Role of endosomal membrane lipids and NPC2 in cholesterol transfer and membrane fusion[s]

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Abstract We examined the effect of Niemann-Pick disease type 2 (NPC2) protein and some late endosomal lipids [sphingomyelin, ceramide and bis(monoacylglycero)phosphate (BMP)] on cholesterol transfer and membrane fusion. Of all lipid-binding proteins tested, only NPC2 transferred cholesterol at a substantial rate, with no transfer of ceramide, GM3, galactosylceramide, sulfatide, phosphatidylethanolamine, or phosphatidylserine. Cholesterol transfer was greatly stimulated by BMP, little by ceramide, and strongly inhibited by sphingomyelin. Cholesterol and ceramide were also significantly transferred in the absence of protein. This spontaneous transfer of cholesterol was greatly enhanced by ceramide, slightly by BMP, and strongly inhibited by sphingomyelin. In our transfer assay, biotinylated donor liposomes were separated from fluorescent acceptor liposomes by streptavidin-coated magnetic beads. Thus, the loss of fluorescence indicated membrane fusion. Ceramide induced spontaneous fusion of lipid vesicles even at very low concentrations, while BMP and sphingomyelin did so at about 20 mol% and 10 mol% concentrations, respectively. In addition to transfer of cholesterol, NPC2 induced membrane fusion, although less than saposin-C. In this process, BMP and ceramide had a strong and mild stimulating effect, and sphingomyelin an inhibiting effect, respectively. ▇▇ Note that the effects of the lipids on cholesterol transfer mediated by NPC2 were similar to their effect on membrane fusion induced by NPC2 and saposin-C.—Abdul-Hammed, M., B. Breiden, M. A. Adebayo, J. O. Babalola, G.Schwarzmann, and K. Sandhoff. **Role of endosomal membrane lipids and NPC2 in cholesterol transfer and membrane fusion.** *J Lipid Res.* 2010. **51:** 1747–1760.

Cholesterol is an important structural and regulatory component of eukaryotic cell membranes, and the endocytic pathways play an important role in cholesterol homeostasis. One of the major cholesterol sources is the uptake via receptor-mediated endocytosis of LDL rich in cholesteryl ester. In the endosomal compartments, cholesteryl esters are hydrolyzed by cholesterol esterase to free fatty acid and cholesterol (1). This cholesterol is continuously recycled between plasma membrane and endosomes (2). Unlike other membrane lipids, unesterified cholesterol is not degraded in lysosomes; it is rapidly transported out of the late endosomes to induce a homeostatic response by downward regulation of the de novo synthesis of LDL-receptor, which regulates the cellular cholesterol uptake and de novo synthesis of cholesterol (2).

Membranes of endocytic organelles contain varying amounts of cholesterol. Recycling endosomes and internal membranes of multivesicular bodies are cholesterol-rich, whereas internal membranes of lysosomes are cholesterol-poor (3). Defects in endocytic cholesterol trafficking and metabolism lead to an imbalance in intracellular cholesterol distribution and an accumulation of cholesterol, resulting in diseases such as hypercholesterolemia (4), Niemann-Pick disease type C (NPC) (5, 6), and Wolman disease (7).

NPC, a rare autosomal-recessive disorder, is a complex lipid storage disease characterized by the accumulation of unesterified cholesterol in the late endosomal/ lysosomal compartment (8). Two genes, *NPC1* and *NPC2*, responsible for this neurodegenerative disorder have been iden-

tified, but their respective functions are yet to be fully elucidated.

NPC1 is a large transmembrane protein located in the perimeter membrane of late endosomes and lysosomes (9–11). It contains 13 putative transmembrane domains, 3 large, highly glycosylated hydrophilic loops projecting into the lumen, as well as 4 small luminal loops, 6 small cytoplasmic loops, and a cytoplasmic tail (12). The N-terminal, luminal loop 1 contains several conserved cysteine residues and a leucine zipper motif and has been identified as the cholesterol and oxysterol binding site (13, 14). Fifty percent of described NPC1 missense mutations are located in the cysteine-rich loop 3 between transmembrane helices 8 and 9. Also a sterol-sensing domain located between the third and seventh transmembrane helices binds photoactivatable cholesterol (15) and is important for a normal cholesterol transfer out of the late endosomal/lysosomal compartments.

NPC2 (previously called HE1) is a small soluble glycoprotein of the late endosomes that comprises 132 amino acids in the mature form (16, 17). It is present at high levels in mammalian epididymal fluid (8), bile (18), and bovine milk (19). It has been shown that NPC2 binds to cholesterol with submicromolar affinity, but not to oxysterols (20–22). It binds also to fatty acids with lower affinity (23). X-ray crystallography studies of ligand-free bovine NPC2 (bNPC2) show an immunoglobulin-like β-sandwich fold consisting of seven β-strands arranged in two β-sheets with an internal ligand binding pocket (21). Variant proteins of NPC2 with a mutation in the binding pocket did not bind cholesterol (23). X-ray crystal structure of bNPC2-cholesterol sulfate complex with a 1:1 stoichiometry shows that the steroid nucleus moiety is deeply buried in a hydrophobic pocket, and the 3β-sulfate ester moiety is exposed to the solvent (24). This orientation is opposite to the orientation of cholesterol bound to the N-terminal loop 1 of NPC1 (25). In a proposed model, soluble NPC2 removes cholesterol from the inner endosomal/lysosomal vesicles and delivers it to NPC1 in the limiting membrane of late endosomes/lysosomes for a cholesterol egress from the late endocytic compartments (26–28). The ability of NPC2 to transfer cholesterol between membranes and/or from a membrane to NPC1 has been demonstrated in several in vitro assays (22, 29–31). It has been shown that NPC2, in contrast to NPC1, binds and releases cholesterol very rapidly and accelerates the cholesterol transport nearly 100-fold between liposomes and NPC1 (30, 31).

In the liver and brain tissue from NPC patients, cholesterol accumulates in the late endosomes and lysosomes. Furthermore, sphingomyelin (SM), neutral glycolipids (e.g., glucosylceramide, lactosylceramide), gangliosides GM3 and GM2 (32, 33), bis(monoacylglycero)phosphate (BMP) (30, 34) and sphingosine (35) also accumulate. This is explained by the type of traffic jam that occurs in the late endosomal/lysosomal compartments when lipids accumulate and by the inhibitory effect of increasing cholesterol levels on the activity of some lysosomal lipid binding and transfer proteins, such as Sap A and B (36, 37).

Along the endocytic pathway, the luminal pH of the organelles decreases from 6.0 to less than 4.5 when going from late endosomes to lysosomes (38). We assume that acid sphingomyelinase, which already is active at pH 5.5 in the presence of BMP (39), generates ceramide (Cer) from SM in the intraendosomal membranes of late endosomes. Cer and SM differ in their affinity to cholesterol. SM interacts with cholesterol because of hydrogen bonds formed between the OH group of cholesterol and the amide group of SM (40, 41), thus keeping cholesterol in the membrane (42, 43). Cer shows a relatively weak affinity to cholesterol. It exhibits a tendency to segregate into lipid ordered domains (44). We assume that an increase in Cer content of the inner late endosomal/lysosomal membranes correlates with a decrease in cholesterol content, which would result in membrane destabilization. BMP is a marker lipid for the inner membranes of late endocytic compartments (3, 45) and was suggested to control the endosomal cholesterol level (46).

We developed an assay for qualitative and quantitative characterization of protein-mediated lipid transfer between donor and acceptor liposomes. We found that NPC2 is a specific and efficient transporter of cholesterol (29). In the present work, we investigate the influence of Cer, SM, and BMP on the cholesterol transfer by bNPC2 at late endosomal pH 5.0 and at lysosomal pH 4.2. With a newly designed fusion assay we could show that sphingomyelin inhibits membrane fusion, whereas ceramide triggers fusion of lipid bilayers in the presence of BMP.

MATERIALS AND METHODS

Materials

1,2-dioleoyl-sn-glycerol-3-phosphocholine (PC), bis(monooleoylglycero)phosphate (C18:1-BMP), and bis(monomyristoylglycero)phosphate (C14:0-BMP) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, cytochrome c (from horse heart), and BSA were obtained from Sigma (Taufkirchen, Germany). N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethyl ammonium salt (NBD-PE) and N-((6-(biotinoyl)amino)hexanoyl-1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolaminetriethyl ammonium salt (Biotin-PE) were from Invitrogen (Carlsbad, CA). C18-Sphingomyelin (SM) and D-erythro-C18-ceramide (Cer) were purchased from Matreya (Pleasant Gap, PA). 4-[14C]cholesterol (58 Ci/mol) was obtained from GE Healthcare (Buckinghamshire, UK). 1-[14C]dioleoyl-L-α-phosphatidylethanolamine (55 Ci/mol) and 1-[14C]dioleoyl-L-α-phosphatidylcholine (80 Ci/mol) were from American Radiolabeled Chemicals (St. Louis, MO).

BioMag streptavidin suspension (5 mg/ml) and the magnetic separation stand MagneSphere were obtained from Qiagen (Hilden, Germany).

Synthesis of radiocarbon-labeled lipids

[14C]sulfatide, [14C]GM3, [14C]galactosylceramide, and [14C]ceramide were synthesized from their corresponding lyso-lipids and sphingosine, respectively, and [1-14C]stearic acid (58 Ci/mol obtained from GE Healthcare), following published procedures (47). Lyso-sulfatide was obtained as described earlier (48).

Protein preparation

Bovine NPC2 was isolated from milk and purified as previously described (21) with some modifications. Sphingolipid activator proteins Sap A–D were expressed in the methylotrophic yeast *Pichia pastoris* (36, 37, 49, 50). Recombinant GM2 activator protein (GM2AP) was expressed in the baculovirus expression vector system and purified as reported previously (51). The recombinant activator proteins contained at the C terminus a hexahistidine-tag. The purity of the protein preparations was better than 95% pure as confirmed by SDS gel electrophoresis and MALDI-MS. The activity of proteins was also tested (29, 37, 51–54).

Preparation of donor and acceptor vesicles

The preparation of donor and acceptor vesicles depends largely on the type of assays (transfer assay, fusion assay, or total fusion control assay) to be employed.

In the transfer assay depicted in **Fig. 1A**, donor and acceptor vesicles were prepared separately. The donor vesicles contained 10 mol% cholesterol, Cer, BMP, and/or SM in a concentration ranging from 0 to 30 mol%, 1 mol% radiolabeled lipid (e.g., [^{14}C]cholesterol or as a control [^{14}C]PE or [^{14}C]PC), 4 mol% Biotin-PE, and were made up to 100 mol% with PC, the host lipid. Biotin-PE enables the separation of donor from acceptor vesicles by the use of streptavidin-coated magnetic beads. The acceptor vesicles contained 10 mol% cholesterol, Cer, BMP, and/or SM in a concentration ranging from 0 to 30 mol%, 4 mol% NBD-PE as marker lipid and fluorescence probe (for monitoring acceptor vesicles recovery), and were made up to 100 mol% with PC.

In the fusion assay, liposome type I and type II were also prepared separately. The type I vesicles contained 10 mol% cholesterol, Cer, BMP, and/or SM in a concentration ranging from 0 to 30 mol%, 4 mol% Biotin-PE, and then were made up to 100 mol% with PC. The type II vesicles contained 10 mol% cholesterol, Cer, BMP, and/or SM in a concentration ranging from 0 to 30 mol%, 1 mol% radiolabeled lipid (e.g., [^{14}C]cholesterol), 4 mol% NBD-PE, and PC. The incorporation of both the radiolabeled and fluorescence probes into the type II vesicle was to measure the extent to which the type II vesicles fuse with type I vesicles in the fusion process. The loss of radioactivity and fluorescence from the type II vesicles in the fusion process should be simultaneous and was taken as a measure of membrane fusion.

In the total fusion control assay, the lipids of liposomes type I and liposomes type II were mixed together before evaporation of the organic solvent under a stream of nitrogen and hydration in the appropriate buffer.

Large unilamellar vesicles were prepared as previously described (29) by mixing appropriate amounts of lipids from stock solutions and drying the mixture under a stream of nitrogen. The lipid mixture was then hydrated in 1 ml of appropriate buffer solution, using either 20 mM citrate buffer (for pH 4.2–6.0) or 20 mM HEPES buffer (for pH 7.4), each containing 150 mM NaCl. The dispersion was vortexed and subjected to eight freeze-thaw cycles to obtain a uniform distribution of buffer solutes across the bilayers. The lipid suspension was then sonicated for 30 s in a Branson sonifier at 120 W followed by extrusion through polycarbonate filters with a pore size of 100 nm mounted in tandem in a mini-extruder (LiposoFast, Avestin, Ottawa, Canada). Samples were subjected to 21 passes.

Determination of the lipid content of liposomes and the final concentration of the liposomal suspension

Aliquots of the stock solution of liposomes, before and after passing through the membranes in the mini-extruder, were evaporated in a stream of nitrogen. The lipids were redissolved in 50 µl of chloroform/methanol (1/1, v/v) and separated by thin layer chromatography with chloroform/methanol/water (60/25/4, v/v/v) as the mobile phase. After development, plates were air-dried, sprayed with 8% (w/v) H3PO4 containing 10% (w/v) copper (II) sulfate pentahydrate, and charred at 180°C for 10 min. The lipids were quantified using densitometry (Camag) at 595 nm. Determination of each lipid content was done in triplicate. The indicated lipid mol% in the figures and in the text corresponds to the content after preparation of liposomes.

Dynamic light scattering

Dynamic light scattering (DLS) size distribution measurements of liposomes were performed with an ALV-NIBS High Performance Particle Sizer (ALV, Langen, Germany) operated at a wavelength of 633 nm and with a detection angle of 172°. For each measurement, 300 µl liposome solutions were used at 25°C.

Lipid transfer/fusion assay

In our previous transfer assay (29), the total amount of lipids in a 200 µl assay volume was 4 and 20 nmol for donor and acceptor vesicles, respectively. In our novel fusion assay depicted in Fig. 1B and in the corresponding transfer assay, we used a donor-to-acceptor vesicle ratio of 1:1, with 4 nmol total lipid each in a total volume of 200 µl of appropriate buffer. The assay samples were kept on ice before addition of the protein under investigation. The experiment was started by the addition of the proteins, and samples were incubated at the desired temperature (usually 25°C) for 10 min in 20 mM citrate buffer (for pH 4.2–6) or 20 mM HEPES buffer (for pH 7.4), both containing 150 mM NaCl. The assay was stopped by adding 75 µl of 1 M Tris buffer (pH 8) on ice, thereby setting the pH of the assay mixture between 7.6 and 7.9. Under these conditions, fusion or transfer of lipids is negligible.

To separate donor from acceptor vesicles, streptavidin-coated paramagnetic particles (BioMag Streptavidin) were added. For routine assays, an appropriate and sufficient amount of BioMag suspension (pre-washed three times with Tris buffer) was used. The mixture was incubated at room temperature for at least 15 min to allow for complete binding of Biotin-PE–containing donor (or type I vesicles in the case of membrane fusion) vesicles to BioMag streptavidin-coated beads.

The tubes were then placed in a magnetic separation stand (MagneSphere) to pull the donor-streptavidin complex to one side of the tube wall. Titration experiments were performed to ensure complete binding of biotinylated liposomes. More than 98% separation efficiency was recorded using 80 µl of 5 mg/ml streptavidin-coated magnetic beads, corresponding to 400 µg of beads in all assays used. After separation, donor vesicles are with the beads, while the supernatant contains the acceptor vesicles.

Aliquots of the supernatant (100 µl) were then measured for radioactivity (R) resulting from [^{14}C]lipid transfer/fusion using Tricarb 2900TR liquid scintillation analyzer (PerkinElmer, Rodgau, Germany), while the fluorescence (F) was measured in another aliquot (150 µl diluted to 400 µl with distilled-deionized water) using a RF-5000 spectrofluorometer from Shimadzu (Kyoto, Japan) (Excitation wavelength 466 nm, emission wavelength 526 nm).

In the transfer assay, the extent of loss of radioactivity from the donor vesicles to the acceptor vesicles (measured in the supernatant as calculated below) indicates lipid transfer while in the fusion assay (when both radio-labeled and fluorescent-labeled lipids were used as markers for vesicle type II), the extent of parallel loss of both radioactivity and fluorescence from the acceptor vesicles (supernatant) to the donor vesicles showed the extent to which fusion process had occurred. For all assays, appropriate controls were performed in the absence of protein. We measured
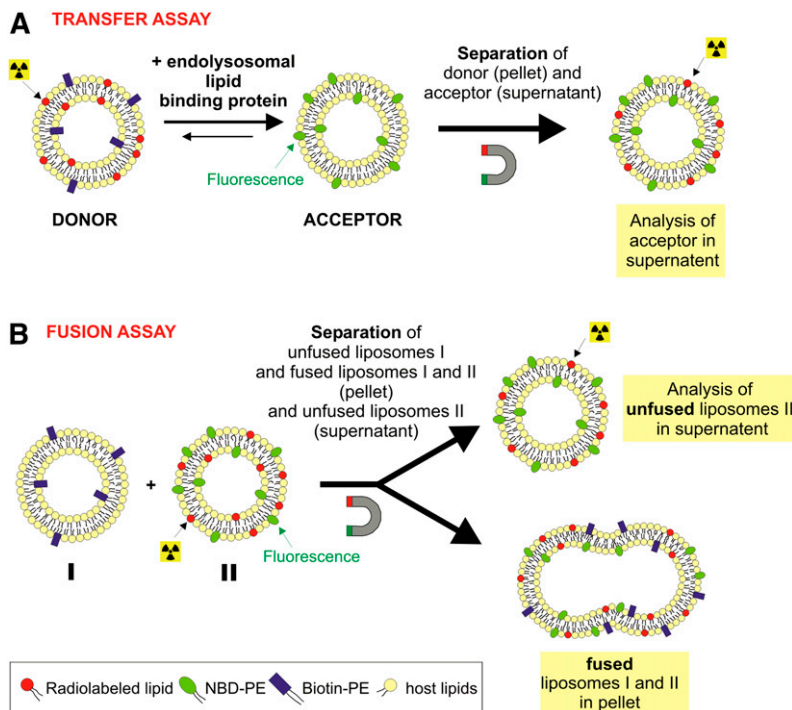
Fig. 1. Schematic diagram for transfer and fusion assay. A: Transfer assay as described (29) based on the use of donor liposomes containing [^{14}C]labeled lipids and a biotin-labeled phosphatidylethanolamine (PE), which allows the separation of these membranes by streptavidin-coated magnetic beads. The acceptor liposomes are marked by fluorescent NBD-PE to control the recovery of these vesicles. After separation of the biotin-labeled liposomes, we measured radioactivity and fluorescence in the supernatant. B: In another in vitro assay, fusion of liposomes was investigated. One kind of liposomes contained NBD-PE and a radiolabeled lipid (II). If these liposomes (II) fused with Biotin-PE labeled liposomes (I), they would be pulled out by streptavidin-coated magnetic beads together with remaining vesicles (I). So we simultaneously measured a decrease of radioactivity and fluorescence in the supernatant. Red: [^{14}C] labeled lipid e.g., cholesterol; green: NBD-PE; blue: Biotin-PE; yellow: PC as a host lipid.

radioactivity and fluorescence of only donor (R^D, F^D) and acceptor (R^A, F^A) vesicles, respectively, as well as in a mixture of both (R^{D+A}, F^{D+A}). These measurements were also done in the presence of BioMag streptavidin (R^{D+B}, F^{D+B} / R^{A+B}, F^{A+B} / R^{D+A+B}, F^{D+A+B}). In the transfer as well as fusion assays, when protein was present, the terms $R^{D+A+P+B}$ and $F^{D+A+P+B}$ designate the measured radioactivity and fluorescence, respectively.

The percentage of lipid transfer mediated by protein (i.e., percentage of total radioactivity left in the supernatant after pulling out donor vesicles) is calculated as follows: $[R^{D+A+P+B} - R^{D+A+B}] \times 100/[R^{D+A} - R^{D+B}]$. The percentage of loss of fluorescence in the acceptor vesicles mediated by protein (i.e., percentage of total fluorescence lost from the supernatant after pulling out donor vesicles) observed in the transfer assay is calculated as follows: $[F^{D+A+B} - F^{D+A+P+B}] \times 100/F^{D+A}$.

The percentage of fusion mediated by protein observed in the fusion assay is calculated either as percentage of total radioactivity lost from the supernatant after pulling out donor vesicles: $[R^{D+A+B} - R^{D+A+P+B}] \times 100/R^{D+A}$, or as percentage of total fluorescence lost from the supernatant after pulling out donor vesicles: $[F^{D+A+B} - F^{D+A+P+B}] \times 100/F^{D+A}$.

The radioactivity and fluorescence measurements are means of four separate measurements ± SEM.

RESULTS

The transfer of cholesterol between intracellular membranes is of great importance for the homeostasis of cholesterol. Our group designed an in vitro assay to determine the amount of cholesterol transferred from the donor to the acceptor liposomes, and measurements were made under conditions that mimic those of the lysosomal compartment (29). This work is now extended to include conditions mimicking that of the late endosomal compartment, and the roles of some endosomal lipids (Cer, SM, and BMP) and NPC2 were examined. Since the lysosomal pH in fibroblasts of NPC patients does not differ from control cells

(55, 56), we assume that the pH value of late endosomes of NPC cells is also in the range of 6.0–5.0 as found for control cells (38). Information on membrane fusion was also deduced from the results of the assay, and a more reliable independent in vitro membrane fusion assay was developed. Fig. 1A shows a schematic diagram of the transfer assay described in "Materials and Methods." Control experiments, apart from those reported in "Materials and Methods," were done to ensure the accuracy and validity of the method. These include experiments to verify and ensure that there were no significant losses of lipids during liposome preparations and in the experiments. Various liposomal compositions were examined for stability using light scattering. The results showed that the liposomes were stable and their size was in the expected range (e.g., the diameter of liposomes containing 10 mol% Chol; 0 mol% SM; Cer and BMP at pH 5.0 directly and 24-h after extrusion were found to be 106 ± 1 nm and 107 ± 1 nm, respectively). The diameters of liposomes of other lipid composition used in the assays were in the same range (data not shown). These liposomes did not fuse before the experiments, and some containing SM were even stable for weeks at 4°C. Nevertheless, all liposomes used were freshly prepared. Streptavidin BioMag titrations for various liposomal compositions showed that 80 µl of the solution (5 mg/ml) was sufficient to completely bind vesicles containing 4 mol% of Biotin-PE used in the experiments (data not shown).

The recovery of acceptor liposomes in the supernatant generated in our transfer assay was monitored by fluorescence of NBD-PE present in acceptor liposomes. Depending on the lipid composition and the pH of the assay, NPC2 also stimulated liposome fusion. To account for fusion processes in the transfer assay, we developed a novel fusion assay (Fig. 1B).
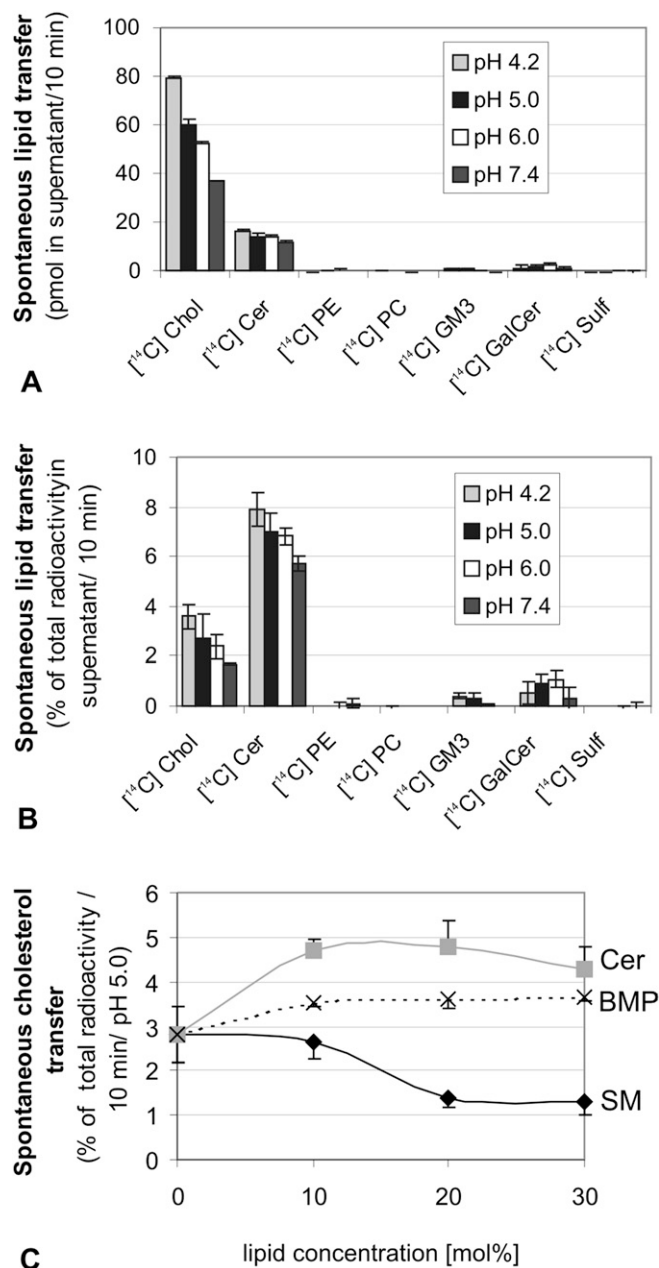
Fig. 2. Spontaneous transfer of cholesterol and ceramide. Spontaneous transfer (defined as the gain of radiolabeled lipid by acceptor liposomes in the supernatant in the absence of added lysosomal lipid-binding proteins) of different [^{14}C]labeled lipids was determined at different pH values for an incubation time of 10 min at 25°C. The complete amount of spontaneously transferred lipid at different pH values (A) and corresponding chart in percentage of transferred radiolabeled lipid (B). Lipid composition of donor liposome: 10 mol% unlabeled cholesterol, 1 mol% [^{14}C]labeled lipid, 4 mol% Biotin-PE. and 85 mol% PC. The acceptor vesicles contained 10 mol% unlabeled cholesterol, 4 mol% NBD-PE and 86 mol% PC. Spontaneous cholesterol transfer (C) depending on the lipid composition (varying Cer, BMP, and SM content) at pH 5.0 (at pH 4.2 and pH 6.0, similar transfer rates were obtained). Liposomes contained 10 mol% unlabeled cholesterol, 1 mol% [^{14}C] cholesterol, 4 mol% marker lipids, varying Cer, SM or BMP content as given, PC added to 100 mol%. Mean ± SEM (n = 4). Biotin-PE, *N*-[6-(biotinoyl)amino]hexanoyl-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; BMP, bis(monoacylglycero)phosphate; Cer, ceramide; Chol, cholesterol; GalCer, galactosylceramide; NBD-PE,

Spontaneous transfer of various lipids and the effects of late endosomal lipids

Spontaneous transfer (i.e., in the absence of transfer protein), although taken into account in our assay/calculation for the lipid under study, can affect our results (e.g., if some marker lipids like NBD-PE or Biotin-PE in the liposomes are also transferred). The results for the spontaneous transfer of various lipids at selected physiological pH values are reported (**Fig. 2A**, B). The spontaneous transfer of cholesterol and ceramide was highest at low pH. The spontaneous transfer of cholesterol is about five and three times higher than that of ceramide at pH 4.2 and pH 7.4, respectively. All other lipids studied did not show significant spontaneous transfer.

The effects of SM, BMP, and Cer, which are found in late endosomal membranes, on the spontaneous transfer of cholesterol are reported (Fig. 2C). Spontaneous transfer of cholesterol between liposomal membranes is slightly increased by Cer and decreased by SM, whereas BMP had almost no effect. If the liposomal membranes contain Cer and SM, the spontaneous transfer was abrogated (data not shown).

The very high levels of free fatty acids in NPC1-deficient late endosomal vesicles (57) led us to investigate the ability of bNPC2 protein to bind and transfer sphingosine, sphinganine, stearic acid, and linoleic acid from donor to acceptor liposomes, but the high off-rate of these molecules made our assay unsuitable for this experiment.

Of all proteins studied, NPC2 is solely and specifically responsible for transfer of cholesterol

NPC2-mediated cholesterol transfer surpassed spontaneous transfer that occurred in the absence of any transfer protein. We studied the lipid transfer of various proteins using the transfer assay (Fig. 1A) previously developed in our laboratory (29). To investigate the influence of some endosomal lipids such as Cer, SM, and BMP, we used liposomes containing 10 mol% cholesterol and PC as a host lipid. **Fig. 3A** shows the cholesterol transferred as mediated by various proteins at varying pH values. Only NPC2 showed a substantial activity in mediating cholesterol transfer. The percentage of cholesterol transferred increased with decreasing pH. GM2AP, which was earlier reported to show some level of activity (29), did not have any significant activity here. This confirms that only NPC2 mediates cholesterol transfer. Sap A, B, and D did not mediate cholesterol transfer (data not shown). It was necessary to check whether NPC2 itself also mediates the transfer of other lipids, especially Cer, in addition to cholesterol. The lipid transfer specificity of NPC2 at varying pH values is shown in Fig. 3B. Of all lipids under investigation (Cer, cholesterol, PE, PC, GM3, sulfatide, and galactosylceramide), only cholesterol was transferred by NPC2. This result also suggests

that the marker lipids Biotin-PE and NBD-PE are not transferred by NPC2. Concentration dependence of NPC2 on the transfer of cholesterol is shown in Fig. 3C. The percentage of cholesterol transferred increased with increasing concentrations and decreasing pH values.

BMP stimulates while sphingomyelin reduces cholesterol transfer by NPC2

BMP is a unique lysophospholipid found predominantly in lysosomes and intravesicular membranes of late endosomes (3, 34). It has been shown previously that the degra-
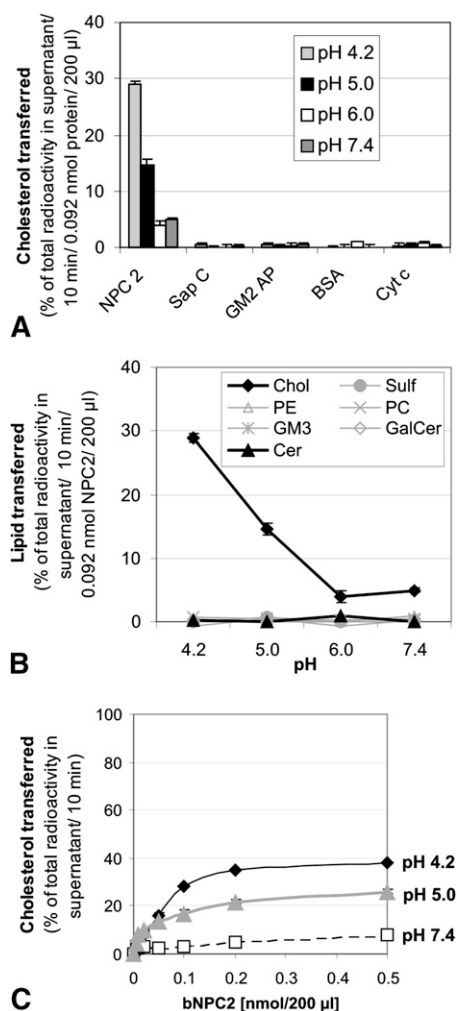

Fig. 3. Bovine NPC2 transfers [^{14}C] cholesterol under different experimental conditions: (A) cholesterol transfer by different lysosomal proteins, (B) NPC2 specificity for cholesterol transfer, and (C) variations of concentration of bNPC2 at pH 4.2 and 7.4. Donor liposomes contained 10 mol% unlabeled cholesterol, 1 mol% [^{14}C] labeled lipid, 4 mol% Biotin-PE, and 85 mol% PC; and the acceptor liposomes contained 10 mol% unlabeled cholesterol, 4 mol% NBD-PE, and 86 mol% PC. Mean ± SEM (n = 4); in (C), SEM <10% (error bars are within the used symbol). Biotin-PE, *N*-[6-(biotinoyl)amino]hexanoyl-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; BMP, bis(monoacylglycero)phosphate; Cer, ceramide; Chol, cholesterol; cyt C, cytochrom C; GalCer, galactosylceramide; GM2AP, GM2 activator protein; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; NPC2, Niemann-Pick disease type 2 protein; PC, dioleoyl-L-α-phosphatidycholine; PE, phosphatidylethanolamine; Sap C, saposin C; Sulf, sulfatide.

dation of (glyco)sphingolipids by lysosomal exohydrolases is stimulated by BMP in the presence of sphingolipid activator proteins (39, 54, 58, 59). It has also been shown to play a crucial role in the transfer of cholesterol in the lysosomal compartment (22, 29, 30). We could show that 20 mol% BMP stimulated the cholesterol transfer mediated by NPC2 at pH 5.0 and 4.2 (**Fig. 4A**). This stimulating effect was observed at various pH values studied. A significant difference is seen in the effect of C14:0 and C18:1 BMP, with BMP C18:1 having a more stimulating effect on protein-mediated transfer of cholesterol than the C14:0. Therefore, we used BMP C18:1 for our experiments. Fig. 4B shows that, unlike the effect of BMP, the inclusion of 10 mol% SM in a protein-mediated cholesterol transfer assay decreased the transfer at pH 4.2, 5.0, 6.0, and 7.4. This effect was more pronounced at pH 4.2 than at pH 6.0. This led us to investigate the effect of the various concentrations of these lipids on cholesterol transfer between liposomes mediated by NPC2.

Fig. 5A shows that an increasing concentration of BMP in the liposomal membranes increased cholesterol transfer under conditions mimicking those of the late endosomal compartment. The cholesterol transfer more than doubled at a 30% molar concentration of BMP. In contrast, the inclusion of 30 mol% of SM more or less completely inhibited the transfer of cholesterol (Fig. 5A). This result supports other lines of evidence that indicate that cholesterol has a higher affinity for SM-rich membranes than for most other types of membranes (60, 61). This accounts for the stabilizing property of the cholesterol-rich membranes, especially the plasma membrane. Fig. 5C shows that the inclusion of BMP, Cer, or Cer/BMP in the liposomal membranes does not prevent SM from inhibiting protein-mediated transfer of cholesterol, although BMP still increased the rate of cholesterol transfer.

Ceramide stimulates cholesterol transfer by NPC2 in the presence of BMP

Cer has been shown to act as a cholesterol competitor and facilitates the exit of cholesterol from membranes (62) by displacing it from bilayers (44, 63), thereby stabilizing lipid bilayers more efficiently than cholesterol (44, 64, 65). Moreover, in late endosomes, SM can be degraded to ceramide by the action of acid sphingomyelinase (39). It is, therefore important to know what effect ceramide has on NPC2-mediated cholesterol transfer. Increase in ceramide concentration, even independent of pH (Fig. 4B), had very little effect on cholesterol transfer (Fig. 5A). However, Fig. 5B shows that ceramide stimulated the BMP-induced cholesterol transfer significantly. This phenomenon, although less pronounced, was also observed in the presence of SM. Thus, the generation of ceramide by the action of sphingomyelinase on SM in the presence of BMP should drastically increase cholesterol transfer between late endosomal and/or lysosomal membranes containing BMP as an essential lipid.

Loss of fluorescence in the transfer assay

The recovery of acceptor liposomes in the supernatant of our transfer assay was monitored by fluorescence of
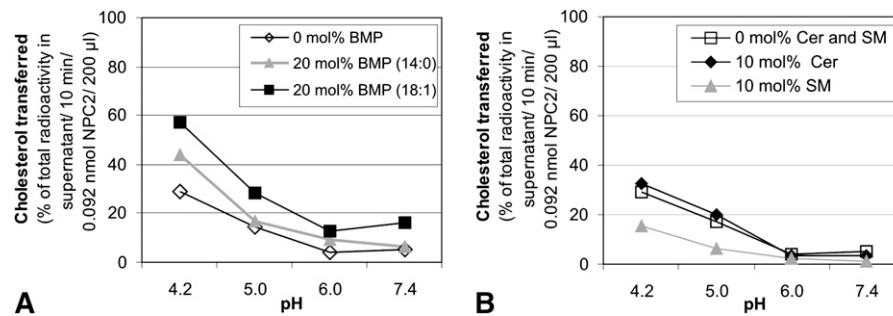
Fig. 4. pH dependence of cholesterol transfer by NPC2 in the presence of different lipids. A: ± 20 mol% BMP with different chain length. B: ± 10 mol% Cer or ± 10 mol% SM. The lipid composition is the same as in Fig. 2. The supplementation of liposomes with BMP, Cer, or SM is on the expense of the host lipid PC. After 10 min incubation time in the presence of 0.092 nmol bNPC2, the donors were separated by BioMag beads, and the radioactivity and fluorescence of the supernatant were measured. Mean ± SEM in the range of 10% (n = 4) (error bars are within the used symbol). BMP, bis(monoacylglycero)phosphate; Cer, ceramide; NPC2, Niemann-Pick disease type 2 protein; PC, dioleoyl-L-α-phosphatidycholine; SM, sphingomyelin.

NBD-PE present in acceptor liposomes. The percentage of cholesterol transferred by NPC2 at pH 4.2 seemed to reach a plateau after 60 min with about 40% radioactive cholesterol originally present in the donor liposomes (supplementary Fig. IA). This seemed to be rather low considering that *i*) the ratio of acceptor to donor liposomes is 5:1 and *ii*) the ability of cholesterol to flip across the bilayers. This unexpectedly low percentage of radioactivity in the supernatant implied either an exchange of NDB-PE and/or Biotin-PE or membrane fusion. Either event would lead to loss of fluorescence. Indeed, loss of fluorescence was observed over time at pH 5.0 and was even more pronounced at pH 4.2 (supplementary Fig. IB).

As demonstrated before, NPC2 was unable to transfer [^{14}C] PE from donor to acceptor vesicles containing NBD-PE (Fig. 3B). Furthermore, when NBD-PE was substituted for [^{14}C] PE in the donor membranes and [^{14}C] PE for NBD-PE in the acceptor membranes, the loss of radioactivity and fluorescence was very similar. This implies that NBD-PE was not transferred by NPC2. We assume that Biotin-PE is also not transferred by this protein. Loss of fluorescence could also result from self-quenching. However, the reverse experiment (fluorescent NBD-PE in donor liposomes and radiolabeled PC in acceptor liposome) clearly suggested that the loss of fluorescence was due to membrane fusion. Therefore, the seemingly low amount of cholesterol transferred seemed to be due to a slow fusion process (supplementary Fig. I) that left fewer unfused acceptor liposomes in the supernatant following magnetic removal of biotinylated membranes. Kinetic experiments showed that 50% of the total cholesterol transfer was achieved after 3 min at pH 4.2 and 10 min at pH 5.0. After 5 min, the loss of fluorescence was 1%–3% at pH 5.0 and about 8% at pH 4.2 compared with a cholesterol transfer of 14% at pH 5.0 and 29% at pH 4.2 (supplementary Fig. I). To verify that our observation of fluorescence loss was due to a fusion process, we substituted Sap C for NPC2 in our experiments as Sap C is known as a strong fusogenic protein (66, 67).

Sap C does not transfer cholesterol but mediates the loss of fluorescence (membrane fusion)

Sap C is required for the lysosomal degradation of membrane-bound glucosylceramide (53). Its fusogenic property had been established by measuring its ability to destabilize lipid vesicles (66–69). The inability of Sap C to mediate cholesterol transfer is reported in **Fig. 6**. Fig. 6A clearly shows that bNPC2 mediates cholesterol transfer both in the absence and presence of BMP at low pH. Measurements from the same experiment showed significant loss of fluorescence (Fig. 6B), especially at pH 4.2 in the presence of BMP. Under the same experimental conditions, unglycosylated Sap C did not mediate the transfer of cholesterol (Fig. 6C) but mediated loss of fluorescence up to 80% in the presence of BMP at low pH (Fig. 6D). NPC2 also mediated loss of fluorescent acceptor vesicles, although to a lesser extent than Sap C. Size measurements on liposomes composed of 10 mol% cholesterol, 4 mol% marker lipid, and 86 mol% PC showed that in the absence of NPC2, the initial signal curve generated by dynamic light scattering persisted for 60 min and represented liposomes with a mean diameter of 105 ± 10 nm. However, in the presence of NPC2, a new signal curve grew with time at the expense of the initial curve. This curve represented diameters in the range 600–900 nm, suggesting that larger aggregates had formed with time (supplementary Fig. II). Because this interesting phenomenon seemed to be worth testing in more detail, we designed a fusion assay.

New assay for membrane fusion

The schematic diagram of the new fusion assay is shown in Fig. 1B. Biotin-PE was incorporated into one type of liposome (I) to allow the separation of these vesicles from the other types of liposomes by streptavidin-coated magnetic beads. Radiolabeled cholesterol and fluorescent NBD-PE were incorporated into the second type of liposomes (II) to determine the extent of membrane fusion by measurement of either radioactivity or fluorescence. Usually, 10 mol% cholesterol was added to each type of liposome to ensure stability while PC served as the host lipid.
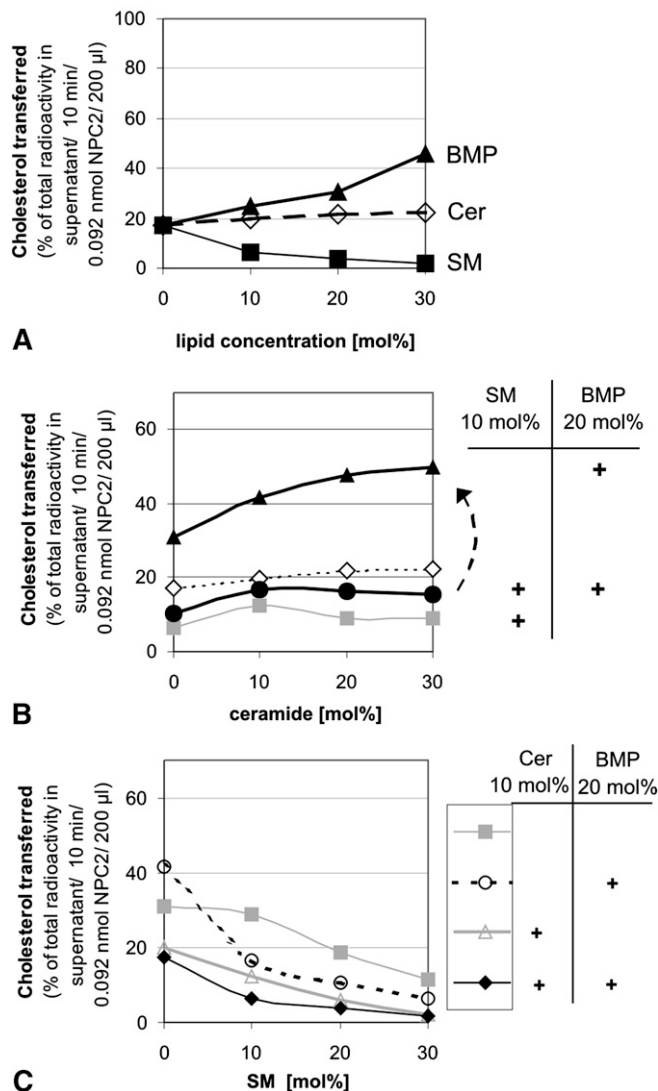
Fig. 5. BMP and ceramide stimulate whereas sphingomyelin inhibits bNPC-2–mediated cholesterol transfer at pH 5. A: Increasing concentrations of BMP, Cer, and SM integrated in both donor and acceptor liposomes at a concentration ranging 0–30 mol% on the expense of the host lipid PC. B, C: Combined effects of different lipids on the NPC2 enhanced cholesterol transfer in the presence of increasing Cer (B) and SM concentrations (C). The broken arrow in B points to the increase of cholesterol transfer rate when SM was absent from vesicle membranes. Mean ± SEM in the range of 10% (n = 4) (error bars are within the used symbols). BMP, bis(monoacylglycero)phosphate; Cer, ceramide; NPC2, Niemann-Pick disease type 2 protein; PC, dioleoyl-L-α-phosphatidycholine; SM, sphingomyelin.

The two liposome types were mixed in the ratio 1:1 in contrast to donor-acceptor ratio of 1:5 in the transfer assay. The fusion experiment was initiated by mixing the two liposome types (for spontaneous membrane fusion) or by the addition of protein (for protein-mediated membrane fusion). The addition of streptavidin-coated magnetic beads allowed the separation of fused liposomes and type I liposomes containing Biotin-PE (in the pellet) from the unfused type II liposomes (in the supernatant). The decrease of either the radioactivity or fluorescence of liposomes (II) indicated the extent of membrane fusion.

Calculation of the percentage of membranes fused was described under "Materials and Methods."

A typical pH dependence (in the presence or absence of BMP) of the percentage loss of radioactivity mediated by both NPC2 and Sap C is shown in **Fig. 7A**, and the loss of fluorescence of the same experiment is shown in Fig. 7B. The percentage of membrane fusion obtained from both were approximately the same, which implies that both are a measure of membrane fusion as expected. Measurement of radioactivity is simpler and more reliable than measurement of fluorescence because fluorescence could be affected by many factors, including quenching or interference by unknown factors. To validate our earlier proposition that the loss of fluorescence in our transfer assay was due to membrane fusion, we carried out a parallel transfer assay (donor-to-acceptor ratio of 1:1) using both NPC2 and Sap C in the presence and absence of BMP. It was observed that only NPC2 transfers cholesterol but to a lesser extent compared with the results of experiments with a donor-to-acceptor ratio of 1:5. As expected, Fig. 7C shows that NPC2 mediated cholesterol transfer while Sap C did not. Fig. 7D shows that the loss of fluorescence in the transfer experiments are identical to those obtained for the membrane fusion using radioactivity measurements (Fig. 7A, B). Before the use of BioMag streptavidin in these experiments, titrations were done to determine the adequate amount of BioMag streptavidin needed to completely pull out Biotin-PE–labeled liposomes with varying liposomal lipid compositions.

Spontaneous vesicle fusion induced by membrane lipids

Spontaneous membrane fusion in the absence of any protein has to be measured before the contribution of individual fusogenic proteins can be evaluated. Just as in the transfer assay, vesicles are expected to fuse to some extent in the absence of proteins. **Fig. 8A** shows the effect of various late endosomal lipids on membrane fusion. In the absence of SM, Cer, or BMP, the membrane fusion is about 17% under the experimental conditions. The inclusion of up to 20 mol% of BMP in the vesicular membranes did not have any significant effect on spontaneous membrane fusion. However, beyond this amount, a significant induction of spontaneous fusion was observed. The inclusion in liposomes of SM up to 10 mol% had no effect on spontaneous fusion. However, increasing the concentration of SM further induced significant spontaneous fusion of liposomes. Ceramide, on the other hand, induced spontaneous fusion even at low concentrations. These results support previous findings that ceramide induces a type of membrane destabilization, i.e., leakage of aqueous solutes from vesicles (70). It is clear from our results that ceramide has a higher tendency to induce spontaneous vesicle fusion (in the absence of protein) than BMP at pH 5.0.

Sap C and NPC2 trigger membrane fusion

It has been shown that some membrane fusion may occur in the absence of proteins. The question then is what will be the effect of various proteins on membrane fusion. Fig. 8B shows protein mediated membrane fusion for vari-
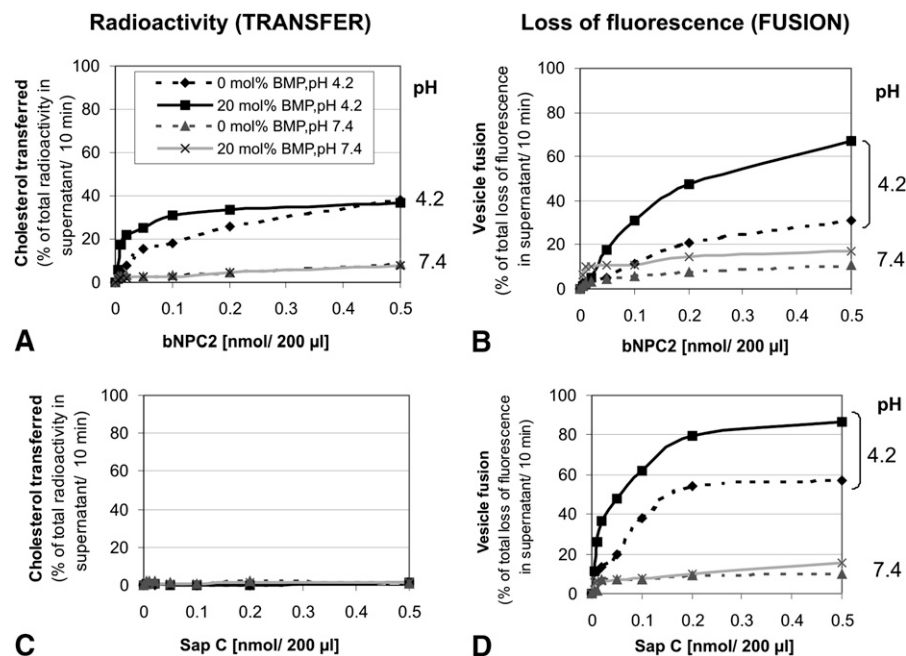
Fig. 6. Transfer of [^{14}C] cholesterol and loss of acceptor liposomes (loss of fluorescence) mediated by NPC2 and fusogenic Sap C. A: Cholesterol transfer depending on the concentration of bNPC2 in the presence or absence of 20 mol% BMP. B: bNPC2-mediated fluorescence loss, which implies membrane fusion. As a control we investigated radiolabeled cholesterol transfer (C) and the loss of acceptor (D) mediated by the fusogenic Sap C, containing a C-terminal hexahistidine tag. Mean ± SEM in the range of 12% (n = 4). BMP, bis(monoacylglycero)phosphate; NPC2, Niemann-Pick disease type 2 protein; Sap C, saposin C.

ous proteins (0.092 nmol) in the absence and presence of BMP in the vesicular membranes. Sap C has the greatest effect (20% and 68%) in both the absence and presence, respectively, of BMP. All the proteins studied trigger membrane fusion to some degree; even NPC2 triggers up to 10% membrane fusion in the absence of BMP and 40% in the presence of BMP. The respective fusogenic properties of Sap C and NPC2 at different pH values were also shown as a measure of a parallel loss of radioactivity and fluorescence from the supernatant (Fig. 7A, B). The extent of loss of fluorescence from acceptor vesicle in the transfer assay correlated with membrane fusion mediated by each of the proteins. This confirms that, in addition to Sap C whose fusogenic activity has been previously reported, NPC2 is also fusogenic at low pH, especially in the presence of BMP.

Ceramide slightly facilitates protein-enhanced fusion at low pH

Ceramide has been identified as an activator of protein function, such as cytosolic phosphatase present in rat T9 glioma cells and rat brain (71), but its effect on any protein in the endosomal/lysosomal compartment has not been reported.

In the presence of ceramide in the liposomal membranes, Sap C and NPC2 induced membrane fusion (Fig. 8C, D, respectively; shaded squares). However, it is important to note that the effect of Sap C is greater than that of NPC2 in the membrane fusion process. The presence of BMP improved the effect of ceramide in facilitating protein enhanced fusion (data not shown).

Effects of increasing contents of BMP, Cer, and SM on fusogenic activity of Sap C and NPC2 at endosomal pH

The effect of BMP, Cer, and SM in the liposomal membranes on cholesterol transfer activity by NPC2 at pH 4.2 has been previously reported (29) as well as its effect at endosomal pH values (Figs. 4 and 5). Fig. 8C, D shows how these membrane lipids affect the fusogenic activities of Sap C and NPC2. The effects they have on the fusogenic activities of these proteins correlate with the trend observed for their respective effects on NPC2-mediated cholesterol transfer. BMP has a strong catalyzing effect, followed by ceramide, on the fusogenic activities of Sap C and NPC2, while SM has an inhibitory effect. The inclusion of 30 mol% BMP enhanced the membrane fusion up to about 5-fold and 4-fold for Sap C– and NPC2-mediated membrane fusion, respectively (Fig. 8C, D). Ceramide seems to have a more pronounced effect on Sap C–mediated membrane fusion than on NPC2-mediated membrane fusion. The inhibitory property of SM was more pronounced on NPC2- than on Sap C–mediated membrane fusion. 20 mol% of SM was enough to stop membrane fusion evoked by NPC2, whereas about 30 mol% was needed to stop Sap C–mediated membrane fusion.

DISCUSSION

The lipid composition of biological membranes is crucial for their functional properties, hence, the sorting of lipids in the endocytic pathway. The composition of lipids in biological membranes also plays an important role during membrane degradation within endosomal/lysosomal
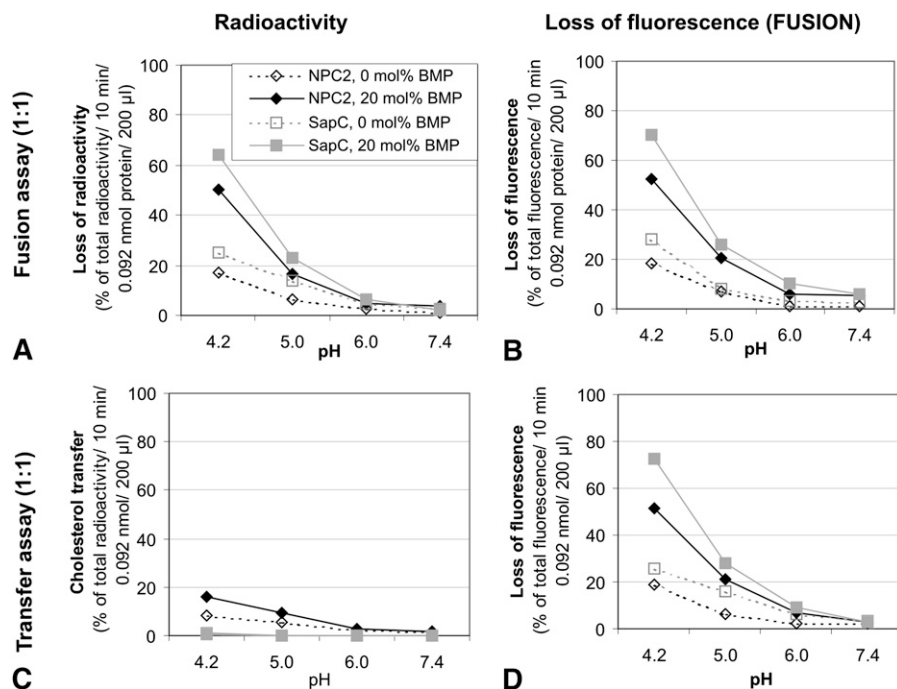
Radioactivity   **Loss of fluorescence (FUSION)**

Fig. 7. Fusion assay of liposomes type I with type II. Liposomes I contained Biotin-PE for separation with strepavidin coated magnetic beads. Liposomes II were composed of fluorescent NBD-PE and radiolabeled cholesterol as marker lipids. The ratio of liposomes I and II was 1:1. In case of vesicle fusion, radioactivity (A) and fluorescence (B) should disappear in parallel. A, B: The pH dependence of fusion rate mediated by bNPC2 and Sap C (containing a C-terminal hexahistidine tag) in the presence or absence of 20 mol% BMP. C, D: The corresponding transfer assay with the donor acceptor ratio of 1:1 instead of a ratio of 1:5 as used before. In this transfer assay, radiolabeled cholesterol is in the donor vesicle, and the fluorescent marker lipid NBD-PE is in the acceptor vesicle. Cholesterol transfer is depicted in C and loss of fluorescence in D. Mean ± SEM in the range of 10% (n = 4). BMP, bis(monoacylglycero)phosphate; NPC2, Niemann-Pick disease type 2 protein; Sap C, saposin C.

compartments. Cholesterol, which is a major steroid constituent of animal tissues, stabilizes plasma membranes, reduces membrane permeability, and enables the liquid-ordered phase of lipid bilayers (72). Failure of intracellular trafficking or accumulation of cholesterol in different intracellular membranes or lipoproteins could result in diseases, such as Wolman disease (7), hypercholesterolemia (4), and Niemann-Pick disease type C, a rare but fatal neurodegenerative disease (5, 6). Sorting of cholesterol in the late endosomal compartment is important because it cannot be degraded. Cholesterol has been shown to be transferred between liposomes by NPC2 (22, 29–31), and a bidirectional transfer of cholesterol between NPC1 and NPC2 has also been reported (31) (**Fig. 9**). Despite this, the dual role of NPC1 and NPC2 proteins in LDL-derived cholesterol trafficking in the endosomal/lysosomal compartments still remains ambiguous.

Cholesterol transfer of NPC2 under the late endosomal condition (pH 5.0) was not as high as under the lysosomal pH 4.2 (Fig. 3B). This may contribute to a higher concentration of cholesterol in the inner membranes of the late endosomal compartment than in the inner membranes of the lysosomal compartment. The difference in cholesterol content of these two compartments is most likely due to their respective physiological pH and lipid composition, especially the higher BMP concentration in the inner lysosomal membranes (3).

Ceramide, a structural component of sphingolipids and glycosphingolipids, occurs in free form as well as covalently bound to proteins in human skin (73). Therefore, ceramide transfer by lipid transfer proteins would be of physiological relevance not only for degradation but also for biosynthetic salvage processes. Since Cer also acts as a cholesterol competitor, we checked whether NPC2 can transfer ceramide in addition to cholesterol. Our data (Fig. 3A) demonstrate that NPC2 does not transfer ceramide, PC, PE, galactosylceramide, sulfatide, or GM1. Its specificity for cholesterol is confirmed. However, NPC2 is known to bind other sterols (24). Nevertheless, in our experiments ceramide was transferred spontaneously in the absence of NPC2, although not to the same extent as cholesterol. The spontaneous transfer of ceramide may be significant as it is unclear whether ceramides are completely degraded by acid ceramidase or, in part, can leave the acidic compartments intact as shown previously for endocytosed, nondegradable analogs of glucosylceramide and gangliosides GM2 and GM1 (74–77).

Ceramides are degraded at pH values below 4.8 by acid ceramidase into fatty acids and sphingoid bases, the final products of sphingolipid degradation in the lysosomes (54). Our data show that oleic acid, linoleic acid, sphinganine, and sphingosine are spontaneously transferred, even within a short experimental period of 3 min. Their abnormally high levels in the liver, spleen, and cerebral cortex of
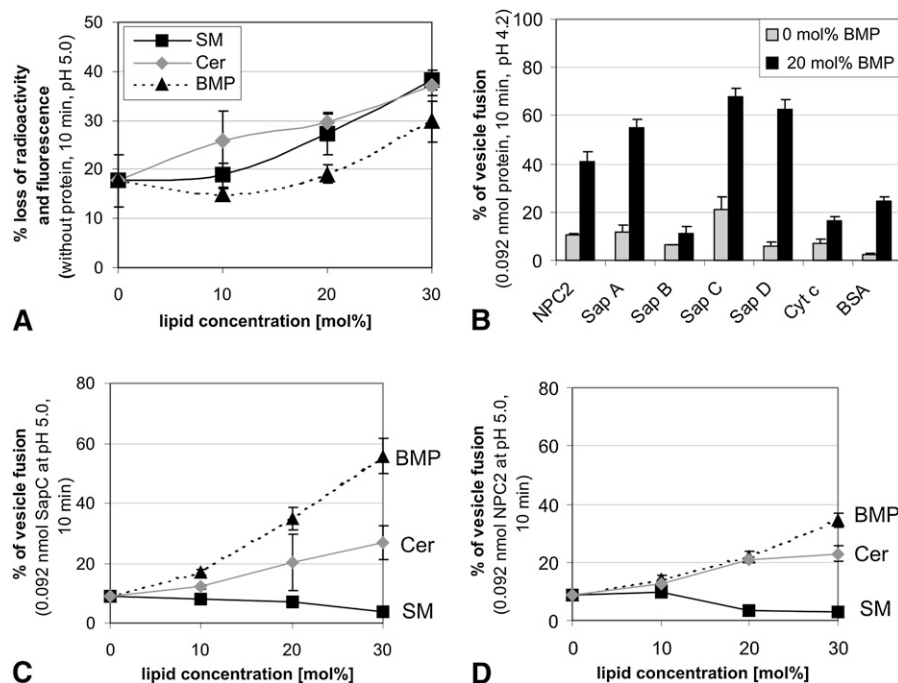
Fig. 8. Fusion assay measurements. A: Spontaneous fusion in the presence of varying concentrations of SM, Cer, and BMP at pH 5.0. B: Fusion mediated by various endosomal/lysosomal proteins in the presence or absence of 20 mol% BMP at pH 4.2. C, D: Effects of various endosomal lipids on fusion mediated by 0.092 nmol each of hexahistidine tag containing Sap C (C) or bNPC2 (D) at pH 5.0. The ratio of liposome I and II is 1:1. Mean ± SEM (n = 4). BMP, bis(monoacylglycero)phosphate; Cer, ceramide; Cyt c, cytochrom C; NPC2, Niemann-Pick disease type 2 protein; Sap A, saposin A; Sap B, saposin B; Sap C, saposin C; Sap D, saposin D; SM, sphingomyelin.

patients with NPC disease and other lysosomal disorders previously reported (35) does not seem to be due to lack of NPC2. With our improved assay conditions, we could clearly demonstrate that, in contrast to NPC2, other lysosomal proteins (GM2AP, Sap C, and others) do not mediate cholesterol transfer at substantial rates, although a slightly faster transfer rate was reported recently (29).

BMP (C18:1) has been reported to enhance degradation of sphingolipids and glycosphingolipids by lysosomal exohydrolases in the presence of sphingolipid activator proteins (39, 54, 58, 59). Its ability to stimulate cholesterol

transfer at lysosomal pH has been previously reported (29). Fig. 4A clearly shows that BMP also stimulates cholesterol transfer at the pH of the late endosomal compartments (around pH 5.0), although to a lesser extent than that previously reported at lysosomal pH of around 4.5 (29). This result is reasonable because it confirms the observation that BMP concentration increases in the inner membranes of the endocytic pathway while cholesterol concentration and the luminal pH decreases (3). However, at a 30% molar concentration of BMP, the rate of cholesterol transfer is more than doubled compared with the choles-
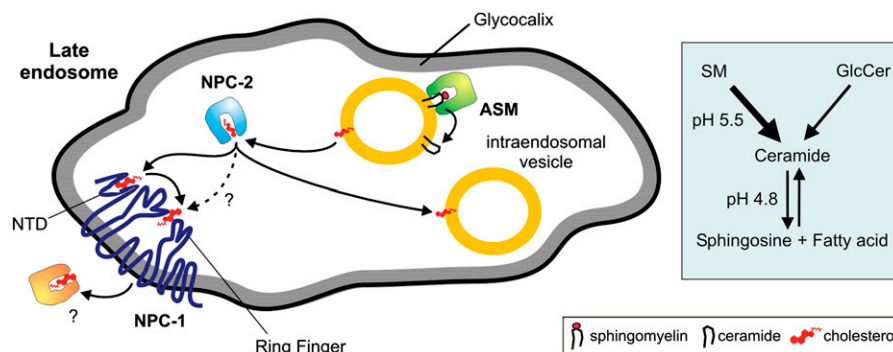
Fig. 9. Model for lipid sorting at the stage of late endosomes (pH 5.0). Acid sphingomyelinase degrades sphingomyelin to ceramide at the surface of intraendosomal vesicles. Decrease of sphingomyelin and increase of ceramide enhances the availability of cholesterol on inner membranes for NPC2 which removes cholesterol from the inner endosomal vesicles (yellow) and transfers it to the N-terminal domain of NPC1 in the limiting membrane of late endosomes (25). NPC1 mediates cholesterol exit through the glycocalix. ASM, acid sphingomyelinase; NPC, Niemann-Pick type C protein, NTD, N-terminal domain.

terol transfer rate recorded in the absence of BMP at pH 5.0. It is noteworthy that BMP (18:1) with native acyl chains stimulates cholesterol transfer much better than BMP (14:0) with unnatural acyl chains. As BMP (18:1) and BMP (14:0) have the same configuration, their effect on cholesterol transport seems to be solely related to the acyl chain length and/or unsaturation. It will be interesting to see what the effect of BMP (22:5) with longer acyl chains will be. There may also be a significant difference between the effects of natural BMPs and the synthetic ones used here. It is obvious from this work and the previous reports (22, 29, 30) that BMP is needed to stimulate cholesterol transfer. The combined presence of BMP and hydrolytic enzymes (e.g., acid sphingomyelinase) might have an important effect on cholesterol transfer. For instance, exogenously added sphingomyelinase reduced accumulation of both SM and cholesterol in cultured fibroblasts of NPC patients (78), which supports the notion above.

The high accumulation of cholesterol in SM storage diseases, such as Niemann-Pick disease type A and B, has been reported to be secondary to SM accumulation (79). As seen in Fig. 5A, the inclusion of increasing amounts of SM into the vesicles inhibited cholesterol transfer nearly completely. This finding is attributed to the high affinity of cholesterol to SM-rich membranes (60, 61). SM interacts with cholesterol by forming hydrogen bonds between the OH-group of cholesterol and the amide group of SM (40, 41), thereby keeping cholesterol in the membrane (42, 43). The inhibitory activity of SM on cholesterol transfer by NPC2 is more pronounced at pH 5.0 than at pH 4.2 (29).

At an optimum pH of 5.5 (39) membrane-stabilizing SM may be degraded to ceramide efficiently by acid sphingomyelinase in the intraendosomal membranes of late endosomes (Fig. 9). This may stimulate the transport of cholesterol within late endosomes. Indeed, as illustrated in Fig. 5B, removal of SM greatly stimulated cholesterol transfer in the presence of BMP (arrow). This shows the important function of acid sphingomyelinase in preventing lipid traffic jam in the presence of BMP in the late endosomal compartments. This is also corroborated by the observation that Hsp 70 stimulates the activity of acid sphingomyelinase and thus the degradation of SM (80). The generation of ceramide in the presence of BMP facilitates the exit of cholesterol from late endosomal compartments, by acting as a cholesterol-competitor, thereby displacing cholesterol from the bilayers (44, 62, 63).

The reported loss of fluorescence in our earlier transfer assay (29) was thought to have arisen from either *i*) the transfer or quenching of the fluorescence probe NBD-PE or the possibility of liposome adherence to the wall of the reaction vessel; or *ii*) the fusion of the donor and acceptor vesicles. We made a significant change in our transfer assay to assess the source of the loss of fluorescence by using a donor-to-acceptor liposome ratio of 1:1. Assuming an even distribution of NBD-PE in liposomes, a significant extent of fluorescence self-quenching at 4 mol% is not very likely. Also the inverse experiment (fluorescence NBD-PE in the donor vesicles and radiolabeled PC in the acceptor lipo-

somes) leads to the same loss of radioactivity as previously of fluorescence, which again argues against self-quenching and a reduction of the NBD group. We also checked the possibility of liposome adherence to the wall of the reaction vessel. For this we used the inverse experiment and measured radioactivity in the absence and presence of NPC2 remaining in the assay volume. The loss of radioactivity observed after 10 min, the incubation time in transfer/fusion assays, was less than 3% of the total radioactivity applied and was within the error of measurement. We conclude, beyond any doubt, that the loss of fluorescence was due to membrane fusion. However, in transfer experiments at pH 5.0 using a 1:5 ratio of donor-to-acceptor liposomes (Figs. 2–6), the rate of fusion did not exceed 1%–3% and 5%–9% of the transfer rate after 10 min in the absence or presence of BMP, respectively.

The extent to which bovine NPC2 and membrane lipids mediate membrane fusion was investigated using the newly designed fusion assay, which is based on the use of streptavidin-coated paramagnetic beads as described in "Materials and Methods." Owing to its fusogenic property, Sap C (66–69) was used as a control in our fusion assays. Our results showed (Fig. 8B) that bNPC2 also catalyzes membrane fusion both in the absence and presence of BMP, although significantly less so than Sap C. The fusogenic property of Sap C has been attributed to the lysine residues, which are located at positions 13 and 17 of the amino acid sequence of the protein (81, 82), A similar mechanism may also be at work for bNPC2 as this protein also contains lysine residues, some of which may be positioned similarly as those in Sap C.

CONCLUSION

The roles of some late endosomal lipids have been determined in vitro and explained. BMP stimulates cholesterol transfer mediated by NPC2 and greatly enhances membrane fusion induced by fusogenic proteins. Its presence also enhanced the stimulating effect of ceramide on cholesterol transfer mediated by NPC2. SM, on the other hand, inhibited NPC2-mediated cholesterol transfer. Therefore, a function of acid sphingomyelinase in living cells in the presence of BMP may be the prevention of cholesterol accumulation in the late endosomal compartment. The combined presence of ceramide and BMP in vitro greatly enhanced the transfer of cholesterol from vesicles at pH 5.0. The specificity of NPC2 as a cholesterol transfer protein has been validated, and NPC2 fusogenic activity is reported. It has been confirmed that Sap C does not mediate cholesterol transfer in vitro but causes membrane fusion. This was ascertained by a newly developed membrane fusion assay which allows various control experiments.

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