NPC2, the Protein Deficient in Niemann-Pick C2 Disease, Consists of Multiple Glycoforms That Bind a Variety of Sterols

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Niemann-Pick C disease is a fatal neurodegenerative disorder characterized by an endolysosomal accumulation of cholesterol and other lipids. One form of the disease is caused by a deficiency in NPC2, a soluble lysosomal glycoprotein that binds cholesterol. To better understand the biological function of NPC2 and how its deficiency results in disease, we have characterized the structural and functional properties of recombinant human protein. Highly purified NPC2 consists of a complex mixture of glycosylated isoforms, similar to that observed in human brain autopsy specimens. Mass spectrometric analysis revealed that of the three potential N-linked glycosylation sites present in the mature protein, Asn-19 is not utilized; Asn-39 is linked to an endoglycosidase H (Endo H)-sensitive oligosaccharide, and Asn-116 is variably utilized, either being unmodified or linked to Endo H-sensitive or Endo H-resistant oligosaccharides. All glycoforms are endocytosed and ameliorate the cholesterol storage phenotype of NPC2-deficient fibroblasts. In addition, the purified preparation contains a mixture of both free and lipid-bound protein. All glycoforms bind cholesterol, and sterol binding to NPC2 significantly alters its behavior upon lipidation. Free and lipid-bound protein. All glycoforms are endocytosed and ameliorate the cholesterol storage phenotype of NPC2-deficient fibroblasts. In addition, the purified preparation contains a mixture of both free and lipid-bound protein. All glycoforms bind cholesterol, and sterol binding to NPC2 significantly alters its behavior upon lipidation.

Cholesterol is important for a wide variety of physiological processes, including serving as a major component of lipid bilayers and droplets and as a precursor in steroid hormone biosynthesis. One of the major pathways by which cholesterol enters cells is through receptor-mediated endocytosis of LDL particles. The LDL is delivered to lysosomes where cholesteryl esters are hydrolyzed by acid lipase and the liberated free cholesterol is then transported to other subcellular sites for further utilization and also for feedback regulation of cholesterol biosynthesis and LDL uptake. Although many of the steps involved in this pathway are well characterized, the mechanism by which free cholesterol exits the lysosome is poorly understood.

Studies of two hereditary childhood neurodegenerative disorders with similar phenotypes, Niemann-Pick C (NPC) type 1 and type 2 diseases, have begun to provide insights into lysosomal cholesterol transport. On a cellular level, both diseases are characterized by lysosomal storage of cholesterol and other lipids, including sphingomyelin, glycolipids, and phospholipids. Fibroblasts from individuals with NPC disease accumulate LDL-derived cholesterol in lysosomes and are impaired in homeostatic responses that normally follow LDL uptake (1, 2). The gene defective in NPC type 1 disease, NPC1, encodes an ~1278-amino acid protein containing 13 transmembrane domains and a putative sterol-sensing domain (3). The NPC1 protein has primary sequence similarity to the bacterial RND permease family (4) and resides in endolysosomal membranes. Although lysosomal cholesterol accumulates in NPC type 1 disease, there is controversy regarding the direct involvement of NPC1 in cholesterol transport (5–7). The gene defective in NPC type 2 disease, NPC2, encodes a 151-amino acid protein with a cleaved 19-amino acid signal sequence that is localized to the lysosome (8). This protein, which is also present at high levels in mammalian epididymal fluid (9) and bovine milk (10), binds cholesterol (11) and functions as a cholesterol transfer protein in vitro (12, 13). Structural features of NPC2 important for sterol binding have been investigated using crystallographic (14) and mutagenesis (15) approaches. Genetic studies...
using NPC1 and NPC2 mutant mice suggest that both proteins function in a common pathway in lysosomal lipid transport (16).

In this study, as a step toward understanding the role of NPC2 in normal and disease processes, we have characterized biochemical and ligand binding properties of functional recombinant human NPC2 produced using a Chinese hamster ovary cell system. The preparation consists of different NPC2 glycoforms that are capable of binding cholesterol, and we find that binding of some ligands induces a marked change in the chromatographic properties of NPC2. Based on this observation, we have developed a new chromatography-based binding assay and have applied this to evaluate ligand structure-activity relationships, to identify potential physiologically relevant ligands, and to evaluate NPC2 as the target of drugs implicated in altered cholesterol transport. Our results suggest that NPC2 may play a role in the cellular transport of a variety of sterols in addition to cholesterol, an observation that may be important in understanding the pathogenic events that take place in the absence of this protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cholesterol was obtained from Nu-Chek (Elysian, MN). Oleate, palmitate, oleoyl lysobisphosphatidic acid (LBPA, also named bis(monacylglycerol)phosphate), and 25-hydroxycholesterol were from Avanti Polar Lipids (Alabaster, AL). 5α-Cholest-3-β-ol, 5β-cholest-3α-ol, and 24-hydroxycholesterol were obtained from Steraloids (Newport, RI). Other sterols, lipids, and drugs were from Sigma. Endoglycosidase H-maltose-binding protein binding protein (Endo H), subsequently referred to as Endo H) and PNGase F were from New England Biolabs (Beverly, MA). Ten percent bis-tris NuPAGE gels were purchased from Invitrogen. Sypro ruby protein gel stain was obtained from Molecular Probes (Eugene, OR). Ezetimibe was obtained by extracting Zetia tablets (Merck/Schering-Plough) with chloroform/methanol followed by preparative TLC.

**Human NPC2 Purification**—Chinese hamster ovary cells were transfected with the human NPC2 cDNA subcloned into the expression vector pMSXND (17), which contains a cDNA expression cassette driven by the mouse metallothionein promoter, a neo expression cassette, and a mouse dihydrofolate reductase expression cassette. Cells were selected with G418 and resistant clones challenged with increasing concentrations of methotrexate. One clone that proliferated in the presence of 800 μM methotrexate, designated NPC2-800 number 7, was used for production of recombinant protein. Serum-free conditioned medium was collected as described (18), except that 50 μM ZnCl2 was included in the medium. For some preparations, cells were cultured in 1050-cm2 roller bottles containing 260 ml of medium at the American Cell Culture Center (Minneapolis, MN). In a typical purification, conditioned media from 33 roller bottles (9 liters) were centrifuged at 5600 × g for 20 min and concentrated to 400 ml using a 5-kDa cut-off tangential flow filtration membrane (CDUF002 LC, Millipore, Bedford, MA). All procedures were conducted at 4 °C unless stated otherwise. The membrane was washed three times with 100 ml of PBS, and the pooled retentate and washes were equilibrated into 100 mM NaCl, 20 mM Tris, pH 7.0, by diafiltration using a stirred cell equipped with a YM10 membrane (Millipore). The material was applied to a DE52 column (5 × 30 cm; Whatman) and an SP-Sepharose C-25 column (5 × 30 cm; GE Healthcare) attached in series to partially deplete the mixture of contaminating proteins. This ion-exchange depletion step was conducted at room temperature. The flow-through was concentrated and buffer exchanged into 12.5 mM ammonium acetate buffer, pH 4.5, using ultrafiltration. NPC2 was purified, and different glycoforms were partially resolved by chromatography on a Mono S 10/100GL column (GE Healthcare) that was eluted using a linear 20-column volume gradient of 12.5 to 512.5 mM ammonium acetate at a 2 ml/min flow rate. To improve the resolution, multiple runs, corresponding to material obtained from ~3 roller bottles, were performed for each preparation. Chromatography was conducted using an Akta Explorer high pressure liquid chromatograph (GE Healthcare). Fractions were pooled to encompass the major peaks described in Fig. 1.

**Glycosylation Site Analysis**—Indicated ion-exchange fractions were incubated overnight with Endo H at room temperature in 50 mM sodium citrate, pH 5.5, under non-denaturing conditions. Parallel samples were denatured in 0.5% SDS, 1% β-mercaptoethanol at 100 °C for 10 min followed by addition of 1% Nonidet P-40 and then incubated overnight with PNGase F at 37 °C in 50 mM sodium phosphate buffer, pH 7.5. Samples were fractionated by SDS-PAGE under reducing conditions, visualized by Sypro ruby and Coomassie staining, and bands of interest excised from the gel. Protein in gel slices was digested with modified trypsin (Promega, Madison, WI) according to the method used by the W. M. Keck Biotechnology Resource Laboratory. Samples were incubated at 37 °C for 24 h and then stored at 4 °C. Digests were mixed 1:1 with a saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 10 mM ammonium phosphate mono-basic or sinapinic acid in 50% acetonitrile, 0.1% trifluoroacetic acid containing internal peptide standards (bradykinin, MH+ 1060.5692 Da; ACTH clip 18–39, MH+ 2465.1989 Da; ACTH clip 7–38, MH+ 3657.9294 Da; Sigma). Peptides were analyzed using a Voyager DE Pro mass spectrometer (Applied Biosystems, Framingham, MA) operated in the reflector mode.

**Analytical Mono S Chromatography**—Samples were brought to a concentration of 1 mg/ml (68 μm) protein in 12.5 ammonium acetate, pH 4.5, using a Centricon YM10. Typically, 50 μg of protein was applied to a Mono S PC 1.6/5 column (GE Healthcare) and eluted using a linear 60-column volume (6 ml) gradient of 12.5 to 512.5 mM ammonium acetate at a 0.2 ml/min flow rate.

**Lipid Analysis and Delipidation**—The purified NPC2 fractions were pooled and concentrated to 10 mg/ml prior to organic solvent extraction. In some cases, the solution was mixed with 2 volumes of acetone, 1 mM HCl to extract bound lipids and to precipitate delipidated protein for further use. In other cases, lipids were isolated by extracting the solution twice with an equal volume of chloroform/methanol (2:1 v/v). Extracts were dried using a stream of N2 gas, and the material was either analyzed by gas chromatography (GC) with flame ionization following trimethylsilylation as described previously.
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(16) or resuspended into a small volume of chloroform/methanol (2:1 v/v) for further analysis by TLC. Extracts or standards (cholesterol oleate, triolein, oleate, sn-1,2-diolein, monoolein, cholesterol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid, lyso phosphatidylcholine, and LBPA) were visualized using iodine vapor after separation using 10 × 20-cm high performance TLC plates (Silica Gel G; Whatman, Hillsboro, OR) and the two-step solvent development method described previously (19).

Cholesterol Clearance Experiments—Fibroblasts were cultured at 37 °C in a humidified 5% CO2 incubator on 16-well glass-bottom slides (Nunc, Naperville, IL) in RPMI/glutamax-1 medium (Invitrogen) supplemented with 1% penicillin/streptomycin and 15% fetal bovine serum. Cells were refed with fresh media containing delipidated NPC2 (100 nM final concentration of protein isolated from the indicated peak) in the presence and absence of 10 mM mannose 6-phosphate. After 24 h, cells were brought to room temperature, washed three times with PBS for 5 min each, fixed with 3% paraformaldehyde in PBS for 30 min, washed three times with PBS, and stained with 50 μg/ml filipin complex (Sigma) and 1 μM SYTOX Green (Invitrogen) in PBS for 60 min. After washing the cells three times with PBS, wells were removed, and the slides were mounted with coverslips in FluoroGuard antifade reagent (Biotra). The slides were viewed under an Olympus-IX81 spinning-disk confocal microscope equipped with X-Cite 120 lamp (EXFO) and Cooke Sensicam QE camera (Applied Scientific Instrumentation). SYTOX Green images were captured using 492/18 nm excitation and 535/40 nm emission filters with a multil wavelength dichroic mirror. Visualization of this nuclear counterstain was used for focusing the microscope prior to visualization of filipin images using 360/40 nm excitation and 465/30 nm emission filters.

Binding Experiments—Delipidated NPC2 in 12.5 mM ammonium acetate, pH 4.5, was incubated with 1.5 mol eq of the compound of interest for 30 min at room temperature and analyzed by analytical cation-exchange chromatography as already described. For calculation of binding stoichiometry, we determined the concentration of DHE from its absorbance at 328 nm and calculated its contribution toward absorbance at 280 nm using its reported extinction coefficients of 1.055 × 104 and 1.686 × 104 M−1 cm−1 at 328 and 280 nm, respectively (20, 21). Control experiments adding DHE directly to methanol or to excess NPC2 in aqueous solution indicated that free DHE in organic solvent and DHE associated with NPC2 had similar extinction coefficients at 328 nm. The concentration of NPC2 was determined using the extinction coefficient of 1.686 × 104 M−1 cm−1 at 280 nm obtained using the Prot Parameter tool on the Expasy website after correcting for the contribution of DHE. The ratio of DHE/NPC2 was essentially constant in the region of co-elution for all traces analyzed.

Competition Binding Assay—Conditions were as described above for the direct binding assay, except that NPC2 (68 μM) was incubated with test ligand (102 μM) for 30 min followed by incubation with DHE (80 μM, 1 μM of a 4 mM solution in ethanol added to the 50-μl reaction mixture). The incubation was continued for another 30 min before conducting analytical cation-exchange chromatography. The integrated peak area of the absorbance at 328 nm was used to estimate DHE associated with NPC2, using three or four independent determinations for each test ligand.

Analysis of Mouse Liver Sterols—Procedures involving live animals were conducted in compliance with approved protocols from the Institutional Animal Care and Use Committee. Genotyping was conducted as described previously (16) on mice that had been backcrossed for 10 generations into a BALB/c background. 79 day-old female mice (genotypes NPC1(+/-)NPC2(+/-)) and NPC1(+/-)NPC2(−/-) were anesthetized with 98 mg/ml sodium pentobarbital containing 12.5 mg/ml phenytoin (1:4 dilution of Euthasol; Delmarva Laboratories, Midlothian, VA) and exsanguinated by transcardiac perfusion with saline. Livers were removed and stored at −80 °C. Analysis of cholesterol, 7β-hydroxycholesterol, 24S-hydroxycholesterol, and 27-hydroxycholesterol was conducted as described previously (22) with the following modifications: coprostanol (50 μg), the anti-oxidant BHT (20 μg), and [1H3]7β-hydroxycholesterol (0.25 μg) (a gift from Dr. Ashok K. Batta) were added to the mixture with 50–100 mg of minced tissue. The mixture was hydrolyzed at room temperature for 1 h in 1 n ethanolic NaOH, and the sterols were extracted exhaustively with hexane. One-tenth of the material was removed and used for cholesterol measurement by GC. The remainder was dried, redisolved in 1 ml of toluene, and applied to a 2-ml Sep-Pak silica cartridge (Waters) that had been preconditioned with 1 ml of hexane. Monohydroxylated steroids (e.g. cholesterol) were removed by washing the cartridge with 8 ml of hexane/propanol (98:2 v/v), and the polyhydroxylated sterols were recovered by flushing the cartridges with 5 ml of hexane/propanol (70:30 v/v) and collecting the eluant. The residue was dried, silylated, and subjected to selected ion monitoring GC-MS using a HP5792A mass detector interfaced to a HP6890 capillary GC (Agilent Technologies, Palo Alto, CA). Monitoring of the positive ions was carried out at m/z 413 (24S-hydroxycholesterol; M-90-43), m/z 456 (27- and 7β-hydroxycholesterol; M-90), and m/z 460 ([1H4]7β-hydroxycholesterol; M-90). Commercial standards of the three oxysterols (Steraloids, Newport, RI) were used to calibrate retention times and system response.

RESULTS

Purification and Glycosylation Analysis of NPC2—Purification of recombinant human NPC2 protein produced using a Chinese hamster ovary cell expression system yielded six major peaks following cation-exchange chromatography at pH 4.5 (Fig. 1). The total recovery of NPC2 protein in all peaks was ~6–7 mg per liter of conditioned medium. Analysis using SDS-PAGE (Fig. 1, inset) and linear MALDI-TOF MS (data not
shown) indicated that peak 1 consisted of a heterogeneous mixture of isoforms with an average molecular mass of 18.9 kDa. Peaks 2–4 consisted of two principal isoforms, each heterogeneous, with average molecular masses of 18.5 and 17.7 kDa. Peaks 5 and 6 consisted of a heterogeneous isoform with an average molecular mass of 16.3 kDa. (Note that NPC2 migrates anomalously by SDS-PAGE. Thus, the molecular weights listed here and in Fig. 1 that were obtained by MS analysis are not in accordance with the predicted molecular weights obtained by comparison with the standards shown in Fig. 2.)

TABLE 1
| Peak | Asn-19 | Asn-39 | Asn-116 |
|------|--------|--------|---------|
| 1    | M      | C      |         |
| 2    | M      | M > C  |         |
| 3    | M      | C > M  |         |
| 4    | M      | M > C  |         |
| 5    | M      |        |         |
| 6    | M      |        |         |

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PNGase F digestion collapsed all isoforms to a uniform mass of 15 kDa, indicating that the heterogeneity observed by SDS-PAGE was because of differences in glycosylation (Fig. 2). This was further investigated using a combination of glycosidase digestion and peptide mass fingerprinting. Our conclusions regarding the glycosylation state of the three potential N-linked glycosylation sequences on NPC2 are summarized in Table 1, and the evidence for these assignments is detailed below.

Our strategy to identify the precise sites and nature of the modifications was based on the following. Human NPC2 contains three asparagines at residues 19, 39, and 116 (numbering based on the sequence of the mature protein) in the context of a potential glycosylation sequence NX(S/T). These residues are encompassed by the theoretical tryptic peptides 17–32, 33–49,
and 116–132 that have calculated monoisotopic MH$^+$ ions of 1843.9, 1859.9, and 2070.0 Da, respectively. If no post-translational modifications are present, PNGase F or Endo H treatment would have no effect on these peptides, and peptides of the predicted masses should be observed. If a potential glycosylation site is occupied, this would shift the mass upward considerably, resulting in the disappearance of the predicted peptide from the observed mass window. However, deglycosylation with PNGase F removes essentially all mammalian $N$-linked oligosaccharides and converts the oligosaccharide-linked Asn to Asp. Thus, a PNGase F-treated glycopeptide will have a mass 1 Da greater than that of the corresponding unmodified peptide. Similarly, if an $N$-linked glycan is susceptible to Endo H, which cleaves high mannose and some hybrid oligosaccharides between the inner two GlcNAc residues, digestion of a glycosylated peptide would yield a GlcNAc-linked Asn, which has a mass 203.1 Da greater than the corresponding unmodified peptide. Thus, by comparing the changes in the migration of the glycosidase-treated NPC2 isoforms following PNGase F and Endo H treatment, and then further digesting the proteins with trypsin and analyzing the peptides using mass spectrometry (MS), it is possible to determine whether a given Asn is glycosylated and also the nature of the modification.

SDS-PAGE analysis revealed that NPC2 isoforms present in peak 1 shifted slightly following Endo H digestion and markedly following PNGase F digestion (Fig. 2). MS analysis of in-gel tryptic digests of all bands derived from untreated, PNGase F-treated, and Endo H-treated peak 1 revealed that the peptide encompassing Asn-19 was present with the predicted MH$^+$ of 1843.9 Da, demonstrating that this potential glycosylation site is not utilized (Fig. 3). In contrast, the unmodified peptide encompassing Asn-39 was not detected, whereas PNGase F and Endo H treatments generated the appropriate MH$^+$ + 1 and MH$^+$ + 203.1 fragments of 1860.9 and 2063.0 Da, respectively, demonstrating that Asn-39 was modified with an Endo H-sensitive oligosaccharide (Fig. 4).

For Asn-116, an ion corresponding to the unmodified tryptic peptide 116–132 was not found (Fig. 5A) in any spectra, but the predicted MH$^+$ + 1 of 2071.0 Da appeared in the PNGase F-treated sample (Fig. 5B). PNGase F-treated peak 1 also contained a 3424.7-Da singly charged ion that corresponds to the MH$^+$ + 1 of peptide 105–132, an incompletely cleaved tryptic fragment that encompasses Asn-116 (Fig. 5D). The unmodified peptide 105–132 was not detected (Fig. 5C). Finally, in the Endo H-treated peak 1 tryptic digests, the predicted MH$^+$ + 203.1 peaks corresponding to GlcNAc-modified peptides 105–132 or 116–132 were not detected (Fig. 6A and data not shown). This indicates that Asn-116 on the NPC2 isoform present in peak 1 is linked to an Endo H-resistant oligosaccharide.

The experiment shown in Fig. 2 reveals that qualitatively peaks 2–4 have similar appearance to each other. The protein present in each undigested peak runs as a doublet by SDS-PAGE analysis. Endo H treatment produced two bands, one with a small shift and the other with a marked shift. The lower band was most prominent for peaks 2 and 4, and the upper band was most prominent for peak 3. PNGase F generated a single band, migrating identically to the band produced by PNGase F treatment of peak 1. Peptide mass fingerprinting analysis of the undigested and PNGase F-di-
gested bands gave results identical to those found for peak 1, demonstrating that Asn-39 and Asn-116 but not Asn-19 were glycosylated (see Figs. 3 and 4). Peptide mass fingerprinting of the upper band generated by Endo H treatment of peaks 2–4 gave results identical to the corresponding peak 1 specimen (Fig. 5). In contrast, analysis of the lower band generated by Endo H treatment of peaks 2–4 revealed that in addition to finding the predicted MH$^+$ + 203.1 peak for the tryptic fragment encompassing Asn-39, we also found a 3626.8-Da ion that corresponded to an incompletely digested tryptic fragment 105–132 that was modified with GlcNAc (Fig. 6B). Interestingly, we did not observe the MH$^+$ + 203.1 ion for peptide 116–132, suggesting that modification of Asn-116 by GlcNAc hindered trypsin cleavage at Lys-115. Nonetheless, these results clearly demonstrate that peaks 2–4 contain NPC2 isoforms with either Endo H-sensitive oligosaccharides at Asn-39 and Asn-116 (major form in peaks 2 and 4 and minor form in peak 3) or with an Endo H-resistant oligosaccharide at Asn-39 and an Endo H-resistant oligosaccharide at Asn-116 (major form in peak 3 and minor forms in peaks 2 and 4).

Peaks 5 and 6 give essentially identical results following glycosidase digestion, eliciting a small shift with both Endo H and PNGase F (Fig. 2). Peptide mass fingerprinting reveals that, as in peaks 1–4, Asn-19 is not modified (Fig. 3), whereas Asn-39 contains an Endo H-sensitive oligosaccharide (Fig. 4). Unlike peaks 1–4, the expected monoisotopic MH$^+$ ions of 2070.0 and 3423.7 for fragments 116–132 and 105–132 were detected for the undigested and glycosidase-digested samples (Fig. 7), demonstrating that Asn-116 is not modified. Thus, peaks 5 and 6 contain a single Endo H-sensitive oligosaccharide at Asn-39.

**Purified NPC2 Consists of Both Free and Ligand-bound Forms—** Although the different NPC2 isoforms can be partially attributed to heterogeneity in glycosylation, further experimentation revealed an additional contributing factor. In the course of developing a sterol-binding assay for NPC2 based on the increase in fluorescence of DHE upon binding to protein, we found that when assaying equivalent amounts of protein, peak 6 gave a significantly higher signal than peaks 1–5 (14). To test the possibility that endogenous ligand co-purified with NPC2 and inhibited binding of DHE, we delipidated the protein in each peak by acetone extraction/precipitation and analyzed the
acetone extract by gas chromatography (see “Experimental Procedures”). Substantial amounts of cholesterol were associated with peaks 1–5 (~0.6 mol of cholesterol per mol of NPC2), with considerably less associated with peak 6 (~0.06 mol of cholesterol per mol of NPC2) (data not shown).

Fig. 8 demonstrates that the presence of bound ligand was largely responsible for the differences in the chromatographic properties of peaks 5 and 6. Material from the preparative ion-exchange column was concentrated and equilibrated with loading buffer, and ~50 µg of protein was fractionated on an analytical cation-exchange column. Although these chromatography conditions revealed additional heterogeneity for each peak from the preparative column, the relative order of elution was preserved, with the majority of the material eluting as doublets centered at about 4.1 and 4.4 ml for peak 5 and at 5.0 and 5.7 ml for peak 6 (Fig. 8, solid traces, A and B). Acetone precipitation and rehydration did not affect the mobility of peak 6 (Fig. 8B, dashed trace), although it shifted the mobility of peak 5 to that of peak 6 (Fig. 8A, dashed trace). Conversely, incubation of peak 6 with cholesterol (Fig. 8B, dotted trace) shifted its elution pattern to that of untreated peak 5, as did incubation of acetone-precipitated peak 5 with cholesterol (Fig. 8A, dotted trace). These results indicate that association of uncharged cholesterol with NPC2 decreases its interaction with the cation-exchange resin, resulting in significantly earlier elution from the column.

Acetone extraction of peaks 1–4 resulted in a similar trend as observed for peak 5, further demonstrating that a substantial portion of these isoforms contained bound ligands (Fig. 9, compare solid and dashed traces). Endo H treatment of the acetone-extracted proteins further increased their retention on the analytical column (Fig. 9, dotted traces). (PNGase F treatment was ineffective under nondenaturing conditions.) Delipidated peak 1, which consists almost exclusively of NPC2 with one Endo H-sensitive and one Endo H-resistant oligosaccharide, was shifted to a later-eluting position (Fig. 9A, dashed trace). Peaks 2 and 4, which consist predominantly of glycoforms with two Endo H-sensitive oligosaccharides, and peaks 5 and 6, which consist of glycoforms with one Endo H-sensitive oligosaccharide, largely elute at a similar retention time following delipidation and deglycosylation. Note that the isoforms containing a single Endo H-resistant oligosaccharide on Asn-116 (e.g. Fig. 9A, dotted trace) elute earlier than the isoforms containing a single Endo H-sensitive oligosaccharide on Asn-39 (e.g. Fig. 9, E and F, dashed traces). Although positional effects might influence elution, this difference would also be consistent with the presence of multiple negatively charged sialic acid residues on the Endo H-resistant oligosaccharides. Furthermore, the heterogeneity apparent after rechromatography of each peak on the analytical column was considerably reduced by Endo H treatment, indicating that much of this heterogeneity was because of differential glycosylation, probably reflecting modifications that would affect net charge such as the presence of one or two mannose 6-phosphate residues (23). Taken together, these results suggest that the isoforms observed in Fig. 1 are attributable to differences in both bound ligand and glycosylation.

Biological Function—To assess the biological function of the different NPC2 isoforms, we cultured primary fibroblasts from a patient with null mutations in the NPC2 gene in the presence or absence of peaks 1–6 and used filipin staining to visualize unesterified sterols (Fig. 10). In the absence of exogenous NPC2, the affected fibroblasts showed a punctate perinuclear staining, reflecting the lysosomal cholesterol storage characteristic of NPC disease (Fig. 10B). Incubation with peaks 1–6 ameliorated the lysosomal cholesterol storage phenotype (Fig. 10, C–H), shifting the pattern from a punctate, perinuclear staining to the diffuse, plasma

**FIGURE 9.** Delipidation and removal of Endo H-sensitive oligosaccharides from peaks 1–6. Material from the preparative cation-exchange column was either left untreated (solid traces), delipidated by acetone extraction (dashed traces), or digested with Endo H following acetone extraction (dotted traces).
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Control experiments demonstrated that inclusion of 10 mM cosylated NPC2 was incubated with 0.1 to 1.5M eq of DHE for receptor-mediated endocytosis and retain biological function. The peak can be delivered to the lysosome by mannose 6-phosphate inhibiting the effect of exogenous NPC2 in reversing the disease phenotype (data not shown). These data demonstrate that the different NPC2 isoforms present in each peak can be delivered to the lysosome by mannose 6-phosphate receptor-mediated endocytosis and retain biological function.

Ligand Binding—We initially attempted to analyze potential ligands that co-purified with NPC2. Analysis of trimethylsilyl-derivatized material from the acetone-precipitated protein by gas chromatography revealed the presence of cholesterol and much lesser amounts (1/15-fold) of the cholesterol precursors desmosterol and lathosterol (data not shown). In addition, we also analyzed both acetone and chloroform/methanol extracts by TLC (see “Experimental Procedures”). This revealed the presence of a lipid that co-migrated with the cholesterol standard and another, more polar molecule that migrated in the same general vicinity as LBPA and phospholipid standards. To date, we have not been able to identify the latter compound using MALDI-TOF, liquid chromatography-MS, or NMR methods (data not shown), but we have ruled out LBPA and some phospholipids (see below).

To search for potential biological ligands of NPC2 and also to explore ligand structure-function relationships, we developed a binding assay based on the previously observed shift in elution on a cation-exchange column after cholesterol binding. We validated our assay using DHE, which is a chromophore and can be detected by monitoring absorbance at 328 nm. A fixed concentration (1 μg/μl, ~68 μM) of apo (acetone-extracted) monoglycosylated NPC2 was incubated with 0.1 to 1.5 M eq of DHE for 30 min at room temperature and then fractionated on the analytical Mono S column, monitoring absorbance at 280 and 328 nm. Fig. 11 demonstrates that DHE was only associated with the NPC2 that shifted to an earlier elution position and that the amount of NPC2 that shifted was proportional to the input DHE up to a saturation ratio of ~1:1 (Fig. 11, lower panel, inset). We also used the ratio of the A(328) and A(300) for the peak that eluted earlier to calculate a binding stoichiometry as described under “Experimental Procedures,” and we found a molar ratio of DHE/NPC2 of 1.13 ± 0.04. This indicates the chromatographic shift is caused by NPC2 binding stoichiometric amounts of ligand.

We surveyed other molecules for their ability to bind to and shift the mobility of NPC2, using acetone-extracted peaks 5 and 6 (Fig. 12A, Apo) and either endogenous ligand-containing NPC2 found in peak 5 (Fig. 12A, Holo) or apoNPC2 incubated with cholesterol (Fig. 12A, CL) as standards. For the two cholesterol precursors, lathosterol and desmosterol, as well as the plant sterols, stigmasterol and β-sitosterol, the observed shift is indistinguishable to that observed with cholesterol (Fig. 12C and Table 2), suggesting that these ligands bind to NPC2 in a similar fashion. In addition, two dihydrocholesterol isomers, 5α-cholestan-3β-ol and 5β-cholestan-3β-ol, were examined, and they both shifted the retention time of NPC2 (Fig. 12B). This indicated that the unsaturated double bond of cholesterol is not required for binding to NPC2. Interestingly, the 5α-isomer produced an essentially indistinguishable shift compared with cholesterol, whereas the 5β-isomer induced a much less marked shift.

To determine the role of the 3-hydroxyl group in ligand binding, we investigated several compounds including 5α-cholestane, epicholesterol, ketalcholesterol, cholesteryl sulfate, cholesteryl esters, and thiocholesterol that have minor modifications of the 3-hydroxyl group. Results showed that the role of the 3-hydroxyl was important, but NPC2 tolerated some changes. Epicholesterol, where the 3-OH is in the α rather than β position, clearly shifted NPC2, indicating binding, but the shift was considerably less dramatic than observed with cholesterol (Fig. 12B). Similarly, ketalcholesterol (5α-cholestan-3β-one) induced a significant shift in NPC2 (Fig. 12A, CL-one), albeit slightly less than that of cholesterol. However, the negatively charged cholesterol sulfate dramatically decreased the retention time of NPC2, probably reflecting the combined effect on both charge and conformation following binding (Fig. 12A). Cholesteryl acetate was capable of binding and shifting the mobility of NPC2 to some extent (Fig. 12A), but longer chain cholesteryl esters (butyrate, palmitate, and olate) did not result in a shift (Table 2). In addition, no shift was observed with 5α-cholestan, thiocholesterol, cholesteryl aniline, or cholesterol bromide (Fig. 12D and/or Table 2), which are cholesterol analogs that lack a hydroxyl at the 3-position.

A number of other compounds were screened for direct binding to NPC2 using the chromatographic shift assay, concentrating on molecules that accumulate in NPC disease and those that affect lysosomal cholesterol transport. Few of these elicited an apparent shift in NPC2 retention (Fig. 12 and/or Table 2). However, we cannot exclude the possibility that the binding of some ligands will not change the net retention time.

**FIGURE 10. Amelioration of cholesterol storage by NPC2 glycoforms.** Filipin staining of unaffected (A) or NPC2-deficient (B–H) primary human fibroblasts. Cells were either not treated (A and B) or incubated in the presence of 100 nM NPC2 (C–H, peaks 1–6, respectively). A 50-μm magnification bar is shown in G. All fields were photographed under identical conditions.
of NPC2. This possibility was underscored by our observations that a small but reproducible shift was observed for 24-hydroxycholesterol (Fig. 12B), whereas 25-hydroxycholesterol (Fig. 12D) and other oxysterols (data not shown) do not appear to display such a shift.

As a complementary method, we monitored the ability of various compounds to inhibit binding of DHE to NPC2 (see “Experimental Procedures”). This protocol was chosen to detect fairly weak ligands, in that 1.5 eq of test ligand were incubated with NPC2 prior to addition of 1.2 eq of DHE. The preincubation step was used to avoid the possibility of a kinetic trap that could cause the binding of the test ligand to be underestimated. Computer simulations conducted using the explicit binding equations described by Wang (24) and a $K_d$ of 0.2 to 0.7 μM for the DHE-NPC2 interaction (14) indicated that if equilibrium were reached under our incubation conditions, a test ligand that bound NPC2 with equal affinity to DHE would inhibit binding by ~55%. In comparison, test ligands with apparent affinities that were 100, 10, 0.1, and 0.01 times that of DHE-NPC2 were predicted to inhibit DHE binding by about 97, 85, 21, and 4%, respectively.

The competition assay was initially validated using cholesterol and cholesterol sulfate. Cholesterol sulfate inhibited DHE binding dramatically (~99% inhibition), indicating an apparent affinity that was considerably greater than that of DHE (Fig. 13). The chromatograms (absorbance at 328 and 280 nm) revealed that the decrease in the signal corresponding to the DHE-NPC2 complex was paralleled by the appearance of the earlier eluting NPC2-cholesterol sulfate complex (data not shown). Cholesterol inhibited DHE binding by ~70%, which is consistent with competition between both ligands for the same binding site on NPC2.

The competition assay detected a small but significant inhibition of DHE binding for some compounds that did not elicit a shift using the direct assay. For instance, of all the oxysterols examined, only 24-hydroxycholesterol elicited a slight shift in the direct assay. Binding of 24-hydroxycholesterol was corroborated by the competition assay, where ~40% inhibition of DHE binding was observed. In addition, cholesterol derivatives that are monohydroxylated at the 7-, 20-, 22-, and 27-positions inhibited DHE binding by 21–35%, with only 25-hydroxycholesterol having no observable effect.

Other molecules such as imipramine and U18666A, two drugs that elicit an NPC-like phenotype in cultured cells, and ezetimibe, a cholesterol absorption inhibitor, also did not induce a shift in the mobility of NPC2 (Fig. 12D and/or Table 2). However, an inhibitory effect by these three drugs was observed in the competition assay (Fig. 13). Similarly, steroid hormones estradiol and testosterone gave a statistically significant decrease of DHE signal but not progesterone, indicating estriadiol and testosterone may interact with NPC2 (Fig. 13).

Additional lipids accumulate in NPC1 and NPC2 disease, and it is possible that some of these may represent primary rather than secondary storage material. We therefore tested various fatty acids (oleate and palmitate), lipids (N-acetyl-D-
sphingosine and LBPA), glycosphingolipids (GM1, GM2, and GM3), the insect hormone 20-hydroxyecdysone, and various bile salts (cholate, taurocholate, and chenodeoxycholate). Results from the direct shift assay and/or the competition assay suggested that none of these compounds bound to NPC2 (Fig. 12, Fig. 13, and Table 2).

As a step toward determining the physiological relevance of these observations, we measured the levels of selected oxysterols in the livers of control and NPC2/H11002/H11002 mice. There was a dramatic elevation of 27-hydroxycholesterol, similar to that of cholesterol, although there was no statistically significant elevation in 24S-hydroxycholesterol or 7β-hydroxycholesterol (Fig. 14).

**DISCUSSION**

In our initial study that elucidated the molecular basis for NPC type 2 disease (8), we isolated ∼7 different NPC2 isoforms by purifying mannose 6-phosphate containing glycoproteins from human brain autopsy specimens. We also demonstrated that the cholesterol storage phenotype of fibroblasts from individuals with NPC type 2 disease could be ameliorated by mannose 6-phosphate receptor-mediated endocytosis of NPC2. In this study, we have developed a robust expression system for producing human NPC2, and we used it to characterize the glycosylation and ligand binding properties of the protein.

Our results demonstrate that recombinant human NPC2 is never glycosylated at Asn-19, which has the sequence NVSP. This is consistent with a glycoproteomic study that revealed that the sequence NX(T,S)P is not glycosylated in vivo (25). We also found that Asn-39 is always linked to an Endo H-sensitive oligosaccharide, whereas Asn-116 is variably glycosylated both in terms of the presence and processing of the oligosaccharide. Given that NPC2 is targeted via the mannose 6-phosphate lysosomal targeting pathway (8, 26, 27), Endo H sensitivity of the NPC2 oligosaccharides is likely to reflect the presence of mannos 6-phosphate (23); this modification occurs temporally prior to and blocks the actions of Golgi α-mannosidase, which is required for conversion of N-linked glycans to Endo H-resistant forms. Thus, Asn-39 represents the primary substrate of lysosomal protein UDP-N-acetylgluosamine phosphotransferase. In addition, in the course of a proteomic analysis of human brain lysosomal proteins (28), we identified mannose 6-phosphorylated NPC2 peptides glycosylated at Asn-39 and at Asn-116, and we also found nonglycosylated peptides containing Asn-116. Thus, the glycosylation state of recombinant human NPC2 produced using the Chinese hamster ovary cell expression system reflects the protein synthesized in vivo.

Our results are in complete accord with a mutagenesis study by Vanier and co-workers (27) that systematically examined the
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### TABLE 2
Interactions between test ligands and NPC2
See supplemental Fig. 1 for selected structures.

| Class           | Compound             | Direct shift | DHE inhibition |
|-----------------|----------------------|--------------|----------------|
| Sterols         | Cholesterol          | ++           | ++             |
|                 | Lathosterol          | ++           |                |
|                 | Desmosterol          | ++           |                |
|                 | 5α-Cholesterol-3β-ol | ++           | ++             |
|                 | 5β-Cholesterol-3β-ol | ++           | ++             |
|                 | 5-Cholesterol-3α-ol  | ++           |                |
| (epicholesterol) |                      |              |                |
| Plant sterols   | Stigmasterol         | ++           | ++             |
|                 | β-Sitosterol         | ++           | ++             |
|                 | DHE                  | ++           | ++             |
| 3-OH modifications | Cholesteryl sulfate | ++           | ++             |
|                 | 5α-Cholestane-3-one  | ++           | ++             |
|                 | Thiocholesterol      | –            | –              |
|                 | Cholesteryl aniline   | –            | –              |
|                 | Cholesteryl bromide   | –            | –              |
|                 | 5α-Cholestane        | –            | –              |
| Cholesteryl esters | Cholesteryl acetate   | +            | –              |
|                 | Cholesteryl butyrate  | –            | –              |
|                 | Cholesteryl palmitate | +            | –              |
|                 | Cholesteryl oleate    | –            | –              |
| Oxyesters       | 27-Hydroxycholesterol | +            | –              |
|                 | 25-Hydroxycholesterol | –            | –              |
|                 | 24-Hydroxycholesterol | +            | +              |
|                 | 22(RS)-Hydroxycholesterol | –            | +              |
|                 | 20-Hydroxycholesterol | –            | –              |
|                 | 7-Hydroxycholesterol  | –            | –              |
| Hormones        | Testosterone propionate | –        | +              |
|                 | Progesterone         | –            | –              |
|                 | Estradiol            | –            | –              |
|                 | 20-Hydroxyecdysone   | +            | –              |
| Bile acids      | Sodium taurocholate  | –            | –              |
|                 | Sodium cholate       | –            | –              |
|                 | Sodium chenodeoxycholate | –        | –              |
| Drugs           | U18666A              | –            | –              |
|                 | Imipramine           | +            | –              |
|                 | Ezetimibe            | –            | –              |
| Fatty acids     | Oleate               | –            | –              |
|                 | Palmitate            | –            | –              |
| Other lipids    | GM1                  | –            | –              |
|                 | GM2                  | –            | –              |
|                 | GM3                  | –            | –              |
|                 | LBPA                 | –            | –              |
|                 | Phosphatidylcholine  | –            | –              |
|                 | N-Acetyl-S-p-sphingosine | –         | –              |

FIGURE 13. Inhibition of DHE binding to NPC2 by test ligands. NPC2 was incubated with the indicated test ligand prior to incubation with DHE and inhibition of the NPC2-DHE complex measured as described under “Experimental Procedures.” The signal of the DHE-NPC2 complex (left axis) is shown as the mean value ± range of three to four determinations, with the solid and dotted lines indicating the mean and range of the no test ligand (DHE alone) control. The right axis shows the percent inhibition of DHE binding. One t test was performed to analyze the differences between the signal obtained in the presence and absence of test ligand. Results were considered significant at the p < 0.05 (*) and p < 0.001 (**) levels.

FIGURE 14. Cholesterol and selected oxysterol levels in control and NPC2-deficient mice. Data are presented as the mean ± range for two independent animals of each phenotype, normalized to the mean of the wild type controls.

utilization and role of each of the three potential N-linked glycosylation sites on human NPC2. In addition to demonstrating the variable glycosylation of Asn-116 in their system, they found that Asn-39 is necessary and sufficient for proper lysosomal targeting of NPC2, whereas Asn-116 is dispensable.

In terms of other effects of glycosylation, monoglycosylated human NPC2 exhibited lipid transfer rates that were ~3-fold faster than that of diglycosylated NPC2 (13). This may reflect greater charge repulsion between the more anionic, earlier eluting peaks 1–4 for acceptor membranes compared with the monoglycosylated forms. However, this difference was small compared with the dependence of transfer rates on acceptor membrane composition and the ionic strength of the medium. Given that many other mammal species lack a potential glycosylation sequence at Asn-116 (e.g. mouse, rat, dog, cow, sheep, and pig), as well as the ability of the different NPC2 isoforms present in each peak to ameliorate cholesterol storage in NPC2-deficient fibroblasts (Fig. 10), this indicates that the variable utilization of this site in human NPC2 is not central to the basic conserved biochemical function of the protein.

Analysis of other lysosomal proteins with multiple potential glycosylation sites indicates that they too exhibit macroheterogeneity in glycosylation site occupancy (29–31). These glycoproteins also exhibit microheterogeneity within different classes of N-linked glycans (e.g. for complex, number of antennae, degree of sialylation, and terminal processing; for high mannose-mannose content, degree and site of mannose 6-phosphorylation; hybrid, contribution of all these factors). Although we have not performed an in-depth analysis of isolated glycans, the heterogeneity observed following rechromatography of delipidated and Endo-H-digested peaks 1–6 (Fig. 9) suggests that there is considerable microheterogeneity within each oligosaccharide. This is also supported by our observations that the proteins present in each ion-exchange peak exhibit heterogeneous mass envelopes when analyzed by linear MALDI-TOF MS (data not shown), with fine structure that suggests differences in number of monosaccharide units.
Thus, although the isolation of the individual peaks is a step toward simplification of the mixture, the extensive microheterogeneity typical of oligosaccharides poses a considerable obstacle toward isolation of truly homogeneous glycoforms. However, given the interspecies differences in glycosylation, as well as heterogeneity of monoglycosylated NPC2 in different tissues of a given species (16, 32, 33), it is possible that there is some variation in the stability, targeting efficiency, and kinetic properties of the individual glycoforms, we think that it is unlikely that such variation has a significant physiological effect on a central, conserved function of the NPC2 protein.

In the course of characterizing the multiple isoforms of purified recombinant human NPC2, we found that cholesterol binding induced a shift in the chromatographic properties of the protein. The chromatographic shift assay provided a convenient means to survey potential binding partners for NPC2, as well as exploring structure-activity relationships, with induction of a shift demonstrating interaction. Although no shift would occur if a molecule did not interact with NPC2, the finding that some sterols induced a less marked shift than cholesterol implied that it was possible that some ligands could bind NPC2 without inducing a chromatographic shift. To search for potential ligands that did not induce a shift, we developed an assay based on inhibition of binding of DHE to NPC2. Here, lack of inhibition indicates that the test ligand does not interact with NPC2 with an affinity comparable with that of DHE. Although inhibition is consistent with binding of the test ligand to NPC2, it is formally possible that the observed inhibition could be due to a secondary effect such as reducing the amount of DHE available to bind to NPC2. One possible mechanism for such an effect would be formation of mixed micelles. For purposes of classification, we define ligands as those molecules that induce a shift and, if assayed, inhibit DHE binding, whereas nonligands are those molecules that neither induce a shift nor inhibit DHE binding. Molecules that fall into the gray zone, representing potential but not demonstrated ligands, are those that do not exhibit a shift but are observed to inhibit DHE binding.

Elucidation of the three-dimensional structure of bovine apoNPC2 (14) revealed a loosely packed hydrophobic core that has three small cavities that are too small to accommodate cholesterol. Inspection of the structure suggests that this represents an incipient pocket that enlarges to accommodate a ligand, and that the protein converts between constricted and open states. We expect that this change in the interior would be accompanied by subtle changes in the exterior of the protein that might affect the interaction of NPC2 with the negatively charged cation-exchange resin. Alternatively, the shift could come from the portion of the ligand molecule that is exposed to the solvent and could influence chromatography. These effects, either singly or in combination, could account for the differences in migration observed for different ligands.

NPC2 clearly can bind a variety of different ligands. Many are closely related to cholesterol, differing in the degree and location of carbon-carbon double bonds, and/or by the presence of additional alkyl substituents on the side chain carbons (e.g. desmosterol, lathosterol, stigmasterol, β-sitosterol, DHE, and 5α-cholestan-3-β-ol) (see supplemental Fig. 1 for structures). Others are more distantly related, differing significantly either in conformation (e.g. 5β-cholestan-3-β-ol) or polarity (e.g. 24-hydroxycholesterol). The versatility of the binding site reflects the malleable nature of the hydrophobic cores of proteins. The hydrophobic core of the ligand-bound form of NPC2 is solidly packed and composed of both protein residues and the hydrocarbon portion of the sterol. Just as the hydrophobic interior of proteins is known to be adaptable to side chain substitutions (34), we envision that NPC2 ligands can be accommodated by compensatory repacking of interior residues. Such rearrangements may also facilitate optimal alignments of polar atoms in ligands (e.g. 24-hydroxycholesterol) and protein side chains within the hydrophobic interior (e.g. Tyr-36, Tyr-100, Trp-109, and Trp-122).

In terms of structure-activity relationships, substituents on the 3-OH had variable effects. Based on the competition assay, cholesterol sulfate had the highest affinity for NPC2 of all compounds tested. It also induced the largest chromatographic shift, which can be attributed in part to the negatively charged cholesterol sulfate having a repulsive interaction with the negatively charged cation-exchange matrix. Analysis of the crystal structure of the bovine NPC2-cholesterol sulfate complex indicates that the sulfate is solvent-accessible and does not interact with NPC2 itself. The relatively strong binding of cholesterol sulfate is likely to arise from its solubility properties, rather than its intrinsic affinity for NPC2. Haberland and Reynolds (35) have reported that cholesterol has a strong propensity to aggregate in aqueous solution, with a critical micelle concentration of ~25 nM, and have discussed how binding of cholesterol to a protein is a balance between the free energy gained from association with that protein and the free energy gained from self-association. It is likely that cholesterol sulfate is less prone to aggregation than cholesterol itself and that this alone may explain the greater apparent affinity of the negatively charged ligand for NPC2. Similar considerations may explain the lower apparent affinity of cholesterol acetate for NPC2 compared with cholesterol, and the lack of binding observed for the longer acyl chain cholesterol esters and other less polar cholesterol analogs.

It is important to stress that although our in vitro assays are a step toward determining what molecules can bind NPC2 and their physiological relevance, given the interplay between solubility of lipids and apparent binding affinity (see above), we do not make any claims regarding binding strength. In vitro assays demonstrate that NPC2 can transfer sterols between membranes (12) and that the lipid composition of the donor and acceptor membranes has an extremely large effect on the kinetics of sterol transfer (13). In addition to lipid composition and structure, other proteins may play a role in loading and unloading of ligand in vivo. The aggregation state of ligand in solution presents a major obstacle for estimating thermodynamic binding parameters in vitro, as the apparent dissociation constants reported for the interaction of NPC2 with cholesterol or DHE are comparable or higher (11, 14, 15) than the reported critical micelle concentration of cholesterol (35).

3. S. Xu, H.-L. Liou, B. Benoff, P. Lobel, and A. M. Stock, unpublished data.

4. K. Sandhoff, personal communication.
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In addition to the factors described above, assay design may also play a factor in estimating affinity as well as ability to screen for potential NPC2 ligands. We chose to use a chromatography-based assay to separate free from bound ligand rather than performing rapid filtration (15) or absorption to activated charcoal (11), as this allowed us to monitor both free and ligand-bound NPC2. Okamura et al. (11) have examined a limited number of compounds for their ability to inhibit binding of radiolabeled cholesterol to porcine NPC2, and they found weak inhibition by cholesterol acetate, lathosterol, and estradiol and no inhibition with cholesterol linolate, bile acids, or testosterone. Except for testosterone, these data are in excellent agreement with our more extensive analysis, indicating that the two different assays are providing similar results. Given these factors, we consider our binding studies to yield qualitative rather than quantitative data, with a major goal of our studies being to survey potential ligands for possible interaction with NPC2, which can then be further scrutinized for physiological significance.

In terms of biological relevance, a number of lipids accumulate in NPC2-deficient mouse liver and/or brain, including cholesterol, plant sterols, gangliosides GM2 and GM3, and LBPA (16). Our finding that stigmasterol and β-sitosterol represent NPC2 ligands suggests that storage of plant sterols in NPC type 2 disease is a direct result of the NPC2 deficiency. Conversely, given that we did not obtain any evidence that GM2, GM3, or LBPA interact with NPC2, storage of these molecules is likely to be secondary to the NPC2 deficiency.

It will be important to determine whether the other biologically relevant molecules that are demonstrated to interact with NPC2 (e.g. cholesterol sulfate and cholesterol precursors) as well as potential NPC2 ligands (e.g. some oxysterols and steroids) also accumulate in NPC type 2 disease and whether they are localized to the lysosome. As a step toward this, we measured levels of 7β-, 24-, and 27-hydroxycholesterol in mouse liver. The finding that 27-hydroxycholesterol accumulates suggests that this potential NPC2 ligand may be transported by NPC2 in vivo. A study by Ory and co-workers (36) reported that production of 25- and 27-hydroxycholesterol following LDL uptake was decreased from impaired lysosomal transport of cholesterol. Previous studies demonstrated that some oxysterols accumulate in NPC1-deficient mouse tissues (37) and that lipid storage is remarkably similar in both NPC1 and NPC2 deficiencies (16, 38). Although little is known regarding the intracellular trafficking of oxysterols (39), it is possible that some of these molecules normally reach the lysosome and are exported via the NPC pathway. This could occur during constitutive processes such as membrane trafficking and autophagy of mitochondria and other organelles. Trapping and accumulation of such molecules in the lysosome might contribute to the pathophysiology of NPC disease, either by direct toxic effects on the lysosome or by the sequestration and unavailability of these molecules or their downstream metabolites for function in some other essential pathway.

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