic analyses. This study was supported by grants from INSERM (CJF 9206), Université Paul Sabatier—Toulouse 3 (JE DRED 174), the association "Vaincre les Maladies Lysosomales" (to TL), the Tournesol program of scientific exchange (to TL and PJC), and the "Interuniversity Attraction Poles" as well as "Action de Recherches Concertées" (to PJC).

Manuscript received 17 June 1996 and in revised form 26 August 1996.

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