

Supporting Information

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SI Text

Materials. We obtained [1,2,6,7-³H]cholesterol (60 Ci/mmol) from American Radiolabeled Chemicals; anti-FLAG M2-agarose affinity beads and monoclonal anti-FLAG M2 antibody from Sigma; QuikChange II XL Site-Directed Mutagenesis kit from Stratagene; Texas red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine from Invitrogen; egg yolk L- α -phosphatidylcholine (chicken) from Avanti Polar Lipids, Inc.; FuGENE 6 and Nonidet P-40 from Roche Applied Sciences; and Hybond-C Extra nitrocellulose filters and all chromatography products (unless otherwise stated) from GE Healthcare Biosciences.

Buffers. Buffer A contained 50 mM Tris-chloride (pH 7.4) and 150 mM NaCl. Buffer B contained 50 mM Mes-chloride (pH 5.5 or pH 6.5) and 150 mM NaCl. Buffer C contained 25 mM Tris-chloride (pH 7.5), 150 mM NaCl, and 0.01% (wt/vol) sodium azide. Buffer D contained 25 mM Tris-chloride (pH 7.5), 50 mM NaCl, and 0.01% sodium azide. Buffer E contained 50 mM sodium citrate (pH 4.5) and 150 mM NaCl.

Plasmid Construction. pCMV-NPC2-His-10 encodes human NPC2, followed sequentially by 10 histidines under control of the cytomegalovirus (CMV) promoter. pCMV-NPC2-FLAG encodes human NPC2, followed sequentially by the FLAG sequence DYKDDDDK. Both plasmids were constructed from pCMV-NPC2 (Origene Technologies) by site-directed mutagenesis (QuikChange II XL kit). The P120S mutant version of NPC2 was produced by site-directed mutagenesis of pCMV-NPC2-His-10. pCMV-NPC1 (1–264)-LVPRGS-His-8-FLAG encodes human NPC1 (1–264), followed by sequentially a 6-amino acid thrombin cleavage site, eight histidines, and a FLAG tag under control of the CMV promoter. This plasmid was constructed from pCMV-NPC1 (1–264)-His-8-FLAG (1) by site-directed mutagenesis using the 5'-oligonucleotide, 5'-CCT GCT CCC TGG ACG ATC CTT GGC TTA GTC CCC CGA GGC AGC CAT CAC CAT CAC CAT CAC CAT CAC GAC TAT AAA-3'; and the 3'-oligonucleotide 5'-TTT ATA GTC GTG ATG GTG ATG GTG ATG GTC GCC TCG GGG GAC TAA GCC AAG GAT CGT CCA GGG AGC AGG-3'. The coding region of each plasmid was sequenced to ensure integrity of the construct.

When this plasmid is expressed in CHO-K1 cells, the resulting protein (after signal peptide cleavage) consists of the N-terminal domain (NTD) of NPC1 (amino acids 25–264) (1). This protein is hereafter referred to as NPC1(NTD).

Purification of Epitope-Tagged NPC2 from Medium of Transiently Transfected CHO Cells. CHO-K1 cells were set up on day 0 at 6×10^5 cells per 100-mm dish in medium A (1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium, 100 units/ml penicillin, and 100 μ g/ml streptomycin sulfate) containing 5% (vol/vol) FCS, grown in monolayer at 37°C in 8–9% CO₂, and transfected on day 2 with 5 μ g of wild-type or mutant pCMV-NPC2-His-10 or 5 μ g of pCMV-NPC2-FLAG as described (2). On day 3, the medium (7 ml per dish) was switched to medium A containing 1% (vol/vol) Cellgro ITS (Fisher Scientific). After 24 h, the medium was collected, and fresh medium A containing 1% ITS was added. This cycle of collection/replenishment of medium was repeated for three consecutive days. All subsequent operations were carried out at 4°C. The medium from each daily collection was centrifuged at $1,800 \times g$ for 5 min, filtered through an Express Plus 0.22- μ M filter

apparatus (Millipore), stored at 4°C for up to 7 days, and then loaded onto a 30-ml column (Bio-Rad) filled with either a 20-ml slurry of Ni-NTA-agarose beads (Qiagen) for purification of wild-type or mutant versions of NPC2-His-10 or a 5-ml slurry of anti-FLAG M2-agarose affinity beads for purification of NPC2-FLAG. Each column was preequilibrated with 4 column volumes of buffer A. A total of 1 liter of medium was applied to each column (flow rate of ≈ 1 ml/min over ≈ 16 h). Each column was then washed sequentially with 5 column volumes of buffer A with 20 mM imidazole and then with 40 mM. Bound protein was eluted with buffer A supplemented either with 250 mM imidazole for Ni-NTA-agarose beads or with 0.1 mg/ml FLAG peptide for anti-FLAG beads. Eluted fractions containing wild-type or mutant versions of NPC2-His-10 or NPC2-FLAG were each concentrated to 0.5 ml in a spin concentrator using an Amicon Ultracel 10K filter device (Millipore). The concentrated material was then subjected to gel-filtration chromatography on a 24-ml Superdex-200 column preequilibrated with buffer A. Fractions containing the peak A²⁸⁰ activity, which eluted between 15.5 and 17.5 ml, were pooled, and their protein content was quantified with the BCA kit (Pierce). Pooled proteins were subjected to 13% SDS/PAGE, followed by Coomassie staining to determine purity.

Stably Transfected CHO Cells Expressing NPC1(NTD). CHO-K1 cells were grown in monolayer at 37°C in 8–9% CO₂. On day 0, cells were plated at a density of 6×10^6 cells per 100-mm dish in medium A containing 5% FCS. On day 3, the cells were cotransfected with 0.3 μ g of pcDNA3.1 and 2.0 μ g of pCMV-NPC1 (1–264)-LVPRGS-His-8-FLAG using FuGENE 6 as described (3). Twenty-four hours after transfection, the medium was switched to medium A containing 5% FCS and 700 μ g/ml G418 to select for cells expressing the *neo*-containing plasmid. Fresh medium was added every 2–3 days until colonies formed at ≈ 14 days. Individual colonies were isolated with cloning cylinders and subcloned by dilution plating. Expression of NPC1(NTD)-LVPRGS-His-8-FLAG was assessed by immunoblot analysis. Once a stably transfected cell line was established, it was grown in roller bottles as described below.

Purification of NPC1(NTD) from Medium of Stably Transfected CHO Cells. The above stably transfected CHO cell line expressing NPC1(NTD)-LVPRGS-His-8-FLAG was set up for experiments on day 0 in 850-cm² roller bottles (Falcon) containing 100 ml of medium A supplemented with 5% FCS and 500 μ g/ml G418. The cells were cultured at 37°C at a CO₂ concentration of 8%. On day 4, the medium was switched to 100 ml of medium A containing 5% FCS and 1% ITS. After 3–4 days, the medium was collected, and fresh medium A containing 5% FCS and 1% ITS was added. This 3–4-day cycle of collecting/replenishing the medium was done over a 3-week period. After each collection, the medium was centrifuged at 2,500 rpm for 5 min at 4°C, filtered through an Express Plus 0.22- μ M filter apparatus, and stored at 4°C for up to 2 weeks.

All subsequent operations were carried out at 4°C. One liter of filtered medium was concentrated, exchanged into buffer C to a final volume of 150 ml by using a 10-kDa Pellicon 2 Ultrafiltration Module (Millipore), loaded onto a 10-ml Ni-NTA-agarose column, and washed sequentially with 100 ml of buffer C and 100 ml of buffer C containing 25 mM imidazole. Bound protein was eluted with buffer C containing 250 mM imidazole and buffer-exchanged into buffer D by successive concentration/

dilution using a 10K Amicon Ultracel Centrifugal Filter device (Millipore). The concentrated protein was loaded onto a 7-ml MonoQ column, washed with 70 ml of buffer D, and eluted by using a linear gradient from 50–500 mM NaCl. Fractions containing NPC1(NTD)-LVPRGS-His-8-FLAG were concentrated and subjected to size-exclusion chromatography using a 24-ml Superdex 200 column equilibrated with buffer C. Fractions containing NPC1(NTD)-LVPRGS-His-8-Flag were pooled and concentrated.

In some cases, the His-8 and FLAG epitope tags of the NPC1(NTD) protein were removed by incubation with 5 units of thrombin (Cat. No. T6634; Sigma) for 1 h at 25°C, followed by 11 h at 4°C. The digest was passed through a Ni-NTA column equilibrated with buffer C to separate cleaved and uncleaved forms of NPC1(NTD). The flow-through was diluted to 50 mM NaCl and purified by MonoQ and Superdex 200 chromatography as described above. The resulting purified protein is referred to as NPC1(NTD)-LVPR.

Isolation of Complexes of [³H]Cholesterol-NPC1(NTD) and [³H]Cholesterol-NPC2. Each reaction contained, in a final volume of 300 μ l of buffer B (pH 5.5), 500 nM [³H]cholesterol (132×10^3 dpm/pmol; delivered in ethanol at final concentration of 3%) and one of the following NPC proteins: 30 μ g of NPC2-His-10, 30 μ g of NPC2-FLAG, 60 μ g of NPC1(NTD)-LVPRGS-His-8-FLAG, or 60 μ g of NPC1(NTD)-LVPR, each delivered in 3–30 μ l of buffer A. The final pH of the reaction was 5.5. After incubation for 24–48 h at 4°C, the mixture was passed through a 24-ml Superdex-200 column preequilibrated with buffer B (pH 5.5). Protein-bound [³H]cholesterol emerged between 15.5 and 17.5 ml for NPC2 and between 13.5 and 15.5 ml for NPC1(NTD). The respective pooled fractions were used for the [³H]cholesterol transfer assays described below.

Preparation of Liposomes. Liposomes were generated by using a standard sonication procedure (4). Briefly, a chloroform solution (1 ml) containing 9.8–10 mg of egg yolk L- α -phosphatidylcholine (PC) and 10 μ g of Texas red dye in the absence or presence of 0.2 mg of [³H]cholesterol (930 dpm/pmol; ≈ 4 mole

%) was added to a round-bottom flask. The solvent was evaporated under a stream of nitrogen, leaving behind a thin uniform film of lipid. The flask was placed under vacuum for at least 24 h to remove any trace of organic solvent, sealed, and stored at –20°C until use. The dry lipid film was hydrated by the addition of 2 ml of buffer A. After incubation at room temperature for 30 min, the large multilamellar vesicle suspension was disrupted with a Branson tip-sonicator until the suspension cleared. Metal particles from the sonicator tip and undisturbed lipid aggregates were removed by centrifugation at $100,000 \times g$ for 30 min at 4°C. The resulting hazy supernatant, composed primarily of small unilamellar vesicles, was stored at 4°C for a maximum of 5 days before use.

The concentration of liposome solutions was determined by a malachite green colorimetric assay of inorganic phosphate released from phospholipids after acidic digestion (5). The location of liposomes in column fractions during nickel agarose chromatography was tracked by measuring the fluorescence of Texas red dye (excitation/emission wavelengths: 595/615 nm).

Immunoblot Analysis. After SDS/PAGE on 13% gels, proteins were transferred to Hybond-C Extra nitrocellulose filters. The filters were incubated at room temperature with one of the following primary antibodies: 1.0 μ g/ml monoclonal anti-FLAG M2 antibody and 1/1,000 dilution of polyclonal antibodies against human NPC1(NTD)-His-8-FLAG and NPC2-His-10 (see below). Bound antibodies were visualized by chemiluminescence (Super Signal Substrate; Pierce) by using a 1:5,000 dilution of donkey anti-mouse IgG (Jackson ImmunoResearch) or a 1:2,000 dilution of anti-rabbit IgG (Amersham) conjugated to horseradish peroxidase. Filters were exposed to Phoenix Blue X-Ray Film (F-BX810; Phoenix Research Products) at room temperature for 1–60 s.

Polyclonal antibodies directed against recombinant human NPC1(NTD)-His-8-FLAG [purified as previously described (1)] and NPC2-His-10 (purified as described above) were produced by immunizing each rabbit s.c. with 500 μ g of the recombinant protein in incomplete Freund's adjuvant, followed by alternating s.c. booster injections every 2 weeks of 250 μ g of the protein.

1. Infante RE, et al. (2008) Purified NPC1 protein: II. Localization of sterol binding to a 240-amino acid soluble luminal loop. *J Biol Chem* 283:1064–1075.
2. Infante RE, et al. (2008) Purified NPC1 protein: I. Binding of cholesterol and oxysterols to a 1278-amino acid membrane protein. *J Biol Chem* 283:1052–1063.
3. Rawson RB, DeBose-Boyd RA, Goldstein JL, Brown MS (1999) Failure to cleave sterol regulatory element-binding proteins (SREBPs) causes cholesterol auxotrophy in Chinese hamster ovary cells with genetic absence of SREBP cleavage-activating protein. *J Biol Chem* 274:28549–28556.

4. Lasch J, Weissig V, Brandl M (2003) in *Liposomes: A Practical Approach*, eds Torchilin V, Weissig V (Oxford Univ. Press, New York), pp 3–29.
5. Chalvardjian A, Rudnicki E (1970) Determination of lipid phosphorus in the nanomolar range. *Anal Biochem* 36:225–226.

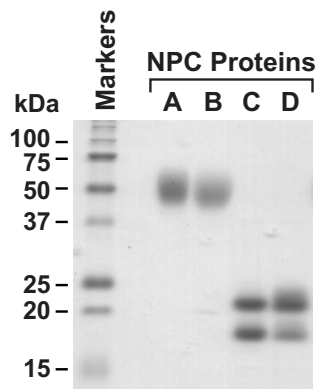


Fig. S1. Coomassie staining of purified NPC proteins. Recombinant human NPC1(NTD) and human NPC2 proteins were purified as described in *Methods*. Aliquots (5 μ g) of NPC1(NTD)-LVPRGS-His-8-FLAG (A), NPC1(NTD)-LVPR (B), NPC2-FLAG (C), and NPC2-His-10 (D) were subjected to 13% SDS/PAGE, and the proteins were visualized with Coomassie Brilliant blue R-250 stain (Bio-Rad). Molecular masses of protein standards are indicated.

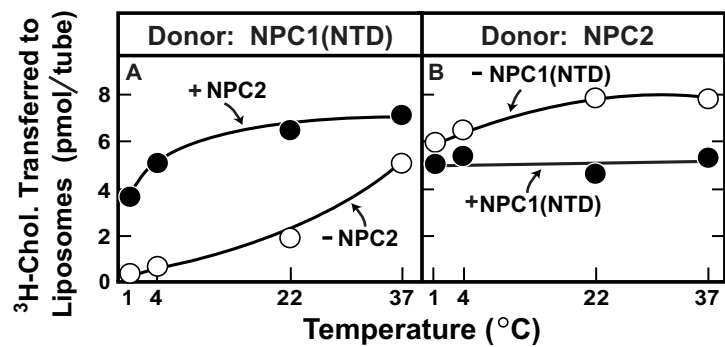


Fig. 54. Transfer of [³H]cholesterol to PC liposomes as a function of temperature. Each reaction, in a final volume of 200 μ l of buffer B (pH 5.5), contained \approx 50 pmol of either NPC1(NTD)-LVPRGS-His-8-FLAG (A) or NPC2-His-10 (B) each complexed to [³H]cholesterol (0.8 and 1.1 pmol, respectively; 132×10^3 dpm/pmol) and 40 μ g of PC liposomes labeled with Texas red dye in the absence (open circles) or presence (filled circles) of 100 pmol of either NPC2-His10 (A) or NPC1(NTD)-LVPRGS-His-8-FLAG (B). After incubation for 30 min at the indicated temperature, the amount of [³H]cholesterol transferred to liposomes was measured in the Ni-NTA-agarose cholesterol transfer assay. Each value is the average of duplicate assays and represents the amount of [³H]cholesterol transferred to liposomes. Blank values in the absence of liposomes (0.07 pmol) were subtracted.