Characterization of Fluorescent Sterol Binding to Purified Human NPC1*

Ronghua Liu, Peihua Lu, Joseph W. K. Chu, and Frances J. Sharom

From the Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Mutations in the NPC1 gene cause Niemann-Pick type C disease, which appears to result from a defect in intracellular cholesterol trafficking. NPC1 is a member of the resistance-nodulation-cell division (RND) permease superfamily and contains a sterol-sensing domain, yet its cellular function and the identity of its substrates remain unknown. FLAG-tagged human NPC1 was purified from NPC1-expressing Chinese hamster ovary cells by solubilization in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), followed by affinity chromatography. Purified NPC1 in detergent solution appeared to be oligomeric as determined by gel filtration fast protein liquid chromatography. The apparent NBD-cholesterol binding affinity was greatly reduced at higher detergent concentration. The stoichiometry of NBD-cholesterol binding to NPC1 was 1. Various sterols, including native cholesterol and 25-hydroxycholesterol, inhibited NBD-cholesterol binding, suggesting that they compete for binding to the protein. Dynamic quenching studies showed that bound NBD-cholesterol was almost completely shielded from the aqueous medium, suggesting that it is buried in a deep hydrophobic pocket in NPC1. The use of fluorescent cholesterol analogs provides novel information on the molecular properties of the sterol-binding site in the full-length NPC1 protein.

Niemann-Pick type C (NPC) disease is an autosomal recessive fatal inherited neurodegenerative disorder of cholesterol homeostasis, which results in accumulation of cholesterol and sphingolipids in the brain and liver (1, 2). Cells displaying the NPC phenotype show accumulation of low density lipoprotein-derived, unesterified cholesterol in the endosomes and lysosomes. NPC disease is also characterized by the subcellular accumulation of other lipids, such as sphingomyelin, sphingosine, and gangliosides (3). Over 95% of cases of NPC disease are caused by mutations in the NPC1 gene (4, 5). NPC1 is a large 1278-amino acid integral membrane protein that shares structural features with several proteins involved in cholesterol homeostasis. It is located primarily in the late endosomes, but it also appears to traffic transiently between other regions of the cell via a network of tubular structures and vesicles (6, 7). The NPC1 protein clearly plays a role in the intracellular trafficking of cholesterol, particularly in the movement of low density lipoprotein-derived cholesterol from the late endosomal and lysosomal compartments to the plasma membrane and trans-Golgi network (8). The other 5% of NPC patients carry a mutation in the gene encoding NPC2 (9, 10), a small 132-amino acid, soluble cholesterol-binding protein found in the lumen of the lysosomes (11–13). Despite the fact that the NPC1 and NPC2 proteins are quite different at the molecular level, the phenotypes displayed by NPC patients carrying mutations in the NPC1 and NPC2 genes are virtually indistinguishable. It seems likely that the two proteins are interacting partners in the intracellular cholesterol trafficking pathway.

NPC1 is a member of the resistance-nodulation-cell division (RND) permease superfamily, a ubiquitous group of proteins found in all the major kingdoms (14). Bacterial RND proteins are generally proton symporters involved in coupled efflux from the cell of substrates such as hydrophobic drugs, fatty acids, detergents, and antibiotics. Eukaryotic RND proteins are largely uncharacterized but fall into two major subclasses as follows: proteins that have an internal duplication, and likely function as transporters, and those that do not, and probably act as sterol-modifying enzymes or sterol sensors. Thus, phylogenetic analysis predicts that the NPC1 protein functions as a proton-coupled transporter, possibly an efflux pump for sterols and/or other amphipathic molecules. A very close mammalian
relative of NPC1, a protein known as NPC1L1 (Niemann-Pick C1 Like 1; 51% amino acid similarity to NPC1) (15), is expressed at the apical surface of intestinal epithelial cells and plays a major role in absorption of cholesterol in the intestine (16, 17). A recent study reported that mutant mice lacking either NPC1 or NPC2 do not show any abnormalities in cholesterol absorption or uptake, indicating that the two proteins do not play a role in these processes in mammals (18).

Investigation of the topology of NPC1 (19) has suggested that it has 13 transmembrane (TM) segments, a cytoplasmic C terminus, a large hydrophilic N-terminal domain located in the lumen of the endosomes, and two additional large luminal loops (see Fig. 1A). The membrane domain of NPC1 contains a cluster of five TM helices known as the sterol-sensing domain (SSD) (4, 20), which was also identified in several other mammalian proteins involved in cholesterol homeostasis (21), including 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the sterol regulatory element-binding protein cleavage-activating protein (SCAP).

In recent years, the assumed role of NPC1 as a cholesterol transporter or sensor has been challenged, and other lipophilic molecules, including fatty acids and hydrophobic drugs such as acriflavine and daunorubicin, have been proposed as putative substrates (22–24). A role for NPC1 in sphingolipid recycling has also been suggested (25), based on the discovery that NCR1, the yeast ortholog of NPC1, is involved in sphingolipid metabolism. However, NPC1 in intact mammalian cells was shown to be labeled by a photoactive cholesterol analog, which was partially inhibited by a 30-fold excess of unlabeled cholesterol (26). Labeling required an intact SSD on the protein but was independent of NPC2, suggesting that NPC1 likely interacts directly with cholesterol, as a substrate and/or allosteric effector. More recently, Infante et al. (27) reported the isolation from rabbit liver of a 25-hydroxycholesterol-binding protein, which analysis showed to be NPC1. Purified recombinant human NPC1 was found to bind various oxysterols, including 24-, 25-, and 27-OH-cholesterol, with high affinity. When a large N-terminal domain (luminal loop-1, residues 25–264) of NPC1 was

### FIGURE 1.

A, model of proposed NPC1 structure showing location of Trp residues; B, chemical structures of the sterols used in this study.
Fluorescent Sterol Binding to Purified Human NPC1

expressed as a soluble protein, it could also bind cholesterol and oxysterols with high affinity (28). Clearly, extensive biochemical characterization of purified NPC1 is needed to characterize its sterol binding and transfer functions, as well as its interactions with putative protein partners, such as NPC2.

In this study, we report the purification of human NPC1 from mammalian cells overexpressing the protein, in milligram amounts sufficient for biochemical and fluorescence characterization. Purified NPC1 can be cross-linked to 3H-labeled photoactive cholesterol analog. Two complementary fluorescence approaches have been used to show a close molecular association between purified NPC1 and various fluorescent sterol derivatives, and to quantitate the binding affinity of the protein for these species. Results show that NPC1 binds various sterols with high affinity, in a deep hydrophobic pocket that is shielded from the aqueous medium.

EXPERIMENTAL PROCEDURES

Materials—CHAPS was purchased from Biosynth (Naperville, IL). Tridecylphosphocholine (fosc-choline 13; FC-13) was obtained from Anatrace (Maumee, OH). M2 anti-FLAG antibody and M2 anti-FLAG-agarose were obtained from Sigma. FLAG peptide was provided by Dalian Biteomics (Dalian, China). Rabbit polyclonal antibody raised against human NPC1 (amino acids 1261–1278) was provided by Dr. Daniel S. Ory (Center for Cardiovascular Research, Washington University School of Medicine) (29). Complete protease inhibitor mixture tablets were obtained from Roche Diagnostics. 22-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) and chol esteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-dodecanoate (cholesteryl-BODIPY; cholesteryl BODIPY® FL C12) were obtained from Molecular Probes (Eugene, OR). Ergosta-5,7,9(11),22-tetraen-3β-ol (DHE), (3β)-3-[2-(diethylamino)ethyl]androst-5-en-17-one dihydrochloride (U18666A), 25-hydroxy-cholesterol, and N-acetyltryptophanamide (NATA) were supplied by Sigma. Δ5,7,9(11) Cholestriene-3β-ol (CTL; cholestatrienol) was provided by Dr. F. Maxfield (Department of Biochemistry, Weil Medical College, Cornell University). 5α-Cholest-3α-ol (epicholesterol) was from Steraloids (Newport, RI). HEPES, acrylamide, KI, and CsCl were from Fisher.

Cell Culture—NPC1+ is a Chinese hamster ovary (CHO) cell line that stably expresses FLAG-tagged human NPC1 in an endogenous NPC1 knock-out background (30, 31). The tag is inserted following Ile-306 in the first cytoplasmic loop of the protein (19). Cells were maintained in Ham’s F-12 medium with 10% bovine calf serum and 20 μg/ml blasticidin (both from Invitrogen). α-Minimum Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone, Logan, UT) was used for large scale cell culture in roller bottles. NPC1+ cells from 30 roller bottles were harvested with trypsin, washed with phosphate-buffered saline (8.1 mM Na2HPO4, 1.3 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 0.3 mM MgCl2, pH 7.4), and centrifuged to obtain a 20-ml cell pellet.

Purification of FLAG-NPC1—NPC+ cells were disrupted using a Yeda press, and a membrane fraction was isolated by sucrose density gradient ultracentrifugation using a procedure described previously (32). To solubilize the membrane fraction, 50 mg of membrane protein was added to 15 ml of 20 mM CHAPS in HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, pH 7.4) and resuspended with a fine needle (26-gauge 5/8), followed by mixing on a nutator for 1 h at 4 °C. The sample was centrifuged at 15,000 × g for 15 min at 4 °C, and the supernatant was collected and diluted with an equal volume of HEPES buffer to a final CHAPS concentration of 10 mM. 10 ml of CHAPS-solubilized membrane extract was loaded onto a 5-ml anti-FLAG-agarose column equilibrated with 10 mM CHAPS in HEPES buffer, and the column flow was stopped when protein started to appear in the run-through. The agarose gel was gently stirred in place, and the column was sealed and placed on a Nutator for 2 h at 4 °C. The column was then re-packed and washed with 2 mM CHAPS in HEPES buffer until the run-through was free of detectable protein (using the Bradford assay). The column was washed with 15 ml of 0.2 mg/ml FLAG peptide in 2 mM CHAPS/HEPES buffer, and the eluate containing NPC1 was pooled and concentrated to a final volume of ~2 ml using an Amicon ultracentrifugation concentrator (100,000-kDa cutoff). The FLAG peptide was removed from purified NPC1 by gel filtration on a 10-ml pre-packed column of Bio-Gel P6 (Bio-Rad), washing with 2 mM CHAPS/HEPES buffer. The purified FLAG-NPC1 (~0.35 mg from 50 mg of membrane protein) was divided into aliquots and stored at ~80 °C. Protein was assayed by the method of Bradford (33).

SDS-PAGE and Western Blotting—Proteins were separated in a 10% (w/v) polyacrylamide gel by SDS-PAGE according to Laemmli (34) and stained with Coomassie Blue. For Western immunoblotting, samples were subjected to SDS-PAGE analysis and electrophoretically transferred onto nitrocellulose membrane, which was then incubated with either anti-NPC1 polyclonal antibody or anti-FLAG antibody. The bound primary antibody was detected using a horseradish peroxidase-conjugated second antibody (Jackson ImmunoResearch) in conjunction with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences), and visualized using a FluorChem IS-8900 imaging system (Alpha Innotech, San Leandro, CA). Kaleidoscope prestained standards were obtained from Bio-Rad.

Photoaffinity Labeling of NPC1 with [3H]Azi-cholesterol—Photoaffinity labeling was carried out by a modification of the method of Loe and Sharom (35). Briefly, purified NPC1 (1 μg) was incubated at 22 °C in 20 mM HEPES, 0.10 M NaCl, 5 mM MgCl2, pH 7.4, with 7-azi-5a-cholestan-3β-ol[3,5,6-3H] (azi-cholesterol; 2.5 μM, 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis) for 2 h under subdued light. The sample was then irradiated at 365 nm (Strategen cross-linker) for 10 min at room temperature at a distance of <1 cm, and analyzed by SDS-PAGE on a 10% polyacrylamide gel. The gel was impregnated with 20% diphenyloxazole in DMSO overnight, washed, dried, and exposed at ~80 °C to Kodak X-Omat LS film, followed by autoradiography after a 12-week exposure.

FPLC Analysis of Purified NPC1—Gel filtration fast protein liquid chromatography (FPLC) was carried out on a Superose 6/300 GL column (10 × 300 mm; 24-ml bed volume) connected to an AKTA FPLC system (both from Amersham Biosciences). The column was equilibrated with buffer (10 mM HEPES, 5.4
respectively. As a control, 0.5 M BODIPY (0.5 μM) was added to purified NPC1 (14 μg in 100 μl) to a final concentration of 1 μM in 2 mM CHAPS/HEPES buffer. BODIPY fluorescence intensity was measured with excitation and emission wavelengths of 490 and 515 nm, respectively. Binding of 2 μM NBD-cholesterol to 15 mM CHAPS detergent micelles was also measured.

Quenching of NPC1 Intrinsic Trp Fluorescence—Binding of fluorescent sterols to NPC1 was measured in 2 mM CHAPS/HEPES buffer by adding 1 μl of increasing concentrations of sterol working solutions in methanol to a series of microcentrifuge tubes containing 59 μl of NPC1 (0.29 μM), followed by incubation at 4 °C for ~3 h for DHE and CTL and 30 min for NBD-cholesterol. Control experiments were performed by adding increasing concentrations of fluorescent sterol to 2 mM CHAPS buffer. The steady-state Trp fluorescence was measured with 290 nm excitation and 330 nm emission. The Trp fluorescence intensity of NPC1 was corrected for light scattering and the inner filter effect (37, 38) using Equation 1,

$$F_{cor} = (F_{i/NPC1} - F_{i/buffer}) \times 10^{0.5(A_{ex} + A_{em})}$$

where $F_{cor}$ is the corrected Trp fluorescence intensity; $F_{i/NPC1}$ and $F_{i/buffer}$ are the experimentally measured fluorescence intensities for either NPC1 samples or buffer controls, and $A_{ex}$ and $A_{em}$ are the absorbance values of the sample at the excitation and emission wavelengths, respectively. Data were fitted to hyperbolic Equation 2 describing binding to a single site,

$$F_{cor} = F_0 - \frac{(\Delta F_{max} \times [S])}{K_d + [S]}$$

where $F_{cor}$ is the corrected Trp fluorescence intensity at any given substrate concentration, and $F_0$ is the initial Trp fluorescence of NPC1 in the absence of sterols. SigmaPlot (Systat Software, Chicago) was used to fit the data, and values for the apparent dissociation constant, $K_d$, and the maximum quenching, $\Delta F_{max}$, were extracted.

The effects of competing compounds on quenching of Trp fluorescence resulting from NBD-cholesterol binding to NPC1 were determined by preincubating NPC1 samples with 20 μM of competitor at 4 °C for 1 h. Then increasing concentrations of NBD-cholesterol were added, followed by incubation at 4 °C for additional 30 min. For control experiments, buffer was used to substitute for purified NPC1 protein. Fluorescence intensities were corrected using Equation 3,

$$\Delta F_{cor} = \{(F_{i/NPC1/S} - F_{NPC1}) - (F_{i/buffer/S} - F_{buffer})\} \times 10^{0.5(A_{ex} + A_{em})}$$

where $F_{i/NPC1/S}$ is Trp fluorescence intensity of NPC1 at any given NBD-cholesterol concentration in the presence of 20 μM competitor; $F_{i/buffer/S}$ is the scattering intensity of NBD-cholesterol in buffer at any given concentration in the presence of 20 μM competitor; $F_{NPC1}$ is the background scatter for NPC1 in buffer, and $F_{buffer}$ is the background scatter for buffer alone. Increasing concentrations of fluorescent sterols were also added to 2 μM NATA in 2 mM CHAPS/HEPES buffer; DHE, CTL, and NBD-cholesterol did not quench the fluorescence of NATA.
Enhancement of Stere fluorescence on Binding to NPC1—The experimental procedures were the same as for quenching of NPC1 Tryp fluorescence. The steady-state fluorescence of DHE/CTL/NBD-cholesterol was measured with 325/325/470 nm excitation and 375/375/520 nm emission. The increased fluorescence intensities of NPC1-bound substrate were calculated using Equation 4,

$$\Delta F_{Iceq} = \frac{(F_{0,NPC1} - F_{NPC1}) - (F_{buffer} - F_{buffer})}{(F_{buffer} - F_{buffer})} \times 10^5 (1 + \frac{1}{A_{max} + A_{ave}})$$  

(Eq. 4)

where $F_{0,NPC1}$ is the fluorescence intensity of sterol at any given concentration in the presence of NPC1; $F_{NPC1}$ is the background scatter of NPC1; $F_{buffer}$ is the fluorescence intensity of sterol in the buffer at any given concentration, and $F_{buffer}$ is the background scatter of buffer alone. Data were fitted to hyperbolic Equation 5 describing binding to a single site using SigmaPlot, and values for the apparent $K_d$ and $\Delta F_{max}$ were estimated.

$$\Delta F_{Iceq} = \frac{(\Delta F_{max} \times [S])}{K_d + [S]}$$  

(Eq. 5)

Subtraction of the fluorescence intensity of DHE and CTL from the intensity in the presence of NPC1 is an approximate approach; however, the values of $K_d$ changed by only 1.7–3% when raw intensities were used compared with corrected intensities.

Stoichiometry of NBD-cholesterol Binding to NPC1—The stoichiometry of NBD-cholesterol binding to NPC1 was determined by methods described previously (39, 40). Binding of NBD-cholesterol was determined at five different NPC1 concentrations ranging from 0.18 to 2.21 μM. The concentration of the NBD-cholesterol working solutions was verified using the molar extinction coefficient of 21,000 cm$^{-1}$ M$^{-1}$ at 537 nm in methanol (41). The protein concentration was verified at the completion of each experiment by a protein assay (33), and the molecular mass of the protein component of NPC1 (139 kDa) was used to calculate its molar concentration. $\Delta F_{max}$ for NBD-cholesterol fluorescence was obtained by fitting each binding curve to a hyperbolic equation as described above. The molar enhancement of NBD-cholesterol fluorescence ($\phi$) was calculated by determining the slope of a plot of $\Delta F_{max}$ versus NPC1 concentration in micromolars. The mass action plot of Dixon and Webb (42) was then applied to the data to determine the binding stoichiometry, using Equation 6,

$$\frac{r}{[L]_{free}} = \frac{n}{K_d} - \frac{r}{K_d}$$  

(Eq. 6)

where $[L]_{free}$ is the concentration of free ligand; $r$ is the molar ratio of bound ligand to total protein; $K_d$ is the apparent dissociation constant, and $n$ is the stoichiometry of ligand binding. The bound ligand concentration was estimated using the molar enhancement of fluorescence ($[L]_{bound} = F/\phi$, and the concentration of free ligand was calculated as $[L]_{free} = [L]_{total} - [L]_{bound}$. A plot of $r/[L]_{free}$ versus $r$ gave a straight line with an x-intercept value of $n$, the binding stoichiometry.

Collisional Quenching of NBD-cholesterol Bound to NPC1 and in Detergent Micelles—Freshly prepared stock solutions of 5 mM acrylamide, KI, and CsCl in 2 mM CHAPS/HEPES buffer were added as 0.5-μl aliquots to 60 μl of 2 μM NBD-cholesterol in the presence of 0.29 μM NPC1 in 2 mM CHAPS buffer. 0.1 mM Na$_2$S$_2$O$_3$ was added to the KI stock solution to prevent I$_3$ formation. Fluorescence intensities were corrected for dilution, and scattering was corrected by titration of buffer alone with the quenchers. Parallel experiments were carried out using 2 μM NBD-cholesterol in 15 mM CHAPS to assess the quenching of NBD-cholesterol in detergent micelles. The data were analyzed using the Stern-Volmer Equation 7,

$$F_0/F = 1 + K_{SV} [Q]$$  

(Eq. 7)

where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of quencher, respectively; [Q] is the concentration of quencher, and $K_{SV}$ is the Stern-Volmer quenching constant. A plot of $F_0/F$ versus [Q] gives a linear plot with a slope of $K_{SV}$.

RESULTS

Purification of Human NPC1 Protein—CHO cells knocked out for endogenous NPC1 and overexpressing FLAG-tagged human NPC1 were used as the starting material for NPC1 isolation. The cell suspension was lysed using nitrogen cavitation, followed by ultracentrifugation on a 35% (w/v) sucrose cushion to collect a purified membrane fraction. Immunodot-blotting and protein assays of the pellet, supernatant, and interfacial protein but contained most of the cellular NPC1. The inclusion of this step greatly increased the lifetime of the antibody affinity column, and reduced NPC1 proteolytic degradation. Treatment of the membrane fraction with a 20 mM concentration of the zwitterionic detergent, CHAPS, solubilized ~85% of the protein. NPC1 was purified from this extract using an anti-FLAG antibody affinity column, eluting with FLAG peptide in buffer containing 2 mM CHAPS, which is below the detergent CMC. After concentration and removal of FLAG peptide by rapid gel filtration on a desalting column, a final yield of 0.35 mg of NPC1 (90–95% purity; see Fig. 2) was obtained from 50 mg of membrane protein starting material. Purified NPC1 was observed as a doublet on SDS-PAGE with Coomassie Blue staining (193 and 182 kDa), and both protein bands reacted with anti-FLAG antibody and anti-NPC1 antibody (Fig. 2) on Western blots, suggesting the presence of heterogeneous glycosylation. The presence of N-linked glycosylation was demonstrated by the reduction in mass of the two bands to ~175 and 150 kDa, respectively, after treatment with N-glycanase (data not shown). NPC1 has 14 potential N-glycosylation sites, and a previous study partially characterized four of these sites at the N terminus (43). Two bands remained after N-glycanase treatment, which suggests that either removal of N-linked chains is incomplete or heterogeneous O-linked chains are also present on NPC1.

Characterization of NPC1 Molecular Size—The molecular size of purified NPC1 was analyzed by gel filtration FPLC, using immunodot-blotting of the column fractions to locate the elution peaks, and the molecular size was estimated by comparison.
to protein standards. In 2 mM CHAPS buffer, most of the NPC1 eluted as a very high molecular weight peak at the void volume of the column, with some small peaks visible at lower molecular weight (higher elution volume) (Fig. 3A). As shown in Fig. 3B, addition of 0.2 mM FC-13 to 2 mM CHAPS buffer resulted in an increase in the amount of NPC1 eluting in the lower molecular weight fractions. When the CHAPS concentration was increased to 15 mM (above the detergent CMC) in the presence of 0.2 mM FC-13, no high molecular weight peak was observed, and all the NPC1 eluted as a series of four peaks corresponding to monomers and oligomers up to tetramers (Fig. 3C). Purified NPC1 solubilized in 0.1% (w/v) digitonin displayed a broad size profile that included monomers and tetramers (Fig. 3D). Thus, depending on the detergent concentration, NPC1 associates into both oligomers up to the size of a tetramer, and larger assemblies, which are reversibly dissociated on addition of more detergent.

**Purified NPC1 Is Photolabeled by [3H]Azi-cholesterol—**Azi-cholesterol is a sterol analog with a photoactivable diazirine ring that closely resembles cholesterol (Fig. 1B), and is likely a functional substitute for it (44). When purified NPC1 was incubated with [3H]azi-cholesterol and cross-linked using UV light, autoradiography showed only a single band corresponding to labeled NPC1 (Fig. 2). Therefore, purified NPC1 retains its sterol-binding function in 2 mM CHAPS buffer. An integral protein of comparable size that has been suggested to interact with cholesterol (ABCB1) showed no labeling by azi-cholesterol under identical conditions.3

**Intrinsic Trp Fluorescence of NPC1—**NPC1 contains 14 Trp residues (see Fig. 1A); 4 residues are predicted to be located in the membrane, and the others are in the large luminal domains (8 residues) and short cytosolic loops (2 residues) (19). The fluorescence emission wavelength maximum of NPC1 was significantly blue-shifted compared with that of the soluble Trp derivative NATA (332 versus 353 nm; Fig. 4A), which indicates that the emitting Trp residues are located in a relatively hydrophobic environment.

3 P. D. Eckford and F. J. Sharom, unpublished data.
Fluorescent Sterol Binding to Purified Human NPC1

**FIGURE 4.** A, intrinsic Trp fluorescence emission spectra of purified NPC1 and NATA. Corrected fluorescence emission spectra for 0.29 μM NPC1 (solid line, $\lambda_{em} = 332$ nm) and 5 μM NATA (dotted line, $\lambda_{em} = 353$ nm) in 2 mM CHAPS/HEPES buffer. Fluorescence emission was recorded at 22 °C following excitation at 290 nm, using 2-nm slits for excitation and 10-nm slits for emission, with a cut-off filter of 305 nm. B and C, corrected fluorescence emission spectra of DHE and CTL in the absence and presence of NPC1. B, fluorescence emission of 1 μM DHE in the presence of 0.29 μM NPC1 in 2 mM CHAPS/HEPES buffer (solid line, $\lambda_{em} = 375$ nm) compared with buffer alone (dashed line, $\lambda_{em} = 375$ nm). C, fluorescence emission of 1 μM CTL in the presence of 0.29 μM NPC1 in 2 mM CHAPS/HEPES buffer (solid line, $\lambda_{em} = 375$ nm) compared with buffer alone (dashed line, $\lambda_{em} = 375$ nm). Fluorescence emission was recorded at 22 °C following excitation at 325 nm, using 2-nm slits for excitation and 10-nm slits for emission.

**Binding of the Fluorescent Sterols DHE and CTL to NPC1—**The fluorescent sterols DHE and CTL closely resemble cholesterol structurally (Fig. 1B). When DHE and CTL were added to NPC1, we observed a large increase in their fluorescence emission intensity (4–5-fold) when compared with that recorded in buffer (Fig. 4, B and C). DHE and CTL in 2 mM CHAPS buffer displayed low fluorescence emission intensity (Fig. 5A), which increased at concentrations up to 10 μM with no evidence of self-quenching (Fig. 5A, inset). Titration of NPC1 with increasing concentrations of DHE and CTL resulted in a saturable concentration-dependent increase of their fluorescence (Fig. 5A). The increase in fluorescence intensity of DHE and CTL in the presence of NPC1 relative to buffer was fitted to an equation for a single high affinity binding site (Fig. 5B), with apparent $K_d$ values of 4 and 2.3 μM, respectively (Table 1). Monitoring of the NPC1 Trp fluorescence showed concomitant saturable quenching (Fig. 5C), which could also be fitted to an equation for a single binding site. The dissociation constants for binding of DHE and CTL to NPC1 estimated using Trp quenching were very similar to those obtained by enhancement of the sterol fluorescence (Table 1). Many key features of the NPC phenotype, including cholesterol accumulation, can be induced by treating intact cells with the cationic amphiphile, U18666A, thus mimicking NPC disease (45). It has been suggested that compounds of this type might act directly on the NPC1 protein (46). Fig. 5C shows that, like fluorescent sterols, U18666A quenches the intrinsic Trp fluorescence of NPC1, suggesting that this drug indeed interacts with the protein with high affinity (Table 1).

**Kinetics of Binding of Fluorescent Sterols to NPC1—**When a sterol is present in aqueous solution as either pure sterol micelles or mixed micelles with detergent, equilibration between micelles and protein, which is slow, may be the rate-determining step for binding. For example, binding of cholesterol to the membrane region of SCAP was observed to take up to 3 h (47). We first determined the kinetics of binding to NPC1 of cholesteryl-BODIPY, a fluorescent cholesterol ester derivative that forms aggregates at concentrations greater than 0.3 μM. Fig. 6A shows that when a 0.5 μM concentration of this compound is added to aqueous buffer containing 2 mM CHAPS, 0.2 mM FC-13, there is an increase in the BODIPY fluorescence, which rises over a time period of up to 3 h. When NPC1 is present in the same detergent-containing buffer, the fluorescence increase is approximately doubled, indicating that the sterol binds to the protein with enhancement of its fluorescence. The same slow time frame for the fluorescence increase is observed, suggesting that the equilibration of cholesteryl-BODIPY from micelles/aggregates to the protein is the rate-limiting step in the binding reaction. In contrast, 2 μM NBD-cholesterol (which is monomeric at concentrations below 4.5 μM) equilibrates into detergent micelles in 15 mM CHAPS buffer relatively rapidly, reaching the maximum fluorescence intensity in ~150 s (Fig. 6B). When NBD-cholesterol is mixed with NPC1 in 2 mM CHAPS buffer, which is below the detergent CMC, the fluorescence increase is very fast and reaches a maximum in ~30 s. Thus, the kinetics of binding of fluorescent sterols to NPC1 appears to depend on the aggregation state of the sterol and the presence of detergent micelles. Rapid binding is seen when sterol monomers associate with the protein in the absence of detergent micelles, whereas very slow binding is
Fluorescent Sterol Binding to Purified Human NPC1

Parameters for binding of fluorescent sterols to NPC1

| Sterol     | Apparent $K_d$ (µM) | $\Delta F_{max}$ | $\Delta F_{max}/F_0 \times 100$ % |
|------------|---------------------|------------------|----------------------------------|
| Enhancement of sterol fluorescence |                     |                  |                                  |
| DHE        | 4.05 ± 0.36         | 3.71 ± 0.13      |                                  |
| CTL        | 2.34 ± 0.16         | 4.83 ± 0.12      |                                  |
| NBD-chol + 2 mM | 0.66 ± 0.06   | 4.87 ± 0.12      |                                  |
| CHAPS      |                    |                  |                                  |
| NBD-chol + 2 mM | 3.80 ± 0.32   | 6.85 ± 0.16      |                                  |
| CHAPS/0.2 mM |                    |                  |                                  |
| FC-13      |                    |                  |                                  |

Quenching of NPC1 Trp fluorescence

| Sterol     | $K_d$ (µM) | $\Delta F_{max}$ | $\Delta F_{max}/F_0 \times 100$ % |
|------------|------------|------------------|----------------------------------|
| DHE        | 5.17 ± 0.12| 42.8 ± 1.7       |                                  |
| CTL        | 1.89 ± 0.49| 41.8 ± 0.6       |                                  |
| U18666A    | 2.33 ± 0.60| 14.6 ± 1.0       |                                  |
| NBD-chol + 2 mM | 0.52 ± 0.14| 27.7 ± 1.8       |                                  |
| CHAPS      |            |                  |                                  |
| NBD-chol + 2 mM | 6.03 ± 1.01| 41.4 ± 2.0       |                                  |
| CHAPS/0.2 mM |            |                  |                                  |
| FC-13      |            |                  |                                  |

* Estimates of the dissociation constant and maximal quenching were obtained by fitting the fluorescence quenching data to an equation for a single binding site as described under "Experimental Procedures"; both are given as values ± fitting error.

FIGURE 5. Binding of fluorescent sterols to NPC1 enhances their fluorescence emission and quenches the intrinsic Trp fluorescence emission of NPC1. Increasing concentrations of DHE, CTL, and U18666A were incubated with 0.29 µM NPC1 in 2 mM CHAPS/HEPES buffer for 3 h at 4 °C. A, uncorrected fluorescence emission of DHE (●) and CTL (□) in the presence of NPC1, measured at 375 nm following excitation at 295 nm. The fluorescence emission intensities of DHE (○) and CTL (□) in buffer alone are shown for comparison (the inset shows the same data using an expanded scale). B, corrected fluorescence emission of DHE and CTL in the presence of NPC1 after subtraction of the fluorescence of the compounds in buffer alone, showing their enhanced fluorescence. The data were fitted to an equation describing binding to a single site. Data points represent the mean ± range (n = 2), and where not visible, error bars are contained within the symbols.

no shift in $\lambda_{em}$ (Fig. 7A). FRET between two fluorophores is possible when there is overlap between the donor emission spectrum and the acceptor absorption spectrum. When the donor is excited, FRET is manifested by quenching of the donor emission and the appearance of sensitized emission from the acceptor. Excitation of NPC1 Trp residues at 290 nm led to sensitized fluorescence emission of bound NBD-cholesterol at 520 nm (Fig. 7A, inset), indicating that the protein and NBD-cholesterol are close enough for FRET to take place. Trp quenching by NBD-cholesterol is saturable (Fig. 7B), and fitting of the data led to an estimate for the apparent $K_d$ of 0.5 µM in 2 mM CHAPS (Table 1). When FC-13 was added to a concentration of 0.2 mM, the apparent $K_d$ was reduced 6–12-fold as indicated by fluorescence enhancement and Trp quenching data, respectively (Fig. 7B and Table 1), indicating that the apparent binding affinity of NPC1 for sterols is substantially lower in the presence of higher detergent concentrations. No binding of NBD-cholesterol could be detected by either Trp quenching or fluorescence enhancement at a CHAPS concentration of 15 mM.

The fluorescence emission spectrum of NBD-cholesterol showed a large increase in intensity in the presence of NPC1 in 2 mM CHAPS (Fig. 7C), together with a blue shift of 38 nm in $\lambda_{em}$ from 558 to 520 nm (Fig. 7C, inset), indicating that the sterol is located in a hydrophobic pocket when bound to the protein. This environment is clearly distinguishable from that of detergent micelles. When NBD-cholesterol is added to 15 mM CHAPS (above the CMC), the fluorescence emission is also increased (Fig. 7C), but the $\lambda_{em}$ blue-shifts by only 9 nm (Fig. 7C, inset). It should be noted that NBD-cholesterol shows no fluorescence increase in 2 mM CHAPS, below the detergent CMC (Fig. 7C). The increase in NBD-cholesterol fluorescence on binding to NPC1 is also saturable (Fig. 7D), and fitting of the data to a binding equation led to an estimate for the apparent $K_d$ almost identical to that determined by Trp fluorescence quenching of NPC1 (Table 1). Similarly, the apparent binding affinity of NPC1 for NBD-cholesterol determined by enhancement of its fluorescence was greatly reduced by the presence of

observed when sterol aggregates must equilibrate with protein in detergent micelles.

Binding of NBD-cholesterol to NPC1—The Trp fluorescence of NPC1 was quenched on addition of NBD-cholesterol, with

JANUARY 16, 2009 • VOLUME 284 • NUMBER 3
Fluorescent Sterol Binding to Purified Human NPC1

**FIGURE 6. Kinetics of binding of cholesteryl-BODIPY and NBD-cholesterol to NPC1.** A shows the time dependence of the fluorescence of cholesteryl-BODIPY (0.5 μM) after addition to 0.29 μM NPC1 in HEPES buffer with 2 mM CHAPS, 0.2 mM FC-13 (○), and to the same buffer without NPC1 (▲). The background fluorescence at the BODIPY excitation and emission wavelengths of NPC1 in buffer (□) and buffer alone (∆) are also shown. Cholesteryl-BODIPY fluorescence was monitored over a period of ~5 h, with excitation and emission wavelengths of 490 and 515 nm, respectively. B shows the time dependence of the fluorescence of NBD-cholesterol (2 μM) after addition to 0.29 μM NPC1 in 2 mM CHAPS/HEPES buffer (solid line), and to 15 mM CHAPS buffer without NPC1 (dashed line). NBD-cholesterol fluorescence was measured for 250 s with excitation and emission wavelengths of 470 and 520 nm, respectively. Data points represent the mean ± range (n = 2), and where not visible, error bars are contained within the symbols.

**FIGURE 7. Binding of NBD-cholesterol to NPC1.** A, Trp emission spectra of NPC1 (0.29 μM) in the absence (long dashed line) and presence (dotted line) of 1 μM NBD-cholesterol in 2 mM CHAPS buffer. The background fluorescence of buffer alone (short dashed line) and 1 μM NBD-cholesterol in 2 mM CHAPS (solid line) are also shown. Fluorescence emission was recorded at 22 °C following excitation at 290 nm. The inset shows the sensitized fluorescence emission of NBD-cholesterol (dotted line) via FRET from NPC1 Try residues following excitation at 290 nm. B, quenching of the Trp fluorescence of NPC1 (0.29 μM) by increasing concentrations of NBD-cholesterol in 2 mM CHAPS (below the CMC, ○), and in 2 mM CHAPS/0.2 mM FC-13 (▲). Fluorescence emission at 330 nm was measured following excitation at 290 nm. The quenching data were fitted to an equation describing quenching to a single site. C, comparison of the emission spectra of 1 μM NBD-cholesterol in 2 mM CHAPS with 0.29 μM NPC1 (solid line), in 15 mM CHAPS/0.2 mM FC-13 (△), and in 2 mM CHAPS (above the CMC, ○), and in 2 mM CHAPS (below the CMC, ○). The background fluorescence of 2 mM CHAPS with NPC1 (long dashed line) and 2 mM CHAPS alone (dashed-dotted line) are also shown. The inset shows the normalized NBD-cholesterol emission in the presence of NPC1 (solid line, λem = 520 nm), 15 mM CHAPS (short dashed line, λem = 549 nm), and 2 mM CHAPS (dotted line, λem = 558 nm). D, increase in NBD-cholesterol fluorescence on binding to NPC1 in the presence of higher detergent concentration (2 mM CHAPS, 0.2 mM FC-13; Fig. 7D and Table 1). Thus measurement of simultaneous Trp quenching and enhancement of NBD-cholesterol fluorescence indicated almost identical behavior, and provided complementary information on NPC1.

**Stoichiometry of NBD-cholesterol Binding to NPC1**—The increase in NBD-cholesterol fluorescence on binding to NPC1 was used to estimate the stoichiometry of binding. Titration of NBD-cholesterol with increasing concentrations of NPC1 led to a proportional increase in the NBD-cholesterol fluorescence (Fig. 8A). A plot of ΔFmax versus NPC1 concentration gave a linear relationship (Fig. 8B), and the slope provided a value of 5.9 arbitrary units/μM for the fluorescence yield φ, which is defined as the increase in NBD-cholesterol fluorescence when bound to 1 μM NPC1. Dixon plots of r/[NBD-cholesterol]free versus r for five different NPC1 concentrations were constructed and yielded straight lines converging at r = 0.94 ± 0.014 (Fig. 8C), which suggests that each NPC1 protein binds approximately one molecule of NBD-cholesterol.

**Accessibility of NPC1-bound NBD-cholesterol to Collisional Quenchers**—To determine whether NBD-cholesterol bound to NPC1 is accessible to the surrounding aqueous solution, dynamic quenching studies were carried out with the collisional quenchers acrylamide (neutral), iodide ion (negatively charged), and cesium ion (positively charged). Stern-Volmer plots indicated that NBD-cholesterol is readily quenched when it is present in detergent micelles (15 mM CHAPS), but when bound to NPC1 it is almost completely shielded from acrylamide and iodide (Fig. 9, A and B) and much less accessible to cesium (Fig. 9C). The calculated Stern-Volmer quenching constants, KSV, for quenching by acrylamide, iodide, and cesium were 22-, 30-, and 3.4-fold lower, respectively, when NBD-cholesterol was bound.
to NPC1 (Table 2), suggesting that the sterol is located in a deep binding pocket within NPC1.

**Competition with NBD-cholesterol for Binding to NPC1**

The ability of other sterols to compete with NBD-cholesterol for binding to NPC1 was investigated by measuring Trp quenching by the fluorescent sterol in the presence of a 20 μM concentration of competitor. As shown in Fig. 10, epicholesterol, DHE, and U18666A reduced the level of Trp quenching by NBD-cholesterol substantially, whereas cholesterol and 25-hydroxycholesterol had a smaller effect. These results suggest that all of these sterols compete to some extent for binding to the same site on NPC1 as NBD-cholesterol. The ability of cholesterol to act as a competitor in these types of

**FIGURE 8.** Stoichiometry of binding of NBD-cholesterol to NPC1. A, increasing concentrations of NBD-cholesterol were titrated with 2 mM CHAPS buffer (○) and NPC1 in the same buffer at five different concentrations as follows: 0.18 μM (●), 0.44 μM (▲), 1.02 μM (▲), 1.57 μM (▼), and 2.20 μM NPC1 (●). Fluorescence emission at 520 nm was measured following excitation at 470 nm. The maximum fluorescence increase, ΔF_{max}, was estimated at each NPC1 concentration by fitting each curve (after background correction) to a binding equation. B, plot of ΔF_{max} versus NPC1 concentration gave a linear relationship with a fluorescence yield, ϕ, of 5.9 arbitrary units (au/mM). C, Dixon plots of the data from A. The lines are fitted to the equation of Dixon and Webb (39, 40, 42) using linear regression, and yielded an average n = 0.94 ± 0.14 from five different NPC1 concentrations. Data points represent the mean ± range (n = 2), and where not visible, error bars are contained within the symbols.

**FIGURE 9.** Dynamic quenching of the fluorescence of NBD-cholesterol bound to 0.29 μM NPC1 in 2 mM CHAPS/HEPES buffer (●) or in detergent micelles (15 mM CHAPS/HEPES buffer (○)) in the presence of increasing concentrations of acrylamide (A), iodide ion (B), and cesium ion (C). NBD-cholesterol fluorescence was measured at 520 nm following excitation at 470 nm. The data were fitted to the Stern-Volmer equation.

**FIGURE 10.** Dynamic quenching of the fluorescence of NBD-cholesterol bound to 0.29 μM NPC1 in 2 mM CHAPS/HEPES buffer (●) or in detergent micelles (15 mM CHAPS/HEPES buffer (○)) in the presence of increasing concentrations of acrylamide (A), iodide ion (B), and cesium ion (C). NBD-cholesterol fluorescence was measured at 520 nm following excitation at 470 nm. The data were fitted to the Stern-Volmer equation.
Fluorescent Sterol Binding to Purified Human NPC1

experiments will likely be underestimated because of its low solubility in aqueous solution.

DISCUSSION

After over a decade of research on NPC1, its physiological substrates remain to be identified, and its mechanism of action at the molecular level is still unclear. It is not known whether NPC1 is involved in the direct molecular transfer of cholesterol, and if it is also involved in transport of sphingolipids, fatty acids, lipophilic molecules, or amphiphatic drugs. A recent report suggests that SNARE-mediated vesicular trafficking is involved in a significant portion of cholesterol transport from the NPC1-containing endosomal compartment to the trans-Golgi network in CHO cells (48). NPC1 was found to play an essential (but unknown) role in this process. However, evidence has begun to accumulate indicating that NPC1 is indeed a sterol-binding protein. 3H-Labeled 7-azi-cholesterol was reported to photolabel NPC1 in intact cells in a manner that required the SSD (26). Similar compounds are known to be good functional substitutes for native cholesterol, and a 6-azi-cholesterol derivative has been shown to specifically label the Torpedo acetylcholine receptor in a 1:1 molar ratio (49). More recently, NPC1 was characterized as an oxysterol-binding protein in a screen intended to identify unknown proteins in this class, and it was also shown to interact with several oxysterols and [3H]cholesterol (27).

In this study, we have used mammalian cells overexpressing human NPC1 as a source to purify the protein in milligram amounts, and have used several different approaches to establish that it binds a variety of cholesterol analogs. Purified NPC1 was robustly photolabeled using an azi-cholesterol derivative. The protein appeared to exist in a reversible equilibrium between very large aggregates in detergent solutions below the CMC, and a mixture of oligomers up to the size of tetramers in detergent solutions above the CMC. The TM domain of SCAP also displayed a monomer-tetramer equilibrium in solutions of FC-13 (47).

Cholesterol has low maximum aqueous solubility of <5 μM and a CMC of 25–40 nM (50), which makes direct measurement of its binding affinity challenging. Fluorescent sterols with higher solubility can be invaluable in exploring protein-sterol interactions. DHE and CTL were observed to bind to NPC1, as indicated by concomitant saturable quenching of the Trp fluorescence of the protein, and enhancement of the fluorescence of the sterol. Both approaches gave similar estimates of the apparent binding affinity of NPC1, in the 2–5 μM range. The kinetics of sterol binding to NPC1 appeared to depend on the presence of micelles, as was observed previously for sterol binding to SCAP (47). It was proposed by Radhakrishnan et al. (47) that sterols must first equilibrate from micelles of sterol/detergent alone into mixed micelles of protein and detergent before binding specifically to the protein. The presence of higher detergent concentrations, and thus increased numbers of micelles, serves to greatly dilute the sterol, and thus reduces the rate of binding. In keeping with this proposal, cholesteryl-BODIPY at concentrations where micelles/aggregates exist displayed very slow binding to NPC1, taking ~3 h to reach equilibrium. In contrast, NBD-cholesterol at concentrations where it is monomeric bound to NPC1 rapidly, reaching equilibrium in less than 1 min. Indeed, binding of NBD-cholesterol to NPC1 in the absence of detergent micelles was found to be substantially faster than equilibration of the sterol into CHAPS detergent micelles alone.

NBD-cholesterol proved to be a very useful fluorescent analog for characterization of NPC1-sterol interactions. High affinity binding to NPC1 (apparent \( K_d = 0.5–0.6 \mu M \)) could be monitored in two different ways as follows: first, by saturable quenching of the Trp fluorescence of the protein, and second, by enhancement of the NBD fluorescence. In addition, we noted that FRET took place between NPC1 Trp residues and the NBD fluorophore, providing further evidence for a close molecular interaction between the sterol and the protein. It was previously shown that the Trp residues of a cholesterol-binding protein (StAR) and NBD-cholesterol are an excellent donor-acceptor pair (51). NBD-cholesterol also displayed a substantial spectral

**TABLE 2**

| Sample                  | Quencher | \( K_{SV} \) **a** |
|-------------------------|----------|-------------------|
| NBD-cholesterol + NPC1 in 2 mM CHAPS | Acrylamide | 0.013 ± 0.004 |
| NBD-cholesterol in 15 mM CHAPS | Acrylamide | 0.29 ± 0.02 |
| NBD-cholesterol + NPC1 in 2 mM CHAPS | KI       | 0.88 ± 0.06 |
| NBD-cholesterol in 15 mM CHAPS | KI       | 26.3 ± 0.47 |
| NBD-cholesterol + NPC1 in 2 mM CHAPS | CsCl    | 0.029 ± 0.006 |
| NBD-cholesterol in 15 mM CHAPS | CsCl | 0.100 ± 0.008 |

**a** NBD-cholesterol was mixed with either NPC1 in 2 mM CHAPS buffer or 15 mM CHAPS buffer and titrated at 22 °C with increasing concentrations of quencher.

**b** Stern-Volmer quenching constant, determined from the slope of a plot of \( F_0/F \) versus concentration of acrylamide, expressed as values ± fitting error.

**FIGURE 10.** Binding of NBD-cholesterol to NPC1 in the presence of competing sterols, monitored by quenching of Trp fluorescence (excitation at 290 nm, emission at 330 nm). NPC1 (0.29 μM in 2 mM CHAPS) was titrated with increasing concentrations of NBD-cholesterol alone (○) or in the presence of 20 μM concentrations of cholesterol (●) and epicholesterol (▲) (A) and 25-OH-cholesterol (25-OH-chol) (■), DHE (▲), and U18666A (▲) (B). The fluorescence quenching data were fitted to an equation describing binding to a single site. Data points represent the mean ± range (n = 2), and where not visible, error bars are contained within the symbols.
Fluorescent Sterol Binding to Purified Human NPC1

sterol binding function. In contrast, Infante et al. (27) for sterol binding to full-length NPC1 were 100 nM for cholesterol and 10 nM for 25-hydroxycholesterol; however, they were highly dependent on detergent concentration (see below). The purified soluble N-terminal domain of NPC1 also showed higher apparent binding affinity for 25-hydroxycholesterol (10 nM) relative to cholesterol (130 nM) (28) Because the regulatory actions of oxysterols are independent of NPC1, the higher apparent binding affinity may result from the use of a detergent-sensitive in vitro assay.

In this study, the apparent affinity of NPC1 for binding NBD-cholesterol was greatly reduced in the presence of higher detergent concentrations and could not be detected in the presence of detergent micelles. Infante et al. (27) used a nickel-chelate affinity column binding assay to show that full-length recombinant NPC1 solubilized using either 1% Nonidet P-40 or 0.1% FC-13 bound [3H]25-hydroxycholesterol with high affinity. However, they were unable to show binding of [3H]cholesterol to the protein unless the detergent concentration was lowered to submicellar levels (0.004% Nonidet P-40). Sterol binding to the soluble N-terminal domain of NPC1 was also markedly detergent-dependent (28). This is also in accordance with previous observations that the binding of [3H]cholesterol to the TM region of SCAP (47) was inhibited by detergent micelles. Given the low aqueous solubility of sterols and the existence of micellar equilibria, measured $K_d$ values for sterol binding to NPC1 are expected to be highly dependent on detergent type and concentration, and "true" binding affinities may be impossible to determine.

The stoichiometry of binding of NBD-cholesterol to NPC1 in this study was ~1, suggesting that the protein fully retains its sterol binding function. In contrast, Infante et al. (27) reported a stoichiometry of 0.25 for the full-length protein (four proteins bound to one sterol) and a stoichiometry of 0.5 for the N-terminal soluble luminal domain of NPC1 (28). These observations suggest that the affinity column approach to measure sterol binding may not provide quantitative results, especially at submicellar detergent concentrations. Our results do not rule out the existence of a second sterol-binding site on NPC1 that is unable to interact with NBD-cholesterol. For example, NPC2 does not bind NBD-cholesterol, likely because the modified side chain cannot be accommodated in the binding pocket.

When other compounds were included with the NBD-cholesterol binding assay, several were able to act as competitors for the fluorescent sterol, including native cholesterol, 25-hydroxycholesterol, and DHE. In contrast, cholesterol was unable to compete for binding of 25-hydroxycholesterol to either full-length NPC1 of the soluble N-terminal domain in the assay of Infante et al. (27). Our results suggest that NPC1 has a relatively broad specificity for binding native sterols, fluorescent sterols, and oxysterols. Epicholesterol was a much poorer competitor than cholesterol itself, suggesting that the NPC1 substrate binding pocket is sensitive to the configuration of the sole hydroxyl group on the sterol, but it can tolerate the addition of an NBD group at the other end of the molecule.

Many key features of the NPC phenotype, including cholesterol accumulation, can be induced by treating intact cells with U18666A and various cationic amphiphiles, thus mimicking NPC disease (45, 52). It has been suggested that these compounds might act directly on the NPC1 protein (46). Certain cationic amphiphiles also induce a conformational change in SCAP, thus mimicking cholesterol, which suggests that they may share the same binding site in this protein (47, 53). In this study, we observed that U18666A quenched the intrinsic Trp fluorescence on NPC1 in a similar manner to the fluorescent sterols DHE and CTL. The higher maximal fluorescence quenching achieved for the latter may reflect their ability to quench Trp residues by a FRET mechanism, which is not possible for U18666A. The drug also competed with NBD-cholesterol for binding to NPC1, suggesting that it interacts directly with the protein. In contrast, Infante et al. (27) did not observe effective competition of U18666A for binding of 25-hydroxycholesterol to full-length NPC1, possibly because of the presence of 1% Nonidet P-40 in the assay.

The molecular details of cholesterol binding to NPC2 are now known from the high resolution x-ray structures of the apoprotein (54), and the protein bound to cholesterol-3-O-sulfate, a high affinity substrate (55). The sterol is bound in a deep hydrophobic pocket with only the sulfate substituent exposed to the solvent. The x-ray crystal structure of the StAR-related lipid transfer domain of the MLN64 protein also shows the existence of a hydrophobic tunnel large enough to accommodate one (or possibly two) cholesterol molecules (56). As indicated by the very low $K_{SV}$ values obtained with the dynamic quenchers, acrylamide and iodide ion, NBD-cholesterol bound to NPC1 has very low accessibility to the aqueous solution. These results suggest that a similar deep pocket to accommodate sterols also exists in the NPC1 molecule, with the side chain bearing the NBD group deeply buried. This binding pocket is also hydrophobic, as indicated by the large blue shift in $\lambda_{em}$ observed for NBD-cholesterol bound to NPC1, and the large enhancement of fluorescence observed for all three fluorescent sterols on interaction with the protein. Not all substrate-binding sites in sterol-binding proteins display these properties. NBD-cholesterol bound to SCP-2 (sterol carrier protein-2) does not show a shift in the fluorescence emission spectrum, suggesting that the NBD group is located close to the entrance of the binding pocket in contact with the aqueous environment (57).

In summary, fluorescent cholesterol analogs interact with purified NPC1 with high affinity, and have provided novel information on the molecular properties of the sterol-binding site. This includes the relative polarity of the binding site, its proximity to other fluorophores (such as Trp residues within the protein), and the accessibility of the bound sterol to the aqueous medium. The location of the sterol-binding site(s) within the NPC1 molecule is not currently known with certainty. Infante et al. (28) reported that the N-terminal domain expressed as a soluble protein could bind cholesterol and oxysterols with high affinity. However, when a Q79A mutation that abolished sterol binding to this domain was introduced into
Fluorescent Sterol Binding to Purified Human NPC1

full-length NPC1, the protein was still able to restore normal cholesterol trafficking in NPC1-deficient cells. Thus, it is not clear that the sterol-binding site located in the N-terminal domain plays a functional role in cholesterol trafficking by NPC1. It is possible that it represents an oxysterol-binding regulatory site. In any event, based on many other studies reported in the literature (for example Refs. 26, 43), it seems likely that the highly conserved SSD of NPC1 must also be involved in cholesterol binding. Indeed, the purified membrane domain of SCAP, which consist of eight TM helices and includes the SSD, was previously shown to bind [3H]cholesterol with high affinity (47). Further work is clearly needed to determine the precise location of the sterol-binding site(s) within the protein.

Acknowledgments—We thank Dr. H. Ninomiya (Tottori University Faculty of Medicine, Yonago, Japan) for the NPC1+ cells, Dr. D. Ory for supplying anti-NPC1 polyclonal antibody, and Dr. F. Maxfield for providing cholestatin.

REFERENCES

1. Pentchev, P. G. (2004) Biochim. Biophys. Acta 1685, 3–7
2. Ory, D. S. (2000) Biochim. Biophys. Acta 1529, 331–339
3. Zervas, M., Somers, K. L., Thrall, M. A., and Walkley, S. U. (2001) Curr. Biol. 11, 1283–1287
4. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymersopor, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., and Tagle, D. A. (1997) Science 277, 228–231
5. Liscum, L., and Sturley, S. L. (2004) Biochim. Biophys. Acta 1685, 22–27
6. Mukhejee, S., and Maxfield, F. R. (2004) Biochim. Biophys. Acta 1685, 28–37
7. Naureckiene, S., Sleat, D. E., Lackland, H., Fensom, A., Vanier, M. T., and Goldstein, J. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6815–6819
8. Malik, M. T., Millard, E. E., Srivastava, K., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2000) J. Biol. Chem. 275, 38445–38451
9. Loe, D. W., and Sharam, F. J. (1994) Biochim. Biophys. Acta 1190, 72–84
10. Muday, D. W., and Sharom, F. J. (2004) Biochim. Biophys. Acta 1685, 14–21
11. Millard, E. E., Srivastava, K., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2000) J. Biol. Chem. 275, 38445–38451
12. Brandt, J. T., Sharam, F. J., and Chang, T. Y. (2004) Science 305, 356–3570
13. Tukey, B. D., and Sharam, F. J. (2004) J. Biol. Chem. 279, 33586–33592
14. Dixit, S. S., Sleat, D. E., Stock, A. M., and Lobel, P. (2007) Biochim. Biophys. Acta 1763, 1–5
15. Davies, J. P., and Ioannou, Y. A. (2000) J. Biol. Chem. 275, 24367–24374
16. Ioannou, Y. A. (2000) Mol. Genet. Metab. 71, 175–181
17. Ioannou, Y. A. (2000) Mol. Genet. Metab. 71, 175–181
18. Lange, Y., and Steck, T. L. (1998) Curr. Opin. Struct. Biol. 8, 435–439
19. Ioannou, Y. A. (2005) Trends Biochem. Sci. 30, 498–505
20. Davies, J. P., Chen, F. W., and Ioannou, Y. A. (2000) Science 290, 2295–2298
21. Gong, Y., Duvvuri, M., Duncan, M. B., Liu, J., and Krise, J. P. (2006) J. Pharmacol. Exp. Ther. 316, 242–247
22. Malathi, K., Higaki, K., Tinkelenberg, A. H., Balderes, D. A., Almanzar-Paramio, D., Wilcox, L. J., Erdeniz, N., Redican, F., Padamsee, M., Liu, Y., Khan, S., Alcantara, F., Carstea, E. D., Morris, J. A., and Sturley, S. L. (2004) J. Cell Biol. 164, 547–556
23. Ohgami, N., Ko, D. C., Thomas, M., Scott, M. P., Chang, C. C., and Chang, T. Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12473–12478
24. Infante, R. E., Abi-Mosleh, L., Radhakrishnan, A., Dale, J. D., Brown, M. S., and Goldstein, J. I. (2008) J. Biol. Chem. 283, 1052–1063
25. Infante, R. E., Radhakrishnan, A., Abi-Mosleh, L., Kinch, L. N., Wang, M. L., Grishin, N. V., Goldstein, J. L., and Brown, M. S. (2008) J. Biol. Chem. 283, 1064–1075
26. Millard, E. E., Srivastava, K., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2000) J. Biol. Chem. 275, 38445–38451
27. Sugimoto, Y., Ninomiya, H., Ohnaki, Y., Higaki, K., Davies, J. P., Ioannou, Y. A., and Ohno, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12391–12396
28. Davis, H. R., Jr., Zhu, L. J., Hoos, L. M., Tetzloff, G., Maguire, M., Liu, J. J., and Saier, M. H., Jr. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 680–685
29. Matsuzawa, M., Thompson, C., Tinkelenberg, A. H., Balderes, D. A., Almanzar-Paramio, D., Wilcox, L. J., Erdeniz, N., Redican, F., Padamsee, M., Liu, Y., Khan, S., Alcantara, F., Carstea, E. D., Morris, J. A., and Sturley, S. L. (2004) J. Cell Biol. 164, 547–556
30. Ohgami, N., Ko, D. C., Thomas, M., Scott, M. P., Chang, C. C., and Chang, T. Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12473–12478
31. Infante, R. E., Abi-Mosleh, L., Radhakrishnan, A., Dale, J. D., Brown, M. S., and Goldstein, J. I. (2008) J. Biol. Chem. 283, 1052–1063
32. Infante, R. E., Radhakrishnan, A., Abi-Mosleh, L., Kinch, L. N., Wang, M. L., Grishin, N. V., Goldstein, J. L., and Brown, M. S. (2008) J. Biol. Chem. 283, 1064–1075
33. Millard, E. E., Srivastava, K., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2000) J. Biol. Chem. 275, 38445–38451