

Regulation of Sterol Transport between Membranes and NPC2[†]Zhi Xu,^{‡,§} William Farver,[‡] Sarala Kodukula,[‡] and Judith Storch^{*,‡,§}

Department of Nutritional Sciences and Rutgers Center for Lipid Research, School of Environmental and Biological Sciences, Rutgers, the State University of New Jersey, New Brunswick, New Jersey 08901

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ABSTRACT: Niemann-Pick disease type C (NPC) is caused by defects in either the NPC1 or NPC2 gene and is characterized by accumulation of cholesterol and glycolipids in the late endosome/lysosome compartment. NPC2 is an intralysosomal protein that binds cholesterol *in vitro*. Previous studies demonstrated rapid rates of cholesterol transfer from NPC2 to model membranes [Cheruku, S. R., et al. (2006) *J. Biol. Chem.* 281, 31594–31604]. To model the potential role of NPC2 as a lysosomal cholesterol export protein, in this study we used fluorescence spectroscopic approaches to examine cholesterol transfer from membranes to NPC2, assessing the rate, mechanism, and regulation of this transport step. In addition, we examined the effect of NPC2 on the rate and kinetic mechanism of intermembrane sterol transport, to model the movement of cholesterol from internal lysosomal membranes to the limiting lysosomal membrane. The results support the hypothesis that NPC2 plays an important role in endo/lysosomal cholesterol trafficking by markedly accelerating the rates of cholesterol transport. Rates of sterol transfer from and between membranes were increased by as much as 2 orders of magnitude by NPC2. The transfer studies indicate that the mechanism of NPC2 action involves direct interaction of the protein with membranes. Such interactions were observed directly using FTIR spectroscopy and protein tryptophan spectral shifts. Additionally, cholesterol transfer by NPC2 was found to be greatly enhanced by the unique lysosomal phospholipid lyso-bisphosphatidic acid (LBPA), suggesting an important role for LBPA in NPC2-mediated cholesterol trafficking.

Niemann-Pick disease type C (NPC)¹ is an inherited lipid storage disorder caused by defects in either the NPC1 or NPC2 gene. In NPC, intracellular trafficking of cholesterol is disturbed, and both cholesterol and glycolipids accumulate in late endosome/lysosomes (1–4). Mutations in NPC1, a 1278-residue transmembrane glycoprotein, account for approximately 95% of the cases (5); mutations in NPC2 account for the remaining 5% (6). While defects in either protein result in the accumulation of cholesterol, the mechanisms of action of NPC1 and NPC2 in lysosomal cholesterol trafficking are not clear.

Cholesterol enters mammalian cells following endocytosis of cholesteryl ester-rich low-density lipoprotein (LDL) via the receptor-mediated endocytic pathway (7). In the endosomal/lysosomal compartments, the cholesteryl ester is hydrolyzed to unesterified cholesterol and free fatty acids by acid lipase. LDL-derived cholesterol is then, by unknown mechanisms, transported primarily to the plasma membrane

or to the endoplasmic reticulum for re-esterification. The egress of cholesterol is important for the induction of homeostatic responses which regulate the cellular cholesterol pool, including LDL receptor expression and *de novo* cholesterol synthesis (8).

NPC1 contains a sterol-sensing domain (SSD) located between the third and seventh of its putative 13 transmembrane domains (9). While the NPC1 protein was suggested to transport fatty acids and not cholesterol (10), others have shown this is likely not occurring in cells (11), and several reports demonstrate sterol binding, if not yet transport, by NPC1 (12–14). The NPC2 gene encodes a 132-residue soluble lysosomal glycoprotein (6), which was characterized as a cholesterol binding protein with a 1/1 stoichiometry (15–17). Depending on the experimental method used, binding affinities have been reported to range from roughly 30 nM to 2 μ M (15, 16, 18); such operational differences are not uncommon for lipid-binding proteins and their small hydrophobic ligands (19). In previous work, we examined the kinetics of cholesterol transfer from NPC2 to model membranes utilizing a fluorescence dequenching assay (20). The results suggested that NPC2 transports cholesterol to phospholipid vesicles rapidly via a collisional mechanism involving direct interaction with acceptor membranes. These studies provided initial evidence of a role for NPC2 in lysosomal cholesterol trafficking and identified lysobisphosphatidic acid (LBPA; also known as bis-monoacylglycerol phosphate) as a key component of the putative protein–membrane “collisional complex” (20).

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* To whom correspondence should be addressed. E-mail: storch@aesop.rutgers.edu. Phone: (732) 932-1689. Fax: (732) 932-6837.

[‡] Department of Nutritional Sciences.

[§] Rutgers Center for Lipid Research.

¹ Abbreviations: NPC, Niemann-Pick disease type C; LDL, low-density lipoprotein; DHE, dehydroergosterol; RCA, *Ricinus communis* Agglutinin; EPC, egg phosphatidylcholine; POPS, palmitoyloleoylphosphatidylserine; LBPA, lysobisphosphatidic acid; dansyl-PE, dansyl phosphatidylethanolamine; CTL, cholestatrienol; FRET, fluorescence resonance energy transfer; FTIR, Fourier transform infrared.

The late endosomal/lysosomal compartment is characterized by an abundance of internal membranes, as well as a limiting outer membrane that borders the cytosolic compartment (21). It is likely that in the absence of efficient egress, the lipase-liberated cholesterol partitions into the abundant internal membranes. To improve our understanding of the mechanisms of endosomal/lysosomal cholesterol transport, in this study we modeled the process of cholesterol transport from membranes to the NPC2 protein, assessing the rate, mechanism, and regulation of this transport step. In addition, we examined the effect of NPC2 on the rate and kinetic mechanism of intermembrane sterol transport, to model the movement of cholesterol from internal lysosomal membranes to the limiting lysosomal membrane. The results support the hypothesis that NPC2 plays an important role in endosomal/lysosomal cholesterol trafficking by markedly accelerating the rates of transport from and between membranes. The mechanism of NPC2 action involves direct interaction of the protein with membranes. NPC2–membrane interaction is supported by results obtained using two spectroscopic approaches, FTIR and fluorescence spectral shift. Additionally, cholesterol transfer by NPC2 is greatly enhanced by LBPA and specifically blocked by an anti-LBPA antibody, further supporting an important role for this unique phospholipid in lysosomal cholesterol trafficking, perhaps working as a localizing site for NPC2 and, thereby, enhancing the efficiency of cholesterol transport by NPC2.

MATERIALS AND METHODS

Materials. Cholesterol, dehydroergosterol (DHE), GM1 and GM2 gangliosides, lactosyl ceramide, and *Ricinus communis* Agent (RCA) were obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), palmitoyl-oleoylphosphatidylserine (POPS), oleoyl and myristoyl lysobisphosphatidic acid (LBPA; also known as bis-monoacylglycerol phosphate or BMP), dansyl phosphatidylethanolamine (dansyl-PE), and dolichols were from Avanti Polar Lipids (Alabaster, AL). [1,2-³H]Cholesterol and cholesteryl [oleate-1-¹⁴C]oleate were from NEN Life Science Products (Boston, MA). Anti-LBPA antibody 6C4 was generously provided by J. Gruenberg (University of Geneva, Geneva, Switzerland). Cholestatrienol (CTL) was generously provided by F. Maxfield (Wiell Cornell Medical College, New York, NY).

Purification of Human and Bovine NPC2 Proteins. Human NPC2 protein was prepared from Chinese hamster ovary cells as previously described (20), using a 5 kDa cutoff flow filtration membrane to concentrate media (CDUF002 LC, Millipore, Bedford, MA). Bovine NPC2 protein was prepared from raw milk as detailed by Friedland et al. (18). Unless otherwise indicated, the monoglycosylated form of NPC2 was used.

Membrane Vesicle Preparation. Small unilamellar vesicles (SUV) were prepared by sonication and ultracentrifugation as described previously (22). Vesicles were maintained at temperatures above the phase transition temperatures of all constituent lipids. The standard vesicles were prepared to contain 100 mol % egg phosphatidylcholine (EPC). For studies of cholesterol transfer from membranes to NPC2, 10% cholesterol was substituted for EPC. For intermembrane sterol transfer experiments, 25% DHE and 3% dansyl-PE were added to donor and acceptor vesicles, respectively, also

substituting for EPC. For some experiments, as indicated in the text, other lipids at 25 mol % were substituted for EPC. Vesicles were prepared in 20 mM sodium citrate, 150 mM NaCl buffer (pH 5.0).

Cholesterol Transfer from NPC2 to Membranes and from Membranes to NPC2. Human NPC2 has two tryptophan residues, at positions 109 and 122. The endogenous tryptophan fluorescence of NPC2 was used to monitor cholesterol transfer from model membranes to NPC2. As described previously, the NPC2 tryptophan signal is quenched by cholesterol binding; therefore, the transfer of cholesterol from NPC2 to membranes can be monitored by the dequenching of the NPC2 tryptophan signal (20). The reverse, i.e., cholesterol transfer from membranes to apo NPC2 protein, can be monitored by the quenching of the NPC2 tryptophan signal, as follows. Donor phospholipid vesicles containing cholesterol are added to apo NPC2, and the transfer of cholesterol from the membranes to NPC2 is monitored directly by the decrease in tryptophan fluorescence over time. For all transfer assays, membranes with a defined phospholipid composition and concentration were mixed with NPC2 using a stopped-flow mixing chamber interfaced with a DX-18MV spectrofluorimeter (Applied Photophysics Ltd.), and the time-dependent change in tryptophan signal was used to obtain the transfer rates. To ensure that unidirectional transfer of cholesterol was being monitored, an equivalent or greater amount of acceptor (NPC2) relative to donor (membrane phospholipid) was used (23, 24). This was determined by calculating the relative partitioning of cholesterol, at equilibrium, between NPC2 and phospholipid using the NPC2 tryptophan signal with stoichiometric cholesterol bound, compared to the signal in the presence of vesicles, as previously described (24, 25). For EPC vesicles, the partition of cholesterol to NPC2 relative to EPC was found to be approximately 50 (NPC2/EPC, molar ratio). Thus, for example, in a mixture of 5 μ M NPC2, 5 μ M cholesterol, and 400 μ M EPC, approximately 3 μ M cholesterol partitions to the vesicles at equilibrium while roughly 2 μ M is bound to NPC2. Transfer was monitored at 25 °C, and controls to ensure the absence of photobleaching were performed before each experiment. The excitation wavelength was 280 nm, and emission was monitored using a 299 nm cutoff filter.

Data were analyzed using software provided with the Applied Photophysics stopped-flow instrument, and the cholesterol transfer rates were obtained by exponential fitting of the curves, all of which were well fit by a single-exponential function. For each experimental condition, at least five replicates were conducted, and the averages \pm the standard deviation for three or more experiments are reported. It is worth noting that these transfer assays use native ligand and native protein with no exogenous probes to acquire kinetic information; thus, the absolute rates obtained are likely to be physiologically relevant.

Intermembrane Transfer of [³H]Cholesterol. We used [1,2-³H]cholesterol to study cholesterol transfer between membranes, as described by Backer and Dawidowicz (26). Briefly, donor SUV were composed of EPC and cholesterol (1/0.4 molar ratio) containing 10% (w/w) lactosoyl ceramide. A trace amount of [³H]cholesterol (25 μ Ci) was added to donor membranes, and 20000 cpm of [¹⁴C]cholesteryl oleate was added as a nontransferring control. Acceptor SUV were 100% EPC. In the experiment, 50 μ M donor phospholipid

SUV were mixed with 1.5 mM acceptor SUV in a total volume of 4 mL. The mixture was incubated at 37 °C, and at specific time points, 100 μ L of the mixture was removed and vortex-mixed with 200 μ L of 2.5 mg/mL RCA. The donor SUV were spun down at 14000 rpm using Eppendorf centrifuge 5415C (Brinkmann Instruments, Westbury, NY), and 200 μ L of the supernatant was counted to determine the amount of cholesterol transferred to acceptor membranes. Values were corrected for volume, and as modified by ^{14}C supernatant counts of the nontransferable cholesteryl oleate, which provides an indication of donor vesicles not removed by centrifugation.

Intermembrane Transfer of DHE. ^3H -labeled cholesterol can be used for monitoring the slow rate of spontaneous cholesterol transfer between membranes. In the presence of NPC2, the cholesterol transfer rates were much faster and we were unable to use the physical separation method. Therefore, fluorescence resonance energy transfer (FRET) between DHE and dansyl-PE was used to study the intermembrane transfer of DHE, a fluorescent analogue of cholesterol (27). This assay uses donor SUV containing DHE and acceptor vesicles containing dansyl-PE. The fluorescence emission of DHE at 370 nm overlaps with the dansyl-PE excitation spectrum; thus, when DHE transfers to acceptor membranes containing dansyl-PE, DHE emission is quenched while the so-called sensitized emission of the dansyl moiety at 510 nm is increased. The transfer of DHE from donor to acceptor membranes is therefore monitored directly by the decrease in DHE fluorescence or the increase in dansyl fluorescence over time. The excitation wavelength was 300 nm, and emission was monitored using a 520 nm cutoff filter for the dansyl emission or a 370 nm narrow band filter for the DHE emission. Essentially identical rates were obtained using either method. Transfer was monitored at 25 °C, and controls to ensure that photobleaching was eliminated were performed before each experiment.

Cholestatrienol Transfer from NPC2 to Membranes: Effect of the Anti-LBPA Antibody. Monoclonal anti-LBPA antibody 6C4 was generously provided by J. Gruenberg. Since the monoclonal antibody itself contains tryptophan residues, it was not possible to use the NPC2 tryptophan quenching method to study cholesterol transfer from NPC2. Therefore, we utilized CTL as a cholesterol analogue and studied its transfer from NPC2 to membranes, in the absence or presence of the anti-LBPA antibody. CTL is a fluorescent sterol that mimics cholesterol behavior (28, 29) and has a fluorescence emission peak at 370–390 nm at an excitation wavelength of 340 nm. As for DHE, there is FRET between CTL and dansyl-PE. In these experiments, 2 μM apo NPC2 was incubated with 4 μM CTL for 30 min at 37 °C, and this donor complex was mixed with 500 μM SUV with the indicated composition and containing 3% dansyl-PE preincubated with 250 ng/mL 6C4, using the stopped-flow spectrofluorimeter. The excitation wavelength was 340 nm, and emission was monitored using a 370 nm narrowband filter. Transfer was monitored at 25 °C.

Fourier Transfer Infrared (FTIR) Assay for Examining Membrane–NPC2 Interactions. FTIR experiments were performed as described previously (30). Briefly, multilamellar vesicles (MLV) were prepared by dissolving POPS in a chloroform/methanol mixture (1/1, v/v), and the solvents were then evaporated under a slow stream of N_2 gas followed

by lyophilization for 3–4 h to remove traces of solvent. MLV were prepared by rehydrating with D_2O buffer [10 mM Tris, 100 mM NaCl, and 100 μM EDTA (pD 7.4)] at a temperature well above the gel/liquid-crystalline phase transition of POPS, in sealed tubes. Throughout the heating cycle, samples were periodically vortexed to ensure complete melting and mixing of all components. After being mixed and hydrated, samples were sandwiched between heated IR windows and mounted in a temperature-controlled transmission cell holder (Harrick Scientific, Ossining, NY). For experiments with NPC2, 0.5 mg of NPC2 was added to 2.0 mg of POPS (1/60 molar ratio). FTIR spectra were collected on a Mattson spectrometer equipped with a broadband mercury-cadmium-telluride (MCT) detector. Spectra were generated by co-addition of 256 interferograms collected at 2 cm^{-1} resolution, apodized with a triangular function, and Fourier-transformed with one level of zero filling. Spectra were routinely acquired at 1 or 2 °C intervals from 4 to 36 °C. Data were analyzed off-line with software provided by the National Research Council of Canada (31).

RESULTS

Cholesterol Transfer from Membranes to NPC2: Effects of Donor Vesicle Composition and NPC2 Concentration. Our previous studies suggested that cholesterol transfer from NPC2 to phospholipid vesicles occurs via a collisional mechanism involving direct interactions between NPC2 protein and the acceptor membranes (20). Here we examined the transfer of cholesterol from membranes to NPC2. To distinguish between collision-based and aqueous diffusion-based mechanisms, transfer was examined as a function of donor membrane composition and as a function of increasing NPC2 concentration. Figure 1A shows that the rate of cholesterol transfer from membranes containing negatively charged phospholipids to NPC2 was markedly faster than transfer from zwitterionic vesicles. The donor membranes containing 25% LBPA, a lysosome, and late endosome-specific phospholipid exhibited the highest transfer rates, ~ 200 -fold greater than rates of transfer from EPC membranes. Figure 1B shows that when cholesterol transfers from a fixed amount of SUV to increasing amounts of NPC2, a linear increase in the cholesterol transfer rate is observed when the quantity of acceptor NPC2 is sufficient for unidirectional transfer rates to be obtained (24, 25). Thus, on the basis of the above-mentioned equilibrium partitioning of cholesterol between NPC2 and SUV, cholesterol transfer was linear from 250 μM EPC SUV at NPC2 concentrations of $>5 \mu\text{M}$. Both the composition dependence and the effect of the acceptor (NPC2) concentration strongly suggest a collisional mechanism of cholesterol transfer from membranes to NPC2. In addition, since egress of cholesterol from this compartment is likely to require movement from lysosomal internal membranes to the limiting endosomal/lysosomal membrane, the results support a physiologically specific role for LBPA in NPC2–membrane interactions and lysosomal cholesterol transport.

Effect of LBPA Acyl Chain Species and LBPA Stereoisomers on Cholesterol Transfer from NPC2 to Membranes. LBPA is uniquely present at high concentrations in the internal membrane network of endosomes/lysosomes (32). Unlike other mammalian phospholipids with an *sn*-3-

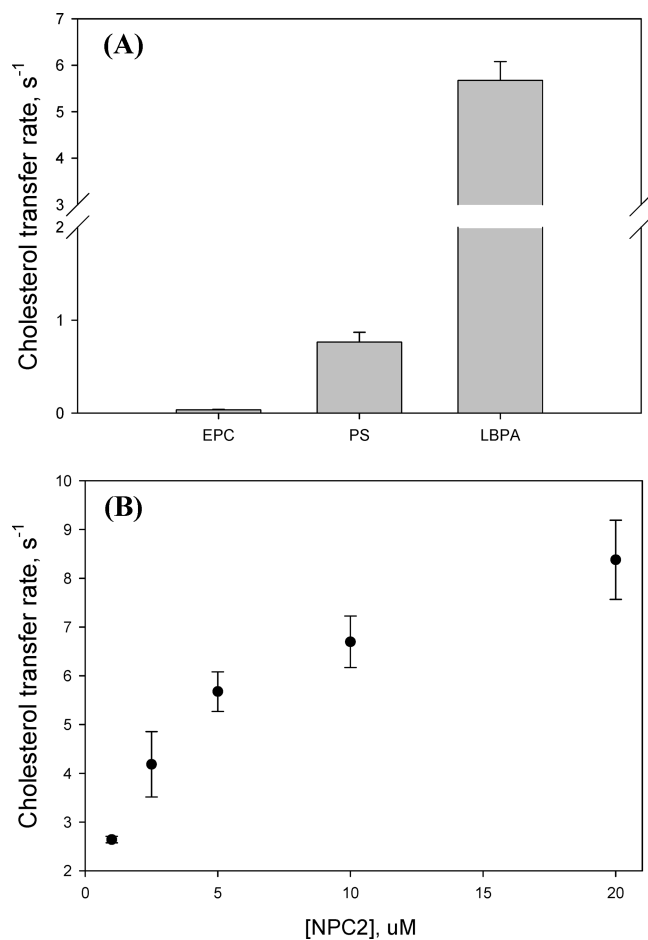


FIGURE 1: Cholesterol transfer from phospholipid membranes to NPC2. (A) Transfer of 2.5 μM cholesterol from 250 μM SUV [EPC (90% EPC/10% cholesterol), PS (65% EPC/25% PS/10% cholesterol), or LBPA (65% EPC/25% LBPA/10% cholesterol)] to 5 μM NPC2 was monitored at 25 °C and pH 5.0 using a stopped-flow fluorescence spectrometer, as described in Materials and Methods. (B) Transfer of 2.5 μM cholesterol from 250 μM LBPA SUV to NPC2 at different concentrations.

glycerophosphate stereoconfiguration, LBPA in mammals favors the *sn*-1:*sn*-1' (*S,S'* isomer) headgroup configuration (33–36), yet the *S,R* isomer was the only commercially available LBPA isomer until very recently and, as such, was used for many of our earlier experiments. With the recent availability of additional isomers, we compared the effect of different LBPA stereoisomers on cholesterol transfer. The results in Figure 2A indicate that the *S,S* and *R,R* isomers of LBPA have essentially the same effect as the *S,R* isomer, with all three species equivalently enhancing the rate of cholesterol transfer from NPC2 to membranes. We also examined whether the acyl chain moieties of LBPA modified its ability to enhance cholesterol transfer from NPC2. Figure 2B shows a comparison of vesicles containing 25 mol % dioleoyl (18:1) LBPA or dimyristoyl (14:0) LBPA. The rates of cholesterol transfer from NPC2 to vesicles containing dimyristoyl LBPA are consistently ~60% slower than the rates of transfer to dioleoyl LBPA vesicles, suggesting that the acyl chain length has an effect on the membrane structure and/or the NPC2–LBPA interaction, such that the oleoyl LBPA is more effective in enhancing the rate of cholesterol transfer from NPC2.

Comparison of Cholesterol and Fluorescent Sterols. For technical reasons noted in Materials and Methods, several

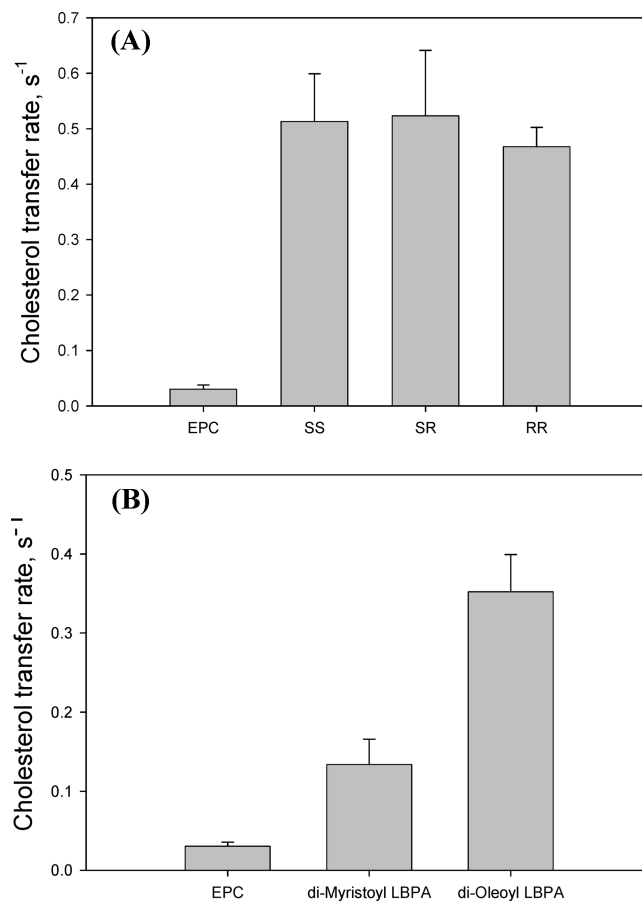


FIGURE 2: Effect of LBPA on cholesterol transfer from NPC2 to membranes. (A) Cholesterol (1 μM) transfer from 1 μM NPC2 to 50 μM SUV containing 100% EPC or with 25% EPC substituted with *S,R* LBPA, *S,S* LBPA, or *R,R* LBPA. (B) Cholesterol (1 μM) transfer from 1 μM NPC2 to 75% EPC/25% dioleoyl LBPA or 75% EPC/25% dimyristoyl LBPA SUV (50 μM).

experiments could not use the endogenous NPC2 tryptophan fluorescence to track the kinetics of cholesterol transfer. A number of fluorescent sterols have been used as cholesterol analogues in biophysical, biochemical, and cellular studies by many investigators (27–29, 37–39). We therefore compared directly the rate of cholesterol transfer from NPC2 to membranes with those of DHE and CTL, two fluorescent sterols which we wished to employ in model membrane studies. DHE has been shown to be a ligand for NPC2 (17), and we found that, as for cholesterol, binding of DHE or CTL resulted in quenching of NPC2 tryptophan fluorescence and, furthermore, was accompanied by an increase in DHE or cholestatrienol fluorescence (not shown). As shown in Figure 3A, the rates of transfer from NPC2 to membranes of both fluorescent sterols, DHE and CTL, are somewhat slower but basically similar to the transfer rates of cholesterol. We also compared the rates of spontaneous transfer of cholesterol between membranes using the RCA-based physical separation method, with the rate of DHE intermembrane transfer using the FRET assay, as described in Materials and Methods. Figure 3B shows that the spontaneous intermembrane transfer rates for cholesterol and DHE are very similar to each other, 0.00023 and 0.00028 s^{-1} , respectively. Thus, the fluorescent sterol analogues were used for several transfer experiments, as they appear to offer a reasonable approximation of the absolute rates of spontaneous and NPC2-mediated cholesterol transfer.

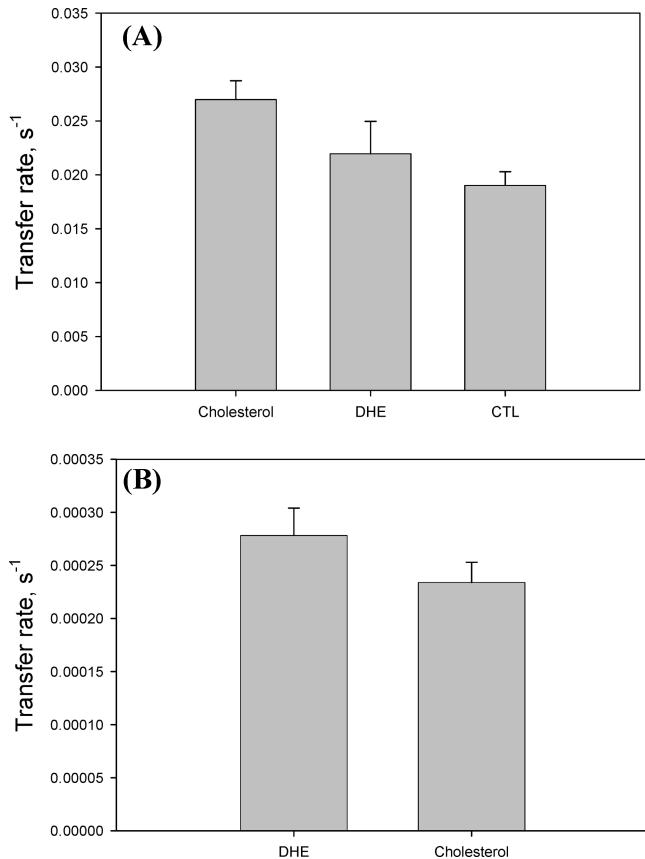


FIGURE 3: Comparison of cholesterol and fluorescent sterols. (A) Transfer of 1 μM cholesterol, DHE, or CTL from 1 μM NPC2 to 100% EPC SUV (50 μM). (B) Comparison of cholesterol and DHE intermembrane transfer rates. [3H]Cholesterol transfer from 65% EPC/25% cholesterol/10% lactosyl ceramide SUV (50 μM) to 100% EPC SUV (750 μM) and DHE transfer from 50 μM SUV (75/25 EPC/DHE) to 97% EPC/3% dansyl-PE SUV (750 μM).

Effect of the LBPA Antibody on Cholesterol Transfer. Kobayashi et al. have shown that treatment of BHK cells with anti-LBPA monoclonal antibody 6C4 results in an NPC-like phenotype, with cholesterol accumulation in late endosomes (40). To further examine the role of LBPA in NPC2-mediated cholesterol transfer, we monitored the transfer of CTL from 1 μM NPC2 to SUV containing 25% LBPA and incubated with 250 ng/mL 6C4. As shown in Figure 4, the anti-LBPA antibody decreased the CTL transfer rate by ~60% relative to that in LBPA vesicles without antibody incubation. Importantly, the antibody had no effect on the rate of CTL transfer to vesicles which contained no LBPA (Figure 4). It is likely that the antibody interferes with LBPA–NPC2 interactions, thereby diminishing the effect of LBPA in enhancing sterol transfer from NPC2 to membranes.

Effect of Dolichol and Gangliosides on Cholesterol Transfer from NPC2 to Membranes. We asked whether other lysosomal lipids, in addition to LBPA, might play a role in the cholesterol transfer processes. Dolichol is a polyisoprenoid compound found in high concentrations in lysosomes, as well as the Golgi and plasma membranes (41). Further, in NPC, not only cholesterol but also other lipids such as GM2 and GM3 gangliosides accumulate in the endosomal/lysosomal compartment (42–44). To examine the effect of dolichol and gangliosides on NPC2-dependent cholesterol transfer, 25% of dolichol, lactosyl ceramide, or

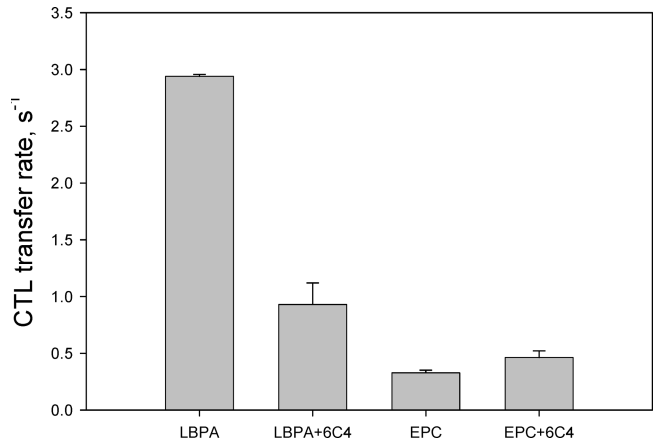


FIGURE 4: Effect of anti-LBPA antibody 6C4 on cholestatrienol transfer from NPC2 to model membranes. Transfer of 1 μM CTL from 1 μM NPC2 to 100% EPC or 25% LBPA SUV (250 μM) with or without incubation (60 min at 37 °C) with 250 ng/mL 6C4. Transfer was monitored at 25 °C.

Table 1: Rates of Cholesterol Transfer from NPC2 to SUV with Differing Compositions^a

SUV		SUV composition	
composition	transfer rate (s ⁻¹)		transfer rate (s ⁻¹)
EPC	0.028 ± 0.008	lactosyl ceramide	0.031±0.014
GM2	0.035 ± 0.002	dolichol	0.036±0.010
GM3	0.104 ± 0.032	LBPA	0.412±0.065

^a Transfer rates of 1 μM cholesterol from 1 μM NPC2 to 250 μM SUV composed of 100% EPC or 75% EPC with 25% indicated lipid (molar ratio). Average transfer rates are from five separate experiments ± the standard deviation.

gangliosides GM2 and GM3 were incorporated into EPC acceptor vesicles. Table 1 shows that the rate of cholesterol transfer from NPC2 was virtually unaffected by the presence of dolichol, lactosyl ceramide, and GM2. A 3-fold increase was observed with GM3-containing membranes, an effect almost 1 order of magnitude smaller than the effects observed for LBPA-containing membranes.

Intermembrane Transfer of DHE: Effects of Acceptor Membrane Concentration, Membrane Composition, and NPC2 Concentration. The spontaneous intermembrane transfer rate of DHE was very slow, in agreement with previous studies of cholesterol transfer between membranes (26, 45), and incorporation of LBPA into the membranes had essentially no effect on the DHE transfer rate (Figure 5). In contrast, addition of NPC2 resulted in a marked increase in the rates of intermembrane DHE transfer, with 40- and 280-fold increases for EPC membranes and LBPA-containing membranes, respectively, relative to rates in the absence of NPC2 (Figures 5 and 6A). Further, in the presence of NPC2, the DHE transfer rate increased as a function of increasing acceptor membrane concentration, whereas in the absence of NPC2, the DHE transfer rate did not change as more acceptor membrane was added (Figure 6B). Addition of increasing concentrations of NPC2 led to proportional increases in DHE transfer rates for both EPC membranes and LBPA-containing membranes, with rates of transfer ~10-fold faster than those for EPC membranes (Figure 6A).

FTIR Study of the Interaction of NPC2 with Membranes. The kinetics studies strongly suggest that the cholesterol transport properties of NPC2 involve protein–membrane interactions. We therefore used FTIR spectroscopy to

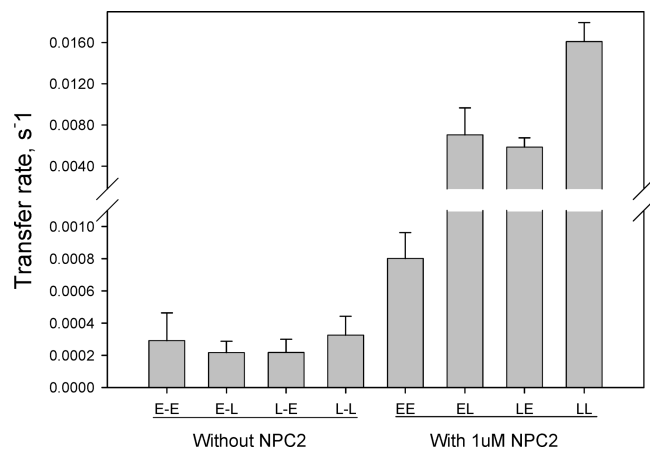


FIGURE 5: Transfer of DHE between membranes with or without NPC2. DHE transfer from 50 μM SUV with LBPA (L) or without LBPA (E) to 250 μM SUV with (L) or without LBPA (E), in the presence or absence of 1 μM NPC2.

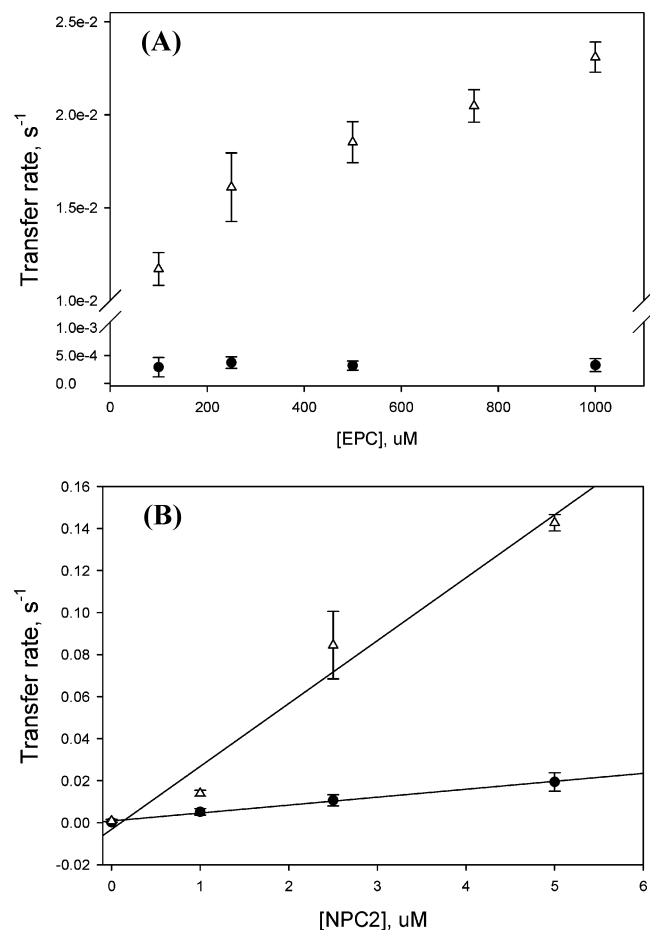


FIGURE 6: DHE transfer between membranes. (A) DHE transfer from 75% EPC/25% DHE SUV (50 μM) to 100% EPC SUV at different concentrations with (Δ) or without (\blacksquare) 1 μM NPC2. (B) DHE transfer from 50 μM SUV (50/25/25 EPC/LBPA/DHE) to 250 μM SUV with (Δ) or without (\blacksquare) LBPA in the presence of different concentrations of NPC2.

examine this hypothesis directly. Figure 7 shows the frequency dependence of the lipid acyl chain symmetric methylene stretching vibration ($\nu_{\text{sym}}, \text{CH}_2$) for POPS MLV as a function of temperature, in the presence and absence of NPC2 protein. The phase transition temperature of POPS, monitored by the shift of the lipid carbonyl stretching vibration, increased by $\sim 2^\circ\text{C}$ (from ~ 15 to $\sim 17^\circ\text{C}$) in the

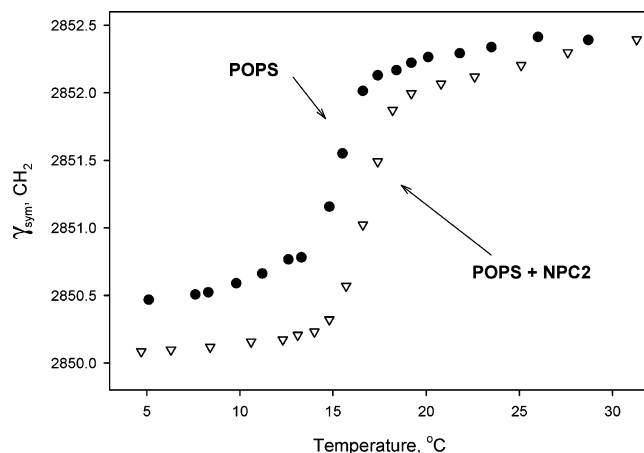


FIGURE 7: Frequencies of the $\nu_{\text{s}}(\text{CH}_2)$ vibrations ($\pm 0.1 \text{ cm}^{-1}$) vs temperature for 2.0 mg of pure POPS multilamellar vesicles and in the presence of NPC2 protein: pure POPS MLV (\blacksquare) and POPS with 0.5 mg of NPC2 (∇) (60/1 POPS/NPC2).

presence of NPC2, indicating an interaction between NPC2 protein and the phospholipid membranes and suggesting that NPC2 stabilizes the bilayer gel phase. The cooperativity of the lipid phase transition was not changed by NPC2 addition.

Tryptophan Fluorescence Detection of NPC2–Membrane Interactions. We examined the tryptophan fluorescence spectrum of NPC2 in the absence or presence of EPC SUV. For monoglycosylated NPC2, vesicle addition quenched the tryptophan signal by approximately 10–15% but did not change the peak position of the tryptophan emission (Figure 8A). For diglycosylated NPC2, tryptophan quenching was also observed, and the emission peak in the absence of SUV (323 nm) shifted to a lower wavelength (317 nm) when EPC vesicles were added (Figure 8B), suggesting an increase in the hydrophobicity of the environment of one or more tryptophan residues and, thereby, indicating an interaction between NPC2 with the phospholipid membranes. Membranes containing 25 mol % LBPA showed the same modifications in tryptophan emission (not shown). Titration of NPC2 with increasing concentrations of phosphatidylcholine vesicles showed that the tryptophan quenching plateaued at a PL/NPC2 molar ratio of approximately 70/1, with an apparent K_d value for phospholipid binding of 30 μM (Figure 8C).

DISCUSSION

Accumulation of cholesterol in the late endosome/lysosome compartment is a major cellular hallmark of Niemann–Pick disease type C. The NPC2 protein, initially identified as a cholesterol binding protein, HE1, in epididymal fluid, was shown to be targeted to the lysosomal compartment via the mannose 6-phosphate receptor (6). The recently determined X-ray crystal structure of holo-NPC2 demonstrates that the ligand binding site closely surrounds the steroid nucleus, with the side chain buried inside the protein interior (46). In this work, we studied the absolute rates and mechanism of cholesterol transfer from model phospholipid membranes to NPC2 and, further, examined the effect of NPC2 on sterol transfer between phospholipid membranes. For many of the experiments, the intrinsic tryptophan fluorescence of NPC2 was used to monitor the transfer of unmodified cholesterol from phospholipid membranes to the

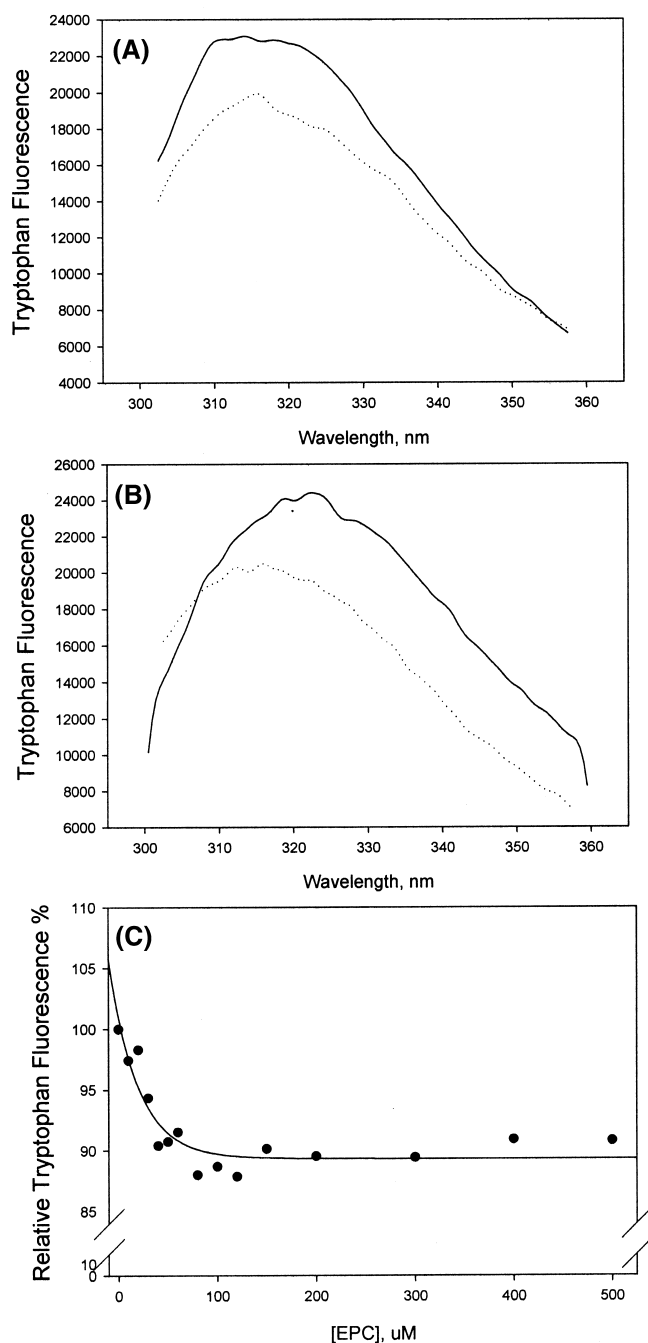


FIGURE 8: Tryptophan fluorescence of NPC2 in the absence (—) or presence (•••) of EPC SUV: (A) 5 μ M monoglycosylated NPC2 with 500 μ M EPC SUV and (B) 5 μ M diglycosylated NPC2 with 500 μ M EPC SUV. (C) Monoglycosylated NPC2 (10 μ M) was incubated with increasing concentrations of EPC SUV, and the peak tryptophan emission was obtained at ≥ 5 min. Results are representative of two separate experiments. The excitation wavelength was 280 nm.

protein. All studies were performed at acidic pH; thus, the rates obtained are likely to be physiologically relevant to endosomal/lysosomal cholesterol transport.

To determine the mechanism of the membrane-to-NPC2 cholesterol transfer process, we monitored the relationship between the cholesterol transfer rates and the acceptor NPC2 concentration. If transfer occurs through aqueous phase diffusion, the rates of transfer from membranes should be constant at different acceptor concentrations, as the rate of dissociation of cholesterol from membranes would be the

limiting step (22, 23, 26). We observed, instead, that the transfer rates increased directly with NPC2 concentration; this increase is likely secondary to the increased frequency of NPC2–membrane interactions, supporting a collisional mechanism of cholesterol transfer from membranes to NPC2. Rates of cholesterol transfer to NPC2 were much faster from membranes containing negatively charged phospholipids than from zwitterionic vesicles. This, too, suggests that cholesterol transfer from membranes to NPC2 involves protein–membrane interactions. Further, the results indicate the likelihood of electrostatic interactions between NPC2 and membranes, which are affected by protein surface properties and membrane structure and composition.

As we initially showed for cholesterol transfer from NPC2 to membranes (20), LBPA-containing membranes exhibit the highest rates of cholesterol transfer to NPC2. Recently, Babalola et al. used an avidin and biotin-based liposome separation method to examine cholesterol transfer between membranes and found that the amount of transfer was strongly stimulated by incorporation of LBPA (47). LBPA is a unique lipid present at high concentrations in the endosome/lysosome compartment, and Kobayashi et al. showed that treatment of BHK cells with an anti-LBPA antibody, 6C4, resulted in cholesterol accumulation and an NPC-like phenotype (40). We found that the 6C4 anti-LBPA antibody substantially weakened the enhancing effect of LBPA on cholesterol transfer by NPC2, suggesting that the cholesterol accumulation in 6C4-treated BHK cells was due, at least in part, to a defect in NPC2-mediated cholesterol transport. Interestingly, LBPA was also shown to increase the extent of glycosphingolipid hydrolysis in an *in vitro* membrane system, acting in concert with sphingolipid activator proteins (48, 49). Taken together, the results suggest that the high level of LBPA present in endosomal/lysosomal membranes is likely to be functionally necessary for the normal trafficking and enzymatic functions of this compartment.

The three dioleoyl LBPA isomers examined [*S,S* (3,3'), *S,R* (3,1'), and *R,R* (1,1')] displayed similar effects in enhancing the rate of cholesterol transport *in vitro*. While the *S,S* (3,3') isomer is considered to be biologically stable in cells (33–36), it was also suggested that 2,2'-diacyl LBPA is dominant in cells, with a dynamic equilibrium between 2,2' and 3,3' (*S,S'*) isomers, but that the 2,2' isomer may be thermodynamically unstable (33, 50). Membranes containing dioleoyl LBPA, which was shown to be the major LBPA species present in BHK cells (40), exhibited a faster rate of cholesterol transfer from NPC2 to membranes than membranes containing dimyristoyl LBPA. We hypothesize that LDL-derived cholesterol accumulates in internal lysosomal membranes, perhaps in LBPA-enriched lateral domains (50–52), and that NPC2 protein may remove cholesterol from these sites by a mechanism involving a direct interaction with these domains. As discussed below, our FTIR results suggest that NPC2 does not access the membrane bilayer acyl chain core but rather interacts at the membrane surface. Thus, the LBPA acyl chain composition is likely to affect the configuration of its polar headgroup, with shorter chains resulting in a configuration with weakened interaction with NPC2. In contrast, the stereochemistry of LBPA does not appear to affect its role in membranes as a putative NPC2 interaction site, suggesting that the structural effects of the isomers at the membrane surface are similar. A direct

comparison between 100% 1,1' (*R,R*) and 3,1' (*S,R*) dimyristoyl LBPA membranes indicated that both form a compact configuration with bent headgroups and closely positioned acyl chains. However, since the capacity for hydrogen bonding was found to be greater for the 3,1' (*S,R*) species and, hence, it showed tighter packing than the 1,1' (*R,R*) isomer (33), we had expected to find differential effects on cholesterol transport by NPC2. The similar effects for the three LBPA stereoisomers that were observed here could have resulted from use of the dioleoyl LBPA species, which may prevent the packing differences found using disaturated LBPA.

The membrane interaction properties of NPC2, suggested by the cholesterol kinetics results, were directly confirmed using two approaches. Titration of NPC2 with SUV resulted in saturable quenching of the tryptophan emission, suggesting protein–membrane interactions. Further, when SUV were incubated with diglycosylated NPC2, the tryptophan emission spectrum displayed a blue shift. This is generally understood as demonstrating an increase in the hydrophobicity of the environment immediately surrounding the emitting tryptophan(s). While one interpretation is that one or both of the two Trp residues on NPC2 are localized in the hydrophobic membrane bilayer, it is also possible that NPC2–membrane interaction causes a conformational change in the protein, which leads to an increased hydrophobicity of the environment surrounding the tryptophan. In either case, the blue shift likely demonstrates an interaction between NPC2 and the membrane. It is not clear why the monoglycosylated NPC2 did not demonstrate a blue-shifted tryptophan spectrum upon membrane incubation; however, given that fluorescence was quenched, that we observed collisional kinetics for both mono- and diglycosylated NPC2 (20), and that the FTIR studies presented here were conducted using the monoglycosylated protein, it is very likely that it, too, is membrane-interactive.

The melting temperature of POPS membranes increased in the presence of NPC2, but the slope of the lipid phase transition was essentially unaltered, suggesting an extrinsic interaction of NPC2 at the membrane surface without penetration into the bilayer acyl chain region. The increase in T_m indicates that binding of NPC2 leads to a stabilization of the gel phase and an increased level of ordering of the membrane phospholipids. Such behavior has been observed for a number of other surface-active proteins, including pediosin (53) and nisin (54). It is possible that the electrostatic interactions between NPC2 and acidic phospholipid headgroups may be stronger than lipid–lipid interactions (54). Promotion of a gel phase configuration rather than a fluid phase might destabilize the membrane near the site of interaction, lowering the free energy for dissociation of cholesterol from the membrane. While these studies imply a long-term interaction between NPC2 and membranes, both used apo NPC2, and we hypothesize that apo and holo NPC2, which have modest conformational differences (46), may interact differently with membranes, leading to binding or release from the membrane surface.

As both cholesterol and glycolipids build up in NPC cells, there has been discussion about whether the accumulation of cholesterol is the initial defect or whether glycosphingolipids such as gangliosides or lactosoyl ceramides accumulate first (55, 56). For example, activities of the

hydrolytic enzymes sphingomyelinase and glycosylceramidase are reduced in NPC cells (57–59), which could contribute to glycosphingolipid accumulation. While the mechanism remains unclear, it has been suggested to be due to enzyme misfolding or mislocalization, or to allosteric regulation of enzyme activities by cholesterol (57, 60). Our results show that incorporation of GM2 and lactosoyl ceramide has no effect on cholesterol transfer rates, while GM3 has a relatively small effect, indirectly supporting the suggestion that cholesterol trafficking is the primary defect in NPC. Since both GM2 and GM3 contain the single *N*-acetylneuraminic acid (NANA) group, the differences found between the two gangliosides are not likely due to differences in net negative charge. The absence of the uncharged *N*-acetylgalactosamine residue that is present in GM2, however, may make the NANA carboxylate group on GM3 more accessible for membrane–NPC2 interaction.

We also examined whether dolichol, a polyisoprenoid compound abundantly present in lysosomes as well as other subcellular compartments (41), might impact the rates of cholesterol transport by NPC2. One report had indicated that NPC2 interacted with the dolichol synthetic enzyme dehydrodolichol diphosphate synthase (61); however, it is worth noting that the enzymes of dolichol synthesis are not localized in the same subcellular compartment as NPC2 (41). No effect of dolichol on cholesterol transfer kinetics was observed.

To model the transfer of LDL-derived cholesterol, much of which is likely intercalated in inner lysosomal lamellae, to the limiting organellar membrane, we developed a fluorescence resonance energy transfer (FRET) assay using the fluorescent analogue DHE as the donor fluorophore, incorporated into donor vesicles, and the dansyl moiety of dansyl-PE as the acceptor fluorophore, incorporated into acceptor membranes. Previous studies of intermembrane cholesterol transfer have been conducted by physically separating the donor and acceptor vesicles containing radio-labeled cholesterol (26, 47, 62). The main shortcoming of the separation approaches is that they limit the resolution of the time scales that can be examined to minutes or longer. For spontaneous transfer, which is very slow, we were able to use a physical separation method and obtained intermembrane transfer rates that were on the order of hours, in agreement with previous literature (26, 62). When NPC2 was added, however, only equilibrium distributions of cholesterol were able to be resolved because transfer was so rapid. Therefore, we designed the FRET method to monitor fluorescent sterol transfer between membranes and showed that the spontaneous intermembrane transfer rates for cholesterol and DHE were basically similar, with DHE transfer ~12% faster than cholesterol transfer. Using the FRET assay, we can monitor sterol transfer in real time through the decrease in DHE fluorescence or the increase in dansyl fluorescence, with a stopped-flow mixing fluorescence spectrometer. The results show that the DHE intermembrane transfer rates in the presence of NPC2 are much faster than the rates of spontaneous transfer and are markedly affected by membrane composition and concentration, supporting a collisional mechanism for sterol transfer between membranes in the presence of NPC2. In the absence of the protein, DHE transfer proceeded via aqueous diffusion, in agreement with previous studies (26, 62), and the slow transfer presumably

reflects the rate of dissociation of DHE from the membrane into buffer. The dramatic, >200-fold increase in intermembrane DHE transfer rates with LBPA in the donor membranes, seen only in the presence of NPC2, further supports the importance of this lipid in sterol transfer by NPC2.

Taken together, these results suggest that NPC2 functions as an intracellular cholesterol transporter, playing an important role in the egress of cholesterol from the endosomal/lysosomal compartment. Mutations or the absence of NPC2 could thus trigger the cholesterol accumulation phenotype of NPC2 disease. The presence of LBPA in the lysosome may be important for NPC2–membrane interactions, perhaps functioning as a localization site for increasing the efficiency of cholesterol transfer by NPC2. A coordinated action of NPC1 and NPC2 is suggested by the similarity in human disease presentation and murine phenotypes caused by depletion or mutation of either gene (8, 63). Thus far, specific interactions between the two proteins have not been reported. While such protein–protein interactions may yet be demonstrated, these results suggest the possibility of a model in which NPC2 could be acting to efficiently deliver cholesterol to NPC1-containing membranes, with NPC1 then binding the sterol following its rapid lateral diffusion in the plane of the membrane.

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