

## Purified NPC1 Protein: I. Binding of Cholesterol and Oxysterols to a 1278-Amino Acid Membrane Protein\*

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The Niemann-Pick, Type C1 protein (NPC1) is required for the transport of lipoprotein-derived cholesterol from lysosomes to endoplasmic reticulum (ER). The 1278-amino acid, polytopic membrane protein has not been purified, and its mechanism of action is unknown. Unexpectedly, we encountered NPC1 in a search for a membrane protein that binds 25-hydroxycholesterol (25-HC) and other oxysterols. A 25-HC binding protein was purified more than 14,000-fold from rabbit liver membranes and identified as NPC1 by mass spectroscopy. We prepared recombinant human NPC1 and confirmed its ability to bind oxysterols, including those with a hydroxyl group on the 24, 25, or 27 positions. Hydroxyl groups on the 7, 19, or 20 positions failed to confer binding. Recombinant human NPC1 also bound [<sup>3</sup>H]cholesterol in a reaction inhibited by Nonidet P-40 above its critical micellar concentration. Low concentrations of unlabeled 25-HC abolished binding of [<sup>3</sup>H]cholesterol, but the converse was not true, i.e., unlabeled cholesterol, even at high concentrations, did not abolish binding of [<sup>3</sup>H]25-HC. NPC1 is not required for the known regulatory actions of oxysterols. Thus, in NPC1-deficient fibroblasts 25-HC blocked the processing of sterol regulatory element-binding proteins and activated acyl-CoA: cholesterol acyltransferase in a normal fashion. The availability of assays to measure NPC1 binding *in vitro* may further the understanding of ways in which

oxysterols regulate intracellular lipid transport.

### INTRODUCTION

Despite intense scientific interest, the mechanism by which cholesterol is transported from one membrane compartment to another in animal cells remains obscure. One transport pathway begins in lysosomes where cholesterol is liberated from plasma lipoproteins that have entered the cell through receptor-mediated endocytosis (1). This cholesterol is transported from the lysosome to the endoplasmic reticulum (ER)<sup>2</sup> where it performs vital regulatory functions, and where excess cholesterol is stored by conversion to cholesteryl esters through the action of the ER enzyme acyl-CoA:cholesterol acyltransferase (ACAT). A clue to the mechanism of this cholesterol movement comes from observations in cells from patients with Niemann-Pick Type C1 (NPC1) disease (2). These individuals accumulate large amounts of cholesterol and sphingolipids in lysosomes throughout the body, particularly the liver and brain. As a result, they suffer from severe neurological disorders and hepatic failure.

Cells from patients with NPC1 disease were shown to have a defect in the exit of lipoprotein-derived cholesterol from lysosomes (3-5). The defect in most of these patients lies in the gene encoding a protein designated NPC1 (6) that is found on membranes of lysosomes and endosomes. The human version contains 1278 amino acids, and its sequence predicts 13 membrane-spanning helices separated by a variety of loops, three of which are quite

large (~240 amino acids) that project into the lumen of endosomes or lysosomes (7). The sequence of five of the transmembrane helices (helices 3 to 7) resembles a sequence that has been observed in other polytopic membrane proteins, including Scap, the protein that transports SREBPs from ER to Golgi; 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase), the rate-controlling enzyme in cholesterol synthesis; and Patched, a regulatory protein in the Hedgehog pathway (8-10). In Scap, this sequence has been shown to bind cholesterol (11), and it has been called the sterol-sensing domain (9). Whether this sequence binds cholesterol in NPC1, HMG CoA reductase, or Patched has not yet been determined.

The mechanism by which NPC1 facilitates cholesterol transport is unknown. The protein has never been purified from native membranes, and recombinant forms of the protein have never been studied *in vitro*. In one study, a photoactivated cholesterol derivative was shown to crosslink to the NPC1 protein when added to intact cells, but the characteristics and specificity of the binding reaction were not determined (12).

In the current paper, we describe the purification of a membrane-bound protein that binds 25-hydroxycholesterol (25-HC). 25-HC belongs to a class of sterols called oxysterols because they contain, in addition to the hydroxyl group on the 3-position, another hydroxyl group, usually on the iso-octyl side chain. We were searching for such a protein because 25-HC and related oxysterols mimic the regulatory actions of cholesterol in the ER of animal cells. Thus, 25-HC blocks the ER-to-Golgi transport of sterol regulatory element-binding proteins (SREBPs), which are transcriptional activators for synthesis of cholesterol and other lipids (13); it accelerates the degradation of HMG CoA reductase (14); and it activates ACAT (15).

Working with membranes from rabbit liver, we purified a protein that binds 25-HC with high affinity. We designated

this protein as “membrane-bound oxysterol-binding protein” (MOBP) to distinguish it from the “soluble oxysterol-binding protein” (OSBP-1), which was purified and its cDNA cloned in our laboratory 20 years ago (16, 17). Peptide sequencing by mass spectroscopy identified MOBP as NPC1. We purified recombinant human NPC1 and confirmed that it binds 25-HC. We also found that the recombinant protein binds cholesterol, but only when the detergent is reduced to submicellar concentrations. We here describe the initial purification of rabbit NPC1 and an analysis of the sterol binding properties of the rabbit and human proteins. This work provides a beginning for the eventual analysis of the mechanism by which NPC1 transports cholesterol from lysosomes to ER.

## EXPERIMENTAL PROCEDURES

**Materials** – We obtained [26,27-<sup>3</sup>H]25-hydroxycholesterol (75-80 Ci/mmol), [1,2,6,7-<sup>3</sup>H]cholesterol (60 Ci/mmol), [3β-<sup>3</sup>H]7,7-azocholistan-3β,25-diol (20 Ci/mmol) (photo [<sup>3</sup>H]25-HC), and [1-<sup>14</sup>C]oleic acid (54.6 mCi/mmol) from American Radiolabeled Chemicals; Fos-Choline 13 from Anatrace; anti-Flag M2-Agarose affinity beads and 6-methylcholesterol from Sigma; all other sterols from Steraloids; FuGENE 6 and Nonidet P-40 (NP-40) from Roche Applied Sciences; and Hybond C nitrocellulose filters and all chromatography products (unless otherwise mentioned) from GE Healthcare Biosciences. Rabbit β-migrating very low density lipoproteins (β-VLDL) (d < 1.006) (18) and human and newborn calf lipoprotein-deficient serum (LPDS, d > 1.215 g/ml) (19) were prepared by ultracentrifugation as described in the indicated reference. Solutions of compactin and sodium mevalonate were prepared as previously described (20, 21). Recombinant hamster His<sub>10</sub>-Scap(TM1-8) was produced in Sf9 cells and purified as previously described (11).

**Buffers** – Buffer A contains 50 mM Tris-chloride at pH 7.4, 50 mM KCl, 10%

(v/v) glycerol, 5 mM dithiothreitol (DTT), 1 mM sodium EDTA, and protease inhibitor mixture (1  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 200  $\mu$ M phenylmethanesulfonyl fluoride). Buffer B contains 50 mM Tris-chloride at pH 7.4, 10% (w/v) NP-40, 1 mM EDTA, and 0.1 mM DTT. Buffer C contains 50 mM Tris-chloride at pH 7.4, 1% NP-40, 1 mM EDTA, and 0.1 mM DTT. Buffer D contains 50 mM MES-chloride at pH 5.5, 1% NP-40, 1 mM EDTA, and 0.1 mM DTT. Buffer E contains 10 mM sodium phosphate at pH 7.4, 1% NP-40, 150 mM KCl, 1 mM EDTA, and 0.1 mM DTT. Buffer F contains 50 mM Tris-chloride at pH 7.4, 1% NP-40, and 150 mM KCl. Buffer G contains 50 mM Tris-chloride at pH 7.4, 1% NP-40, and 100 mM KCl. Buffer H contains 50 mM Tris-chloride at pH 7.4, 0.1% (w/v) Fos-Choline 13, and 100 mM NaCl. Buffer I contains 50 mM Tris-chloride at pH 7.4, 0.004% NP-40, and 150 mM NaCl.

**Plasmid Construction** – pCMV-NPC1-His<sub>8</sub>-Flag encodes wild-type human NPC1 followed sequentially by eight-histidines and a Flag tag under control of the cytomegalovirus (CMV) promoter. This plasmid was constructed from pCMV-NPC1 (Origene Technologies) by site-directed mutagenesis (QuickChange II XL kit, Stratagene). The coding region of pCMV-NPC1-His<sub>8</sub>-Flag was sequenced to ensure integrity of the construct.

**Solubilization of Sterols in Detergent or Ethanol** – For solubilization of sterols in detergent, an ethanol solution containing 4-5 nmol of [<sup>3</sup>H]25-hydroxycholesterol ([<sup>3</sup>H]25-HC) at 75-80 Ci/mmol was evaporated to dryness on the sides of a microcentrifuge tube in a SpeedVac concentrator. Detergent-containing buffer G or buffer H (0.5 ml) was then added to the tube, after which it was agitated for 4 h on a vortex mixer and centrifuged at 20,000g for 30 min at room temperature. As determined by scintillation counting, the concentration of solubilized [<sup>3</sup>H]25-HC in the supernatant ranged from 0.4-1.5  $\mu$ M. Stock solutions of [<sup>3</sup>H]cholesterol at 60 Ci/mmol were

prepared in a similar manner. Saturated solutions of unlabeled sterols in buffer G or buffer H were prepared by evaporating an ethanolic solution of each sterol (50  $\mu$ g) in a microcentrifuge tube in a SpeedVac concentrator. The dried sterol was solubilized by the same procedure as above. The recovery was estimated by adding tracer amounts of [<sup>3</sup>H]25-HC or [<sup>3</sup>H]cholesterol, which allowed calculation of the concentrations of various sterol solutions. The choice of the labeled tracer sterol was based on structural similarity to the bulk sterol.

Sterols not solubilized in detergent were dissolved in 100% ethanol and delivered to reaction mixtures at a final ethanol concentration of 1-4%. In a given experiment, all reactions received the same amount of ethanol.

**Filter Assay for [<sup>3</sup>H]25-HC Binding** – Each reaction, in a final volume of 120  $\mu$ l of buffer C, contained 50 nM [<sup>3</sup>H]25-HC (165-180 dpm/fmol) delivered in ethanol and the indicated amount of liver extract, column fraction, or purified protein. After incubation for 2 h at 4°C, the mixture was loaded onto a pretreated glass-fiber filter (G4, Fisher) placed on a vacuum apparatus that contained 1 ml of buffer F. The filter was pretreated by soaking in 0.3% (w/v) polyethyleneimine for at least 1 h. After application of the vacuum, the free [<sup>3</sup>H]25-HC was removed by washing with 2.5 ml of buffer F. The filter was then dried and immersed in 8 ml of scintillation fluid (3a70B, Research Product International). Bound [<sup>3</sup>H]25-HC was measured by scintillation counting. Nonspecific binding was determined by incubating a duplicate reaction with 10  $\mu$ M unlabeled 25-HC delivered in ethanol.

**Ni-NTA Agarose Assay for [<sup>3</sup>H]Sterol Binding** – Each reaction was carried out in a final volume of 80-140  $\mu$ l of buffer G, buffer H, or buffer I containing the indicated amount of purified NPC1-His<sub>8</sub>-Flag and 10-400 nM [<sup>3</sup>H]25-HC (165-180 dpm/fmol) or [<sup>3</sup>H]cholesterol (130 dpm/fmol) delivered in buffer G, buffer H, or ethanol. After

incubation for 3 h at 4°C, the mixture was loaded onto a column packed with 0.3 ml of Ni-NTA agarose beads (Qiagen). Each column was washed for ~15 min with 6 ml of buffer G. The protein-bound  $^3\text{H}$ -sterol was eluted with 200 mM imidazole in buffer G and quantified by scintillation counting as previously described (11). For competition experiments with unlabeled sterols, the standard assays were carried out in the presence of buffer G, buffer H, or ethanol containing the indicated unlabeled sterol (0–10  $\mu\text{M}$ ).

*Solubilization of MOBP from Rabbit Liver Membranes* – All operations were carried out on ice or at 4°C. Frozen rabbit liver (200–300 grams, Pel-Freez Biologicals) was thawed in 300 ml of cold buffer A supplemented with 25  $\mu\text{g}/\text{ml}$  N-acetyl-leucinal-leucinal-norleucinal (ALLN). The thawed tissue was shredded into small pieces in an Oster blender, homogenized with a Polytron homogenizer, and filtered through a triple-layered cheese cloth. The filtrate was again homogenized with a 100-ml Dounce homogenizer and then centrifuged at 100,000g for 1 h. The resulting 100,000g pellet was washed in three sequential steps, each followed by centrifugation at 100,000g. The first and third steps employed 300 ml of buffer A supplemented with 450 mM KCl and protease inhibitors; the second step employed 300 ml of 100 mM  $\text{Na}_2\text{CO}_3$  (pH 11.3) and protease inhibitors. The final washed membrane pellet was resuspended in 300 ml of buffer C containing the protease inhibitor mixture. Protein concentration was measured using a BCA kit (Pierce), and the final detergent (NP-40) to protein ratio was adjusted to 4.5 (w/w) with buffer B. This mixture was rotated overnight at 4°C and centrifuged at 100,000g for 30 min, after which the supernatant was collected.

*Purification of MOBP from Detergent-solubilized Rabbit Liver* – All operations were carried out at 4°C. The filter binding assay described above was used to follow [ $^3\text{H}$ ]25-HC binding activity through each of the seven steps in the

purification (Table 1). The content of OSBP-1 was followed by immunoblotting. A solubilized extract of rabbit liver membranes was prepared as described above (*Step 1*). As described in Fig. 1, the soluble extract was loaded onto a 20-ml Q-Sepharose ion-exchange column (Hi Prep 16/10 Q FF, pH 7.4) (Table 1, *Step 2*). Active fractions that were devoid of immunoblot reactivity for OSBP were pooled from multiple runs and frozen at -80°C. Pooled material was thawed and dialyzed against buffer D for 6–12 h and then loaded onto a 20-ml SP-Sepharose ion-exchange column (Hi Prep 16/10 SP FF) pre-equilibrated with buffer D (*Step 3*). The flow-through was collected and loaded onto a 5-ml Q-Sepharose ion exchange column (HiTrap Q HP, pH 5.5) pre-equilibrated with buffer D (*Step 4*). The column was washed with 8 column volumes of buffer D, and bound proteins were eluted with a continuous KCl gradient (0–500 mM) in buffer D. The fractions containing binding activity were dialyzed against buffer E for 6–12 h. The dialyzed material was loaded onto 5 to 10 2-ml RCA Lectin columns (EY Laboratories) pre-equilibrated with buffer E (*Step 5*). Each column was washed with 3 column volumes of buffer E, and proteins were eluted with buffer E plus 250 mM  $\alpha$ -Lactose. The eluted fractions containing binding activity were pooled, dialyzed into buffer C, and concentrated by loading onto a 1-ml Mono-Q ion-exchange column pre-equilibrated in buffer C. The column was washed with 8 column volumes of buffer C and eluted with a continuous KCl gradient (0–500 mM). The fractions containing binding activity were loaded onto a 320-ml Sephacryl 300 gel filtration column (HiPrep 26/60 S-300) pre-equilibrated with buffer C containing 100 mM KCl (*Step 6*). The fractions eluting between 112 and 140 ml (containing peak binding activity) were pooled and mixed with an equal volume of buffer C to decrease the salt concentration. The diluted fractions (containing ~50 mM KCl) were concentrated over a 1-ml Mono-Q ion-exchange column. After washing



with 8 column volumes of buffer C, proteins were eluted with a continuous KCl gradient (0-500 mM). The eluted fractions containing 25-HC binding activity were pooled and loaded onto a 2-ml Reactive Blue Dye 72 column (Sigma) that was pre-equilibrated with buffer C (Step 7). The column was washed with 4 column volumes of buffer C, and the bound protein was eluted with a step KCl gradient (100, 250, and 500 mM KCl) in buffer C. Binding activity was found primarily in the 250-mM KCl fraction.

**Photoaffinity Labeling of MOBP** – All experiments were performed in the dark. Each reaction, in a final volume of 3 ml, contained 0.133 mg of purified protein (fraction eluting from Reactive Blue 72 column (Table 1) and 500 nM of photo [<sup>3</sup>H]25-HC in the absence or presence of 10 μM unlabeled 25-HC. After overnight incubation at 4°C, each reaction was subjected to 20 min of UV irradiation (13) and loaded onto a 1-ml Mono-Q ion-exchange column pre-equilibrated with buffer C. After washing with 12 column volumes of buffer C, bound protein was eluted with a salt gradient (0-500 mM KCl). Radioactivity of each fraction was quantified by applying 20-μl aliquots into a scintillation vial filled with 8 ml of scintillation fluid 3a70b. Samples with the highest radioactivity were subjected to 8% SDS-PAGE. Individual gel lanes were cut into 3-mm slices and placed into scintillation vials. Two ml of tissue solubilizer (TS-2, Research Product International) were added to each vial. After incubation overnight at 50°C, each sample received 400 μl of glacial acetic acid, after which it was shaken and then filled with 8 ml of scintillation fluid. The vials were stored in the dark for 6 h before radioactivity was measured.

**LC-MS/MS Analysis of Photo [<sup>3</sup>H]25-HC Labeled Proteins** – Protein samples were separated by SDS-PAGE and then subjected to in-gel tryptic digestion followed by HPLC/tandem mass spectrometry analysis as previously described (22).

**Purification of Epitope-tagged NPC1 from Transfected CHO Cells** – Stock cultures of CHO-K1 cells were grown in monolayer at 37°C in an atmosphere of 8-9% CO<sub>2</sub> and maintained 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 μg/ml streptomycin sulfate) containing 5% (v/v) fetal calf serum (FCS). On day 0, CHO-K1 cells were set up for experiments in medium A containing 5% FCS at 6x10<sup>5</sup> cells/100-mm dish. On day 2, each dish was transfected with 4-5 μg of pCMV-NPC1-His<sub>8</sub>-Flag in medium A with 5% FCS, using FuGENE 6 reagent as described (23). After incubation at 37°C for 24 h, the cells were harvested, washed, and resuspended in ice-cold buffer A containing 25 μg/ml ALLN. Cells were homogenized with a 15-ml or 40-ml Dounce homogenizer, and then subjected to 100,000g centrifugation for 30 min at 4°C. The membrane pellet was resuspended by Dounce homogenization in buffer C containing the protease inhibitor mixture and 25 μg/ml ALLN (4 dishes of cells per 1 ml of buffer), incubated overnight at 4°C to solubilize membrane proteins, and centrifuged at 100,000g for 30 min. The resulting 100,000g supernatant (containing detergent-solubilized membranes) was dialyzed against buffer G for 6-12 h at 4°C, after which imidazole was added at a final concentration of 20 mM. This material was then loaded onto a 1-ml His Trap HP nickel column pre-equilibrated with buffer G. The column was washed sequentially with 10 column volumes of buffer G, 10 column volumes of buffer G plus 25 mM imidazole, and 20 column volumes of buffer G plus 50 mM imidazole. Bound protein was eluted in 1.5-ml fractions with buffer G plus 200 mM imidazole. The eluted fractions containing anti-Flag (NPC1) immunoblot reactivity were then loaded onto a column containing 1-ml Anti-Flag M2-Agarose beads (Sigma), which had been pre-equilibrated with buffer G. The column was washed with either 10 column volumes of buffer G (1% NP-40) or 25 column volumes of buffer H (0.1% Fos-

Choline 13). Bound protein was eluted with 0.1 mg/ml of Flag peptide in 9 column volumes of either buffer G or buffer H. Protein concentration of purified NPC1 was estimated by silver staining and densitometric scanning of an 8% SDS-PAGE gel in which known amounts (50 to 400 ng) of bovine serum albumin (Pierce) was used as a reference.

*Preparation of Epitope-tagged NPC1 at Low Detergent Concentration.* The fractions containing eluted protein (in 1% NP-40) from the His Trap HP nickel column as described in the above section were loaded onto a 1-ml anti-Flag M2-Agarose affinity column and then washed with 6 column volumes of buffer I (in 0.004% NP-40). Bound protein was eluted with 0.1 mg/ml of Flag peptide in 9 column volumes of buffer I.

*SREBP-2 Processing in Cultured Cells* – Skin fibroblasts from a normal subject and a patient with NPC1 disease (obtained from American Type Culture Collection, ATTC No. GM3123) were immortalized with the catalytic subunit of telomerase (hTERT) by retroviral infection of the cells with the Babepuro-hTERT vector as previously described (24, 25). The resulting immortalized cell lines are designated hTERT-Control and hTERT-NPC1. The cells were grown in monolayer at 37°C in 5% CO<sub>2</sub> and maintained in medium B (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 µg/ml streptomycin sulfate) supplemented with 10% FCS. On day 0, hTERT-Control and hTERT-NPC1 fibroblasts were set up in medium B containing 10% FCS at 6x10<sup>4</sup> and 7x10<sup>4</sup> cells/60-mm dish, respectively. On day 2, cells were washed once with phosphate-buffered saline and switched to medium B containing 5% human LPDS. On day 4, cells were switched to medium C (medium B containing 50 µM compactin, 50 µM sodium mevalonate) supplemented with 10% human LPDS and various concentrations of 25-HC or β-VLDL. After incubation for 5 h, cells were treated with 25

µg/ml ALLN for 1 h and then harvested. Six dishes for each condition were pooled for preparation of nuclear extract and membrane fractions, which were analyzed by immunoblotting for SREBP (described below).

Primary fibroblast cultures from a wild type BALB/c mouse and a mutant BALB/c *npc<sup>nih</sup>* mouse (6) were established from explants of skin taken at 7 to 8 weeks of age (26). Cells were cultured and set up for experiments as described above for the human fibroblasts except that the density for plating was 4 x 10<sup>4</sup> cells/60-mm dish. The wild type and mutant mice were obtained from Drs. Stephen Turley and John Dietschy (UT Southwestern Medical Center, Dallas, TX).

*Immunoblot Analysis* – Column fractions from liver purification of MOBP and nuclear extract and membrane fractions from cultured cells were subjected to 8% or 12% SDS-PAGE, after which the proteins were transferred to nitrocellulose filters. The immunoblots were performed at room temperature using the following primary antibodies: 1 µg/ml of a rabbit polyclonal antibody against human NPC1 (Novus); 5 µg/ml of a mouse monoclonal anti-Flag (IgG fraction; Sigma); 4 µg/ml of monoclonal IgG-11H9 directed against rabbit OSBP (IgG fraction) (16); 8 µg/ml of a rabbit polyclonal antibody (IgG-R139) directed against hamster Scap (27); and 10 µg/ml of a rabbit polyclonal antibody (IgG-1819) directed against amino acids 1-100 of human SREBP-2. The latter antibody was raised by injecting rabbits with a His-tagged recombinant version of the antigen. Bound antibodies were visualized by chemiluminescence (SuperSignal Substrate; Pierce) using a 1:5000 dilution of anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) or a 1:2000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences). The filters were exposed to Kodak X-Omat Blue XB-1 film at room temperature.

*ACAT Assay* – The rate of incorporation of [<sup>14</sup>C]oleate into cholesteryl

[ $^{14}\text{C}$ ]oleate and [ $^{14}\text{C}$ ]triglycerides by intact cell monolayers was measured as described previously (19).

## RESULTS

*Purification and Sterol Binding Characteristics of MOBP from Rabbit Livers* – In an attempt to isolate a membrane-bound oxysterol binding protein (MOBP), we carried out a biochemical purification of MOBP from rabbit liver membranes as described in “Experimental Procedures.” MOBP activity was followed during each step of the purification by a filter assay for [ $^3\text{H}$ ]25-HC binding as described in “Experimental Procedures.” The overall scheme and enrichment of MOBP through the various purification steps is shown in Table 1. Fig. 1A shows the result of Q-Sepharose chromatography of detergent-solubilized liver membranes (*Step 2* of the purification scheme in Table 1). The [ $^3\text{H}$ ]25-HC binding activity separated into two discrete peaks (*filled circles*). As shown by the immunoblot in *Inset*, the second peak (fractions 39-51) co-eluted with OSBP-1, a soluble protein with nanomolar affinity for 25-HC (17). The fractions comprising the first peak (fractions 30-35) do not contain OSBP-1 immunoreactivity and were postulated to contain a novel MOBP, which then became the focus of all subsequent purification steps. Gel filtration studies were consistent with a molecular mass of ~440 kDa for this MOBP•detergent complex (Fig. 1B). Fractions 30-35 from several Q-Sepharose chromatography runs were combined, and the 25-HC binding activity was further purified through 5 additional chromatography steps as described in “Experimental Procedures” and Table 1, resulting in an overall enrichment of ~14,000-fold and an 8% recovery of activity relative to the starting solubilized membrane fraction (i.e., *Step 1* in Table 1).

Purified MOBP from *Steps 4* and *5* (see *Table 1*) was used to define the specificity of sterol binding. We tested 13 sterols (listed in Fig. 2B) with modifications either to the tetracyclic sterol nucleus or the

iso-octyl side chain for their ability to compete for binding of 50 nM [ $^3\text{H}$ ]25-HC to MOBP. These assays were carried out in micellar concentrations of NP-40 (1%). As shown in Figs. 2C-2E, sterols 1-3 showed complete competition at 10  $\mu\text{M}$ , whereas sterols 4-13 competed only weakly at 10  $\mu\text{M}$ . Thus, under these assay conditions the presence of a hydroxyl group on the iso-octyl side chain is crucial for sterol binding to MOBP.

*Identification of MOBP as NPC1* – The final purified MOBP fraction from *Step 7* contained 5-8 proteins that could be visualized on silver-stained SDS-PAGE gels. In order to determine which of these bands accounted for the [ $^3\text{H}$ ]25-HC binding activity, we carried out cross-linking studies with photo [ $^3\text{H}$ ]25-HC. When photo [ $^3\text{H}$ ]25-HC is activated by UV light, the diazirine ring is cleaved, generating a carbene radical that crosslinks covalently to interacting proteins (13). The eluate from *Step 7* was incubated with photo [ $^3\text{H}$ ]25-HC in the absence or presence of unlabeled 25-HC, exposed to UV light, and then subjected to Mono-Q chromatography. As shown in Fig. 3A, the eluate showed a distinct peak of radioactivity (*closed circles*), which was diminished by 86% when the incubation was conducted in the presence of unlabeled 25-HC (*open circles*). The peak fraction (number 32) in Fig. 3A was subjected to 8% SDS/PAGE. As shown in the top panel of Fig. 3B, silver staining of the gel revealed 3 distinct and 2 faintly visible protein bands, labeled *Band 1-5*. A duplicate gel lane was cut into 18 equal slices, each of which was assayed for radioactivity (see “Experimental Procedures”). The radioactivity from the crosslinked photo [ $^3\text{H}$ ]25-HC localized almost exclusively to *Band 1* (*filled circles*). Mass spectrometry analysis of *Band 1* yielded sequences of 19 peptides, 17 of which corresponded to NPC1 (Supplementary Table 1). The other 2 peptides corresponded to sialoadhesin. A complete summary of the mass spectrometry results for *Band 1* and *Bands 2-5* can be found in Supplementary Table 1. NPC1 was



chosen for further studies because of its abundance and because it contains a putative sterol-sensing domain, which is found in other proteins involved in cholesterol homeostasis, i.e., Scap and HMG CoA reductase (8-10).

*Purification of Recombinant Human NPC1 and Demonstration of Oxysterol Binding in Presence of 1% NP-40* – To confirm that NPC1 binds oxysterols, we used CHO-K1 cells to express recombinant human NPC1 with a tandem His<sub>8</sub>-tag and Flag-tag at the COOH-terminus. The recombinant protein, designated NPC1-His<sub>8</sub>-Flag, was solubilized with 1% NP-40 from the 100,000g pellet of CHO-K1 membranes. The solubilized NPC1-His<sub>8</sub>-Flag was purified to near homogeneity using Ni-chromatography (Fig. 4A), followed by Flag affinity chromatography (Fig. 4B). Oxysterol binding activity was measured by the aforementioned filter assay (*closed circles*), and protein concentration was measured with the BCA method (*open circles*). The [<sup>3</sup>H]25-HC binding activity co-eluted with NPC1 on both columns as judged by immunoblots in Fig. 4A and 4B. Silver-stained gels of the final purified product subjected to 8% and 12% SDS/PAGE are shown in Fig. 4C and 4D, respectively. The major protein (indicated by *asterisks*) migrated near the 250-kDa marker. This protein reacted with anti-Flag antibody (see immunoblots in Figs. 4A and 4B) and was confirmed to be NPC1 by mass spectrometry. The less intense band above the 250-kDa marker (denoted by arrows in Fig. 4C) also stained with anti-Flag antibody; it most likely represents an oligomerized form of NPC1 that is resistant to SDS denaturation. The faint band between 37-50 kDa is non-muscle  $\gamma$ -actin as indicated by mass spectrometry (data not shown); it likely represents contamination by this abundant cellular protein.

*Binding of 25-HC but not Cholesterol to Recombinant NPC1 in Presence of 1% NP-40 or 0.1% Fos-Choline 13* – We used an assay developed in earlier work on Scap (11) with slight modification

to investigate the binding of <sup>3</sup>H-labeled sterols to NPC1-His<sub>8</sub>-Flag. <sup>3</sup>H-Sterols were solubilized in NP-40, the same detergent used for the solubilization and purification of NPC1-His<sub>8</sub>-Flag. Free and NPC1-bound <sup>3</sup>H-sterols were separated using a column packed with nickel beads. Fig. 5A shows a saturation curve for binding of [<sup>3</sup>H]25-HC to NPC1-His<sub>8</sub>-Flag. The binding was saturable and was diminished in the presence of excess unlabeled 25-HC. Under the conditions of this assay, [<sup>3</sup>H]cholesterol did not bind to NPC1-His<sub>8</sub>-Flag in a saturable fashion (Fig. 5B). Consistent with these results, unlabeled 25-HC but not unlabeled cholesterol competed for binding of [<sup>3</sup>H]25-HC to NPC1 (Fig. 5C).

In previous studies, we showed that [<sup>3</sup>H]cholesterol binds with high affinity and specificity to solubilized Scap (11, 28). Scap resembles NPC1 in that both proteins contain a sterol-sensing domain (9, 10). The binding studies with Scap were carried out in Fos-Choline 13 detergent, whereas the studies with NPC1 used NP-40. In order to assess the possibility that [<sup>3</sup>H]cholesterol is not delivered efficiently to NPC1 in NP-40, we exchanged NPC1-His<sub>8</sub>-Flag into Fos-Choline 13 detergent. Gel filtration studies showed that NPC1 in Fos-Choline 13 detergent was not aggregated and migrated as a ~350 to 400-kDa species (data not shown). We also dissolved [<sup>3</sup>H]cholesterol, [<sup>3</sup>H]25-HC, and their unlabeled versions in Fos-Choline 13 as described in “Experimental Procedures.” Fig. 5D shows that NPC1 solubilized in Fos-Choline 13 bound [<sup>3</sup>H]25-HC with high affinity, but not [<sup>3</sup>H]cholesterol. Under the same detergent conditions Scap bound [<sup>3</sup>H]cholesterol with high affinity, but not [<sup>3</sup>H]25-HC (Fig. 5E). It should be noted that the maximal amount of binding of [<sup>3</sup>H]25-HC to NPC1 in Fig. 5D (after detergent exchange from 1% NP-40 to 0.1% Fos-Choline 13) is several-fold lower than that in Fig. 5A (no detergent exchange), yet the affinity of binding under these two different conditions is similar. This is a consistent finding that we have observed when NPC1 is subjected to the extensive



column washings associated with the detergent exchange.

*Sterol Specificity for Purified Recombinant NPC1 in Presence of 1% NP-40* – We tested the binding specificity of NPC1-His<sub>8</sub>-Flag by measuring the ability of unlabeled oxysterols and cholesterol to compete for binding of 100 nM [<sup>3</sup>H]25-HC (Figs. 6A and 6B). As shown in Figs. 6C and 6D, sterols with hydroxyl groups at positions 24, 25, or 27 competed with [<sup>3</sup>H]25-HC for binding to NPC1-His<sub>8</sub>-Flag. On the other hand, sterols with a hydroxyl group on the 19 or 20 position did not compete for [<sup>3</sup>H]25-HC binding to NPC1-His<sub>8</sub>-Flag. We also tested the ability of various sterol precursors in the cholesterol synthesis pathway, analogs of cholesterol, and other sterols to compete for binding of [<sup>3</sup>H]25-HC, when added at a 50-fold excess concentration. Under the conditions of this *in vitro* assay, none of these precursor sterols, cholesterol analogs, or other sterols competed effectively for binding of [<sup>3</sup>H]25-HC to NPC1-His<sub>8</sub>-Flag (Fig. 6E).

*Binding of Cholesterol to Recombinant NPC1 in Low Concentrations of Detergent* – The failure of NPC1 to bind [<sup>3</sup>H]cholesterol *in vitro* was surprising since NPC1 functions in cholesterol transport and since photoactivated cholesterol binds to NPC1 when added to living cells (12). We speculated that the failure of binding *in vitro* may have been due to the presence of high concentrations of detergent in the assay. Previous studies of cholesterol binding to Scap showed that high concentrations of detergent slowed the binding reaction, but they did not abolish it (11). To lower the concentration of detergent, we solubilized doubly-tagged recombinant human NPC1 and purified the protein on nickel agarose in the standard way in the presence of 1% NP-40. We then bound the protein to anti-FLAG beads, washed the beads extensively in a solution containing 0.004% NP-40, and then eluted the protein in 0.004% NP-40. The NPC1 remained in solution at this low detergent concentration. On gel filtration the protein emerged with an apparent

molecular mass of ~400 kDa, which was the same as its apparent molecular mass in 1% NP-40 (Fig. 7A).

To measure cholesterol binding in low detergent concentrations, we added various concentrations of [<sup>3</sup>H]cholesterol in ethanol to the isolated NPC1 in the presence of 0.004% NP-40. Under these conditions recombinant NPC1 bound [<sup>3</sup>H]cholesterol with saturation kinetics with an apparent  $K_d$  of ~100 nM and a  $B_{max}$  of 1 molecule of [<sup>3</sup>H]cholesterol bound per ~2 NPC1 dimers (or 4 monomers) (Fig. 7B, *left panel*). In 0.004% NP-40, the  $K_d$  for [<sup>3</sup>H]25-HC was ~10 nM, and the  $B_{max}$  was 1 molecule of [<sup>3</sup>H]25-HC bound per ~2 NPC1 dimers (or 4 monomers). In the low detergent concentration (0.004%), recombinant NPC1 bound [<sup>3</sup>H]25-HC with a higher affinity than at a higher detergent concentration (1%) where the  $K_d$  was ~80 nM (compare Fig. 7B, *right panel*, with Fig. 5A).

Competition studies show that binding of [<sup>3</sup>H]cholesterol was inhibited by 25-HC and by 27-HC, but not by epicholesterol, which has a 3 $\alpha$ -hydroxyl group, or by androstenol, which lacks a side chain (Fig. 7C, *left panel*). Inasmuch as epicholesterol is a structural isomer of cholesterol and presumably has the same solubility, the inability of epicholesterol to compete for [<sup>3</sup>H]cholesterol binding suggests that binding specificity and affinity are determined by NPC1's binding pocket rather than differences in solubility of the sterol ligands.

Binding of [<sup>3</sup>H]25-HC was competed by 25-HC and by 27-HC (Fig. 7C, *right panel*). Remarkably, binding of [<sup>3</sup>H]25-HC was not inhibited by cholesterol, even though 25-HC potently inhibited the binding of [<sup>3</sup>H]cholesterol. Binding of [<sup>3</sup>H]25-HC was also not inhibited by epicholesterol or androstenol.

To explore the relation between micellar concentrations of NP-40 and [<sup>3</sup>H]cholesterol binding, we first determined the critical micellar concentration (CMC) for NP-40 at 4°C in the buffer solution used for assays. Using a dye encapsulation assay

(29), we estimated the CMC for NP-40 to be ~0.0015% (data not shown). We then measured [ $^3\text{H}$ ]cholesterol binding to recombinant NPC1 at detergent concentrations ranging between 0.0007% and 0.01% (Fig. 8A). Binding of [ $^3\text{H}$ ]cholesterol remained constant up to 0.002% NP-40 which roughly matches the CMC. Binding declined precipitously as the detergent exceeded the threshold for micelle formation. Fig. 8B shows that the binding of [ $^3\text{H}$ ]25-HC was not decreased by NP-40 at concentrations up to 0.1%, which greatly exceeded the CMC.

#### *NPC1 Not Required for Oxysterol-mediated Inhibition of SREBP-2 Processing*

Oxysterols such as 25-HC are potent inhibitors of SREBP-2 processing in mammalian cells (13). To test whether NPC1 is required for this processing event, we analyzed the proteolytic processing of SREBP-2 in mutant fibroblasts that lack functional NPC1. The first set of experiments was performed with telomerase-immortalized human fibroblasts from an individual with NPC1 disease (GM3123). Previous studies have shown that NPC1 is required for the export of lipoprotein-derived cholesterol from the lysosome to the ER where the cholesterol activates ACAT, thereby causing an increase in the incorporation of radiolabeled fatty acids into cholesteryl esters (2, 4). However, NPC1 is not required for the stimulation of cholesterol esterification by 25-HC (4). To deliver lipoprotein cholesterol to cells, we used cholesterol-rich rabbit  $\beta$ -VLDL, which binds with high affinity to human LDL receptors (30) and reaches lysosomes through receptor-mediated endocytosis. We tested the ability of increasing concentrations of  $\beta$ -VLDL to stimulate the incorporation of [ $^{14}\text{C}$ ]oleate into cellular cholesteryl [ $^{14}\text{C}$ ]oleate. As shown in Fig. 9A (*left panel*),  $\beta$ -VLDL markedly increased the amount of cholesteryl [ $^{14}\text{C}$ ]oleate formed in control fibroblasts, but not in NPC1 fibroblasts. On the other hand, 25-HC increased cholesteryl

[ $^{14}\text{C}$ ]oleate formation in both control and NPC1 cells (*right panel*).

If NPC1 is required for the oxysterol-mediated inhibition of the proteolytic processing of SREBPs, then the telomerase-immortalized NPC1 fibroblasts should show a decreased nuclear accumulation of the cleaved form of SREBP. Fig. 9B shows an immunoblot analysis of SREBP-2 processing in control and mutant fibroblasts. As a positive control for NPC1 function, we added  $\beta$ -VLDL, which inhibited cleavage in control cells (lanes 2-4), but not in NPC1 cells that fail to transport lipoprotein-derived cholesterol to the ER (lanes 9-11). In contrast, 25-HC blocked the generation of nuclear SREBP-2 at 0.3-1  $\mu\text{g/ml}$  both in control (lanes 5-7) and NPC1 (lanes 12-14) cells.

To verify the above result in another cell culture system, we carried out similar studies of SREBP-2 processing in fibroblasts from a mouse homozygous for a nonfunctional *NPC1* gene (6). Fig. 9C shows that the mouse NPC1 cells responded similarly to the control cells in terms of inhibition of SREBP-2 processing by 25-HC (lanes 2-4, 9-11), but not in terms of their response to  $\beta$ -VLDL (lanes 5-7, 12-14).

## DISCUSSION

In the present studies we set out to purify a hypothesized membrane-bound oxysterol binding protein (MOBP) that we predicted would differ from the previously described oxysterol-binding proteins of the cytosol (OSBP-1 and ORPs). Our ultimate goal was to find a membrane protein that binds oxysterols and in turn regulates cholesterol synthesis and esterification. We used rabbit liver as a source because of the commercial availability of frozen rabbit livers. We succeeded in identifying and purifying by 14,000-fold a membrane protein that bound [ $^3\text{H}$ ]25-HC with high affinity and specificity. When peptides from this protein were sequenced by mass spectrometry, we were surprised to find that the oxysterol-binding protein was NPC1, a previously described protein with a known

role in intracellular transport of cholesterol. There was no prior evidence to indicate that NPC1 binds oxysterols.

To confirm the oxysterol binding activity of NPC1, we prepared the human protein through recombinant DNA methodology and purified it through use of affinity tags. The recombinant human NPC1 bound oxysterols with specificity that was similar to that of the rabbit liver protein. Both proteins showed a preference for sterols with a hydroxyl group near the distal end of the 8-carbon side chain, i.e. at positions 24, 25, or 27. Hydroxyl groups elsewhere on the sterol (positions 7, 19, or 20) failed to confer binding.

In the current experiments, recombinant human NPC1 also bound cholesterol. Binding was inhibited when the concentration of NP-40 exceeded the micellar threshold. We hypothesize that detergent micelles block [ $^3$ H]cholesterol binding not because of an effect on the protein, but rather because the detergent micelles have a higher affinity and capacity for binding cholesterol than does the NPC1 protein. In this regard, Cheruku et al. (31) performed informative studies with NPC2 protein, a soluble cholesterol binding protein that participates together with NPC1 in intracellular cholesterol transport. NPC2 rapidly transferred cholesterol to phospholipid bilayers. It will be of interest in the future to determine whether phospholipid bilayers also steal cholesterol from NPC1 and whether this relative affinity is important in the ability of NPC1 to transfer cholesterol between membranes and in the ability of sphingolipids or phospholipids to regulate this process.

A remarkable feature of NPC1 sterol binding was the non-reciprocal effect of cholesterol and 25-HC. Whereas 25-HC potently blocked [ $^3$ H]cholesterol binding, the reverse was not true, i.e., cholesterol failed to block [ $^3$ H]25-HC binding. This result might be explained by a one-site or two-site model. In the former, NPC1 would have a single sterol binding site, and 25-HC

would have a much higher affinity for this site than cholesterol. In the two-site model, 25-HC binding to the oxysterol site would allosterically alter the separate cholesterol binding site, blocking the binding of cholesterol. Distinguishing these possibilities will require purification of larger amounts of recombinant NPC1 than are available presently. These studies may be aided by the identification of the domain of NPC1 that binds cholesterol and 25-HC (see accompanying paper) (32).

As described in the Introduction, we initiated the search for a MOBP in an attempt to explain the ability of oxysterols to inhibit cholesterol synthesis by blocking the proteolytic processing of SREBPs. However, we have been unable to find any evidence that NPC1 is required for this regulatory action. While these studies were in progress, we demonstrated that the effect of 25-HC on SREBP processing is mediated by 25-HC binding to Insig proteins (28), which in turn prevents SREBPs from exiting from the ER (33). Thus, we have no reason to postulate a direct role for NPC1 in mediating the oxysterol regulation of SREBP activity.

The question now arises as to whether 25-HC or other oxysterols regulate the activity of NPC1. In cultured fibroblasts, NPC1 is required primarily for transport of LDL-derived cholesterol from its site of liberation in endosomes and lysosomes to its sites of function in the ER and plasma membrane (5, 34). As yet, there is no evidence that oxysterols regulate this function. However, in humans and animals, NPC1 deficiency leads to accumulation of gangliosides and other lipids in brain (2). The brain converts cholesterol to 24-HC, which is then transported to the liver (35, 36). It is possible that oxysterols such as 24-HC or 25-HC play some role in regulating the transport function of NPC1 in neurons and glial cells. This and other possibilities are open to experimentation now that the sterol binding activity of NPC1 has been characterized.

## FOOTNOTES

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<sup>2</sup>The abbreviations used are: ACAT, acyl-CoA: cholesterol acyltransferase; ALLN, *N*-acetyl-leucinal-leucinal-norleucinal; CMC, critical micellar concentration; CMV, cytomegalovirus; DTT, dithiothreitol; ER, endoplasmic reticulum; FCS, fetal calf serum; 25-HC, 25-hydroxycholesterol; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPDS, lipoprotein-deficient serum; MOBP, membrane-bound oxysterol binding protein; OSBP, oxysterol binding protein; ORP, OSBP-related protein; NP-40, Nonidet P-40; NPC1, Niemann-Pick, Type C1; photo [<sup>3</sup>H]25-HC, [3 $\beta$ -<sup>3</sup>H]7,7-azocholistan-3 $\beta$ ,25-diol; SREBP, sterol regulatory element-binding protein;  $\beta$ -VLDL,  $\beta$ -migrating very low density lipoproteins.

<sup>3</sup>Ye, J., Adams, C., Goldstein, J.L., and Brown, M.S., unpublished observations.

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## FIGURE LEGENDS

**Fig. 1 Representative steps in purification of rabbit MOBP.** A, separation of MOBP from OSBP-1 by Q-Sepharose chromatography. The 100,000g supernatant of NP-40 solubilized membrane proteins from rabbit liver (181 mg) was applied to a 20-ml Q-Sepharose column pre-equilibrated with buffer C. The column was washed with 5 column volumes of buffer C, and bound proteins were eluted with a continuous gradient of 0-500 mM KCl in buffer C. Fractions (5-ml) were collected and assayed for [<sup>3</sup>H]25-HC binding activity (●) and protein concentration (○) using the filter assay and BCA method, respectively. Fractions 26-54 were also subjected to SDS/PAGE and OSBP-1 immunoblot analysis using monoclonal antibody 11H9 as described under "Experimental Procedures." B, estimation of molecular mass of MOBP by gel filtration. Partially purified MOBP (Q-Sepharose fraction, 5 mg protein), which was devoid of detectable OSBP-1 immunoblot reactivity, was loaded onto a 320-ml Sephacryl 300 gel filtration column pre-equilibrated with buffer C containing 100 mM KCl. Fractions (2-ml) were collected and assayed for [<sup>3</sup>H]25-HC binding activity (●) and protein concentration (○) as described in (A). Standard molecular weight markers (thyroglobulin, 670 kDa; γ-globulin, 158; ovalbumin, 44;

and myoglobin, 17) were chromatographed on the same column under identical buffer conditions. The apparent molecular mass of MOBP is ~440 kDa.

**Fig. 2 Specificity of sterol binding to partially purified rabbit MOBP.** *A*, chemical structure of cholesterol. *B*, list of unlabeled sterols tested for their ability to compete with binding of [<sup>3</sup>H]25-HC to MOBP. Blue and red colored sterols denote those that compete and those that do not compete for [<sup>3</sup>H]25-HC binding, respectively. *C*, each assay tube, in a final volume of 120 µl of buffer C, contained partially purified MOBP (Q-Sepharose (pH 5.5) fraction), 50 nM of [<sup>3</sup>H]25-HC delivered in ethanol, and varying concentrations of the indicated unlabeled competitor sterol, delivered in ethanol. After incubation for 2 h at 4°C, the amount of [<sup>3</sup>H]25-HC binding was measured by the filter assay. The “100% of control” value in tubes with no unlabeled sterol was 50 fmol/filter. Blank values of 3.3 fmol (obtained in parallel assays of tubes containing no protein) were subtracted from each point. Each value is the average of triplicate incubations. *D* and *E*, each assay was performed as in (*C*) except that a different partially purified preparation of MOBP was used (RCA lectin fraction). The “100% of control” value in tubes with no unlabeled sterol was 71 fmol/filter for both (*D*) and (*E*). Blank values of 1.4 (*D*) and 2.5 (*E*) fmol were subtracted from each point. Each value in (*D*) is the average of triplicate incubations; each value in (*E*) represents a single incubation.

**Fig. 3. Photolabeling and SDS-PAGE of purified rabbit MOBP.** *A*, Mono-Q chromatography of photolabeled MOBP. Highly purified rabbit MOBP (0.13 mg of the Reactive Blue Dye 72 fraction) was incubated in a final volume of 3 ml with 500 nM of photo [<sup>3</sup>H]25-HC in the absence (●) or presence (○) of 10 µM unlabeled 25-HC. After overnight incubation at 4°C, the photo crosslinking group was activated by UV irradiation for 20 min. Each sample was loaded onto a 1-ml Mono-Q ion exchange column, washed, and eluted with a salt gradient. Radioactivity was determined by direct counting of each fraction. *B*, SDS-PAGE of Mono-Q purified material. Fractions 32 (with and without unlabeled 25-HC) from (*A*) were subjected to 8% SDS-PAGE, after which the gel was cut into 18 slices (3-mm each) that were then subjected to scintillation counting as described under “Experimental Procedures.” A silver-stained gel (done in parallel with the cut gel) is shown at the top.

**Fig. 4. Purification of recombinant human NPC1 from transfected CHO-K1 cells.** *A*, nickel column chromatography. CHO-K1 cells (50 100-mm dishes) were transfected with 5 µg/dish of human NPC1-His<sub>8</sub>-Flag as described under “Experimental Procedures.” Detergent-solubilized membranes (100,000g fraction, 22 mg protein in buffer G containing 20 mM imidazole) was loaded onto a 1-ml His Trap HP nickel column pre-equilibrated with buffer G and eluted stepwise with 4 imidazole washes (25, 50, 200, 300 mM) as indicated. Fractions (1.5 ml) were tested for protein concentration (○), [<sup>3</sup>H]25-HC binding activity (●), and immunoblot analysis with monoclonal Flag antibody (shown below fraction numbers). *B*, anti-Flag chromatography. Fractions 27 and 28 from (*A*) were pooled, loaded onto a 1-ml M2 anti-Flag M2-Agarose affinity column, washed with buffer G, and eluted with 0.1 mg/ml of Flag peptide in buffer G. Fractions (1 ml) were tested for protein concentration (○), [<sup>3</sup>H]25-HC binding activity (●), and immunoblot analysis with monoclonal anti-Flag antibody. Protein measurements for fractions 14-21 are not shown since they contain the eluted Flag peptide. *C* and *D*, SDS-PAGE of purified recombinant NPC1. An aliquot of fraction 15 from the anti-Flag M2-Agarose column in (*B*) was subjected to 8% (*C*) and 12% (*D*) SDS-PAGE along with bovine serum albumin (BSA) standards and then stained with silver. The two bands at the top of the 8% gel (*C*) in the lane designated “Flag Peak” (~200 kDa and > 250 kDa) were shown by mass spectrometry to be NPC1.

**Fig. 5. Reciprocal [ $^3\text{H}$ ]25-HC and [ $^3\text{H}$ ]cholesterol binding activities of purified recombinant NPC1 and Scap in presence of 1% NP-40 (A-C) or 0.1% Fos-Choline 13 (D-E).** A and B, saturation curves for NPC1 binding of [ $^3\text{H}$ ]25-HC and [ $^3\text{H}$ ]cholesterol. Each reaction, in a final volume of 120  $\mu\text{l}$  of buffer G, contained  $\sim 250$  ng of purified human NPC1-His<sub>8</sub>-Flag and 10-400 nM of either [ $^3\text{H}$ ]25-HC (A) or [ $^3\text{H}$ ]cholesterol (B) (both solubilized in NP-40) in the absence ( $\bullet$ ) or presence ( $\circ$ ) of 10  $\mu\text{M}$  unlabeled 25-HC (A) or cholesterol (B) (both solubilized in NP-40). After incubation for 3 h at 4°C, bound [ $^3\text{H}$ ]25-HC (A) or [ $^3\text{H}$ ]cholesterol (B) was measured using the Ni-NTA agarose binding assay as described under “Experimental Procedures.” Each data point represents total binding without subtraction of blank values. C, competitive binding of [ $^3\text{H}$ ]25-HC to NPC1 in presence of unlabeled sterols. Each reaction, in a total volume of 140  $\mu\text{l}$  of buffer G, contained  $\sim 150$  ng of purified NPC1-His<sub>8</sub>-Flag, 100 nM of [ $^3\text{H}$ ]25-HC, and the indicated concentration of unlabeled 25-HC ( $\bullet$ ) or cholesterol ( $\blacksquare$ ). After incubation for 3 h at 4°C, bound [ $^3\text{H}$ ]25-HC was measured as described above. The “100% of control” value with no unlabeled sterol was 26 fmol/tube. A blank value of 1.2 fmol was subtracted from each point. D and E, comparison of direct sterol binding activities of NPC1 and Scap. Each reaction, in a final volume of 140  $\mu\text{l}$  of buffer H, contained  $\sim 400$  ng of purified human NPC1-His<sub>8</sub>-Flag in 0.1% Fos-Choline 13 (D) or 200 ng of purified hamster His<sub>10</sub>-SCAP(TM1-8) in 0.1% Fos-Choline 13 (E) and the indicated concentration of [ $^3\text{H}$ ]25-HC ( $\bullet$ ) or [ $^3\text{H}$ ]cholesterol ( $\blacktriangle$ ) (both solubilized in 0.1% Fos-Choline 13). After incubation for 3 h at 4°C, binding was measured as described above. Each data point represents total binding without subtraction of blank values. The experiments in (A)-(E) were done in two or more independent studies with similar results.

**Fig. 6. Ability of unlabeled oxysterols and various sterols to compete for binding of [ $^3\text{H}$ ]25-HC to purified recombinant human NPC1 in presence of 1% NP-40.** A, chemical structure of cholesterol. B, list of unlabeled sterols tested for their ability to compete with binding of [ $^3\text{H}$ ]25-HC to NPC1. Blue and red colored sterols denote those that compete and those that do not compete for [ $^3\text{H}$ ]25-HC binding, respectively. C and D, competitive binding of [ $^3\text{H}$ ]25-HC to NPC1. Each reaction, in a total volume of 120  $\mu\text{l}$  of buffer G, contained 250 ng NPC1-His<sub>8</sub>-Flag, 100 nM [ $^3\text{H}$ ]25-HC, and varying concentrations of the indicated unlabeled sterol. After incubation for 3 h at 4°C, bound [ $^3\text{H}$ ]25-HC was measured by the Ni-NTA agarose binding assay. Each data point represents the amount of [ $^3\text{H}$ ]25-HC bound relative to that in the control tube, which contained no unlabeled sterol. The “100% of control” value was 60 (C) and 68 (D) fmol/tube, respectively. A blank value of 1 fmol/tube was subtracted from each point. E, competitive binding of [ $^3\text{H}$ ]25-HC to NPC1 by unlabeled oxysterols, cholesterol precursors and analogues, and other sterols. Competitive assays were performed as in (C) and (D) except that each reaction contained  $\sim 150$  ng NPC1-His<sub>8</sub>-Flag and each unlabeled sterol was tested at one concentration (5  $\mu\text{M}$ ). The “100% of control” value in the absence of unlabeled sterol was 37 fmol/tube. A blank value of 1 fmol/tube was subtracted from each point. The experiments in (C)-(E) were done in two or more independent studies with similar results.

**Fig. 7 [ $^3\text{H}$ ]Cholesterol and [ $^3\text{H}$ ]25-HC binding activity of purified recombinant human NPC1 in presence of 0.004% NP-40.** A, Gel filtration chromatography of purified NPC1. Recombinant human NPC1-His<sub>8</sub>-Flag (1  $\mu\text{g}$ ) purified in either 0.004% or 1% NP-40 was loaded onto a 24-ml Superdex 200 column preequilibrated with buffer I (0.004% NP-40) or buffer G (1% NP-40), respectively. Fractions of 1 ml were collected. Aliquots of each fraction (40  $\mu\text{l}$ ) were subjected to SDS/PAGE and NPC1 immunoblot analysis using monoclonal anti-Flag antibody. Standard molecular weight markers (thyroglobulin, 670 kDa;  $\gamma$ -globulin, 158; ovalbumin, 44; myoglobulin, 17) were chromatographed on the same column under identical



buffer conditions. Filters were exposed for 0.5 to 3 s. *B*, saturation curves for binding of [ $^3$ H]cholesterol and [ $^3$ H]25-HC to NPC1 in presence of 0.004% NP-40. Each reaction, in a final volume of 80  $\mu$ l of buffer I, contained  $\sim$ 100 ng of purified human NPC1-His<sub>8</sub>-Flag, 1  $\mu$ g of bovine serum albumin, and 10-300 nM of either [ $^3$ H]cholesterol (*left panel*) or [ $^3$ H]25-HC (*right panel*) delivered in ethanol. After incubation at 4°C for 16 h, bound  $^3$ H-sterol was measured using the Ni-NTA agarose binding assay as described under “Experimental Procedures.” Each data point represents the average of duplicate assays of total binding without subtraction of blank values. *C*, competitive binding of [ $^3$ H]cholesterol (*left panel*) and [ $^3$ H]25-HC (*right panel*) to NPC1 in presence of unlabeled sterols. Each assay tube, in a total volume of 80  $\mu$ l of buffer I, contained  $\sim$ 100 ng of purified NPC1-His<sub>8</sub>-Flag, 1  $\mu$ g of bovine serum albumin, either 200 nM [ $^3$ H]cholesterol (*left panel*) or 40 nM [ $^3$ H]25-HC (*right panel*) delivered in ethanol, and the indicated concentration of unlabeled sterols. After incubation at 4°C for 16 h, bound  $^3$ H-sterol was measured as described above. The “100% of control” values in the absence of unlabeled sterol were 197 and 194 fmol/tube for [ $^3$ H]cholesterol and [ $^3$ H]25-HC, respectively. Blank values of 11 and 12 fmol/tube were subtracted from each of the [ $^3$ H]cholesterol and [ $^3$ H]25-HC data points, respectively.

**Fig. 8. Effect of detergent concentration on [ $^3$ H]cholesterol and [ $^3$ H]25-HC binding to NPC1.** *A*, [ $^3$ H]cholesterol binding at NP-40 concentrations from 0.0007 to 0.01%. *Arrow* denotes the measured value for the CMC of NP-40 (0.0015%). *B*, cholesterol and [ $^3$ H]25-HC binding at NP-40 concentrations from 0.001 to 0.1%. *A* and *B*, each reaction, in a final volume of 80  $\mu$ l of buffer I, contained  $\sim$ 80 ng of purified NPC1-His<sub>8</sub>-Flag, 200 nM [ $^3$ H]cholesterol or 200 nM [ $^3$ H]25-HC as indicated, and the indicated concentration of NP-40. After incubation at 4°C for 16 h, the total amount of bound  $^3$ H-sterol was measured using the Ni-NTA agarose binding assay as described under “Experimental Procedures.” The “100% of control” value in the absence of unlabeled sterol were 218 and 108 fmol for [ $^3$ H]cholesterol and [ $^3$ H]25-HC, respectively. No blank values were subtracted. Each value is the average of duplicate incubations.

**Fig. 9. NPC1 does not regulate oxysterol-mediated inhibition of SREBP-2 processing in cultured cells.** *A*, cholesterol esterification assay in telomerase-immortalized NPC1 human fibroblasts. On day 0, h-TERT-Control ( $\blacktriangle$ ) and h-TERT-NPC-1 ( $\bullet$ ) fibroblasts were set up at in medium B containing 10% FCS and grown as described under “Experimental Procedures.” On day 4, after incubation for 2 days in LPDS, each dish received sterol-depleting medium C supplemented with 10% human LPDS the indicated concentration of  $\beta$ -VLDL (*left*) or 25-HC (*right*). After incubation for 5 h at 37°C, each monolayer was pulse-labeled for 2 h with 0.2 mM [ $^{14}$ C]oleate (7681 dpm/pmol). The cells were then harvested for measurement of their cholesteryl [ $^{14}$ C]oleate and [ $^{14}$ C]triglyceride content as described under “Experimental Procedures.” Each value is the average of triplicate incubations. The cellular content of [ $^3$ H]triglycerides (data not shown in figure) was similar for control and NPC1 cells incubated with 10  $\mu$ g protein/ml  $\beta$ -VLDL (25 and 21 nmol/h per mg, respectively) or 5  $\mu$ g/ml 25-HC (39 and 29 nmol/h per mg, respectively). *B*, immunoblot analysis of SREBP-2 cleavage in NPC1 human fibroblasts. hTERT-Control (*left*) and h-TERT-NPC1 (*right*) fibroblasts were set up for experiments as described under “Experimental Procedures.” On day 4, after incubation for 2 days in LPDS, the cells were incubated with medium C supplemented with 10% human LPDS the indicated concentration of  $\beta$ -VLDL or 25-HC. After 6 h at 37°C, the cells were harvested. The nuclear extract and 100,000g membrane fractions were subjected to immunoblot analysis of SREBP-2. *C*, immunoblot analysis of SREBP-2 cleavage in NPC1 mouse fibroblasts. On day 4, after incubation for 1 day in 5% newborn calf LPDS, the cells were incubated with medium C

supplemented with 5% newborn calf LPDS and the indicated concentration of 25-HC or  $\beta$ -VLDL. After 5 h at 37 C, the cells were harvested, and processed as in *B*. All filters in *B* and *C* were exposed to X-ray film for 1-5 s except for the Scap and NPC1 filters, which were exposed for 20 s.

**TABLE 1**  
**Purification of MOBP from rabbit liver**

Step	Fraction	Protein <sup>a</sup> (mg)	Specific Activity (pmol/mg)	Total Activity (pmol)	Purification (fold)	Recovery (%)
1	Solubilized Membranes <sup>b</sup>	29,008	0.01	300	-----	100
2	Q-Sepharose (pH 7.4)	1,727	0.19	332	19 (74) <sup>c</sup>	110
3	SP-Sepharose	846	0.35	295	34 (135)	98
4	Q-Sepharose (pH 5.5)	92	1.6	144	150 (601)	48
5	RCA Lectin → Mono-Q	9	3.6	31	347 (1388)	10
6	S-300 Gel Filtration → Mono-Q	9	3.2	29	309 (1237)	10
7	Reactive Blue 72	0.7	37.4	25	3618 (14471)	8

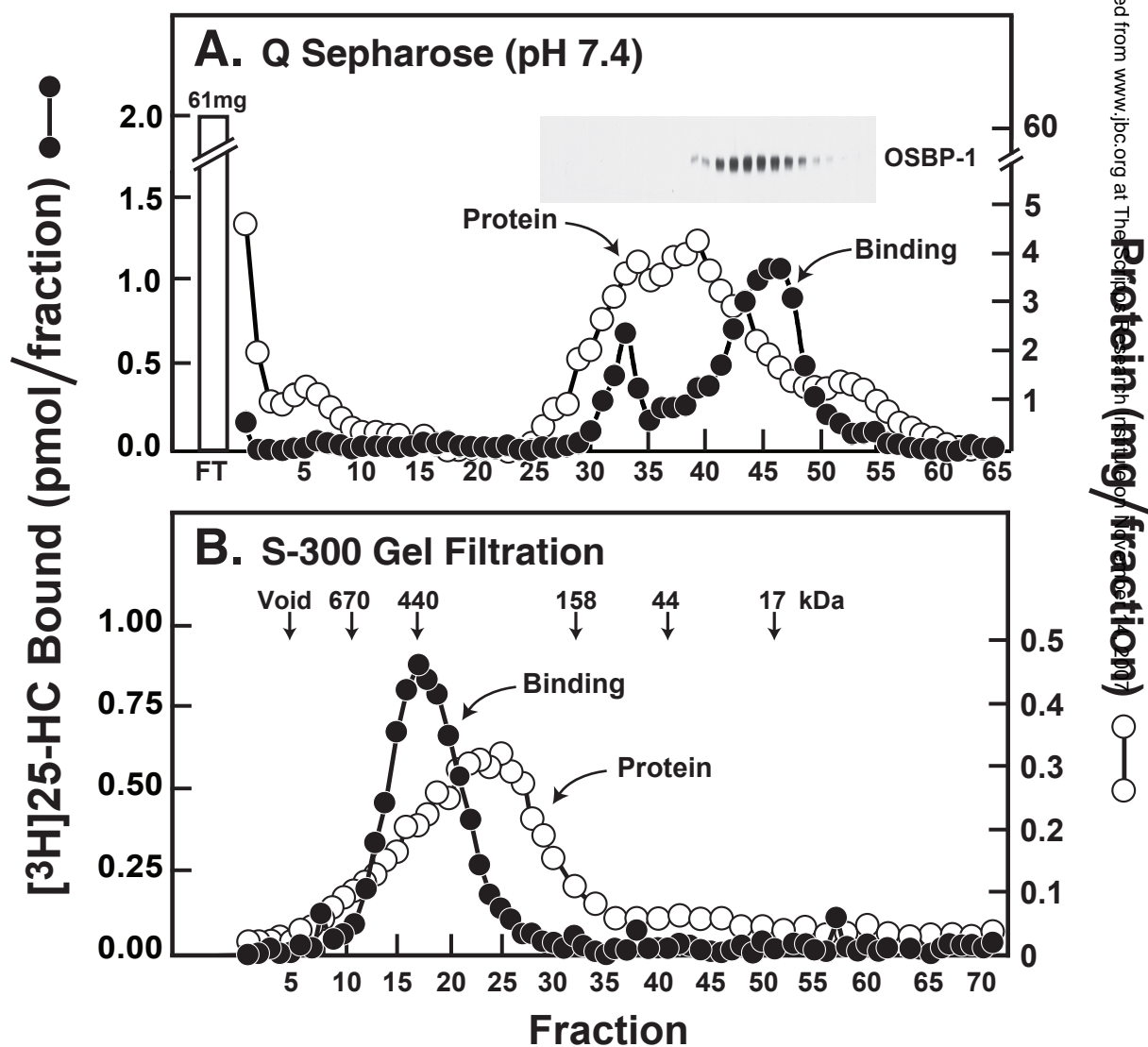
[<sup>3</sup>H]25-HC binding activity was assayed under standard conditions in the presence of 50 nM [<sup>3</sup>H]25-HC. Incubations were carried out for 90 min at 4°C as described under “Experimental Procedures.”

<sup>a</sup>Protein concentration of the various fractions was determined as described under “Experimental Procedures.”

<sup>b</sup>This starting fraction contains two proteins that bind [<sup>3</sup>H]25-HC: MOBP (~25% of total binding) and OSBP-1 (~75% of total binding).

<sup>c</sup>Numbers in parenthesis denote the *fold*-purification of MOBP when the contribution of OSBP-1 binding in the starting fraction is subtracted.

Fig. 1

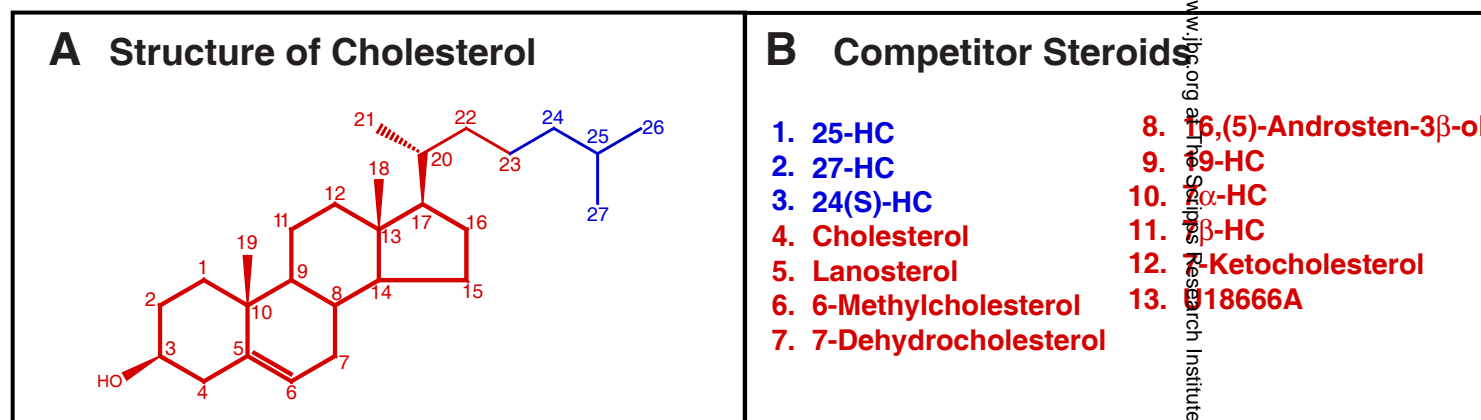


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Fig. 2

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[<sup>3</sup>H] 25-HC Bound

(% of control)

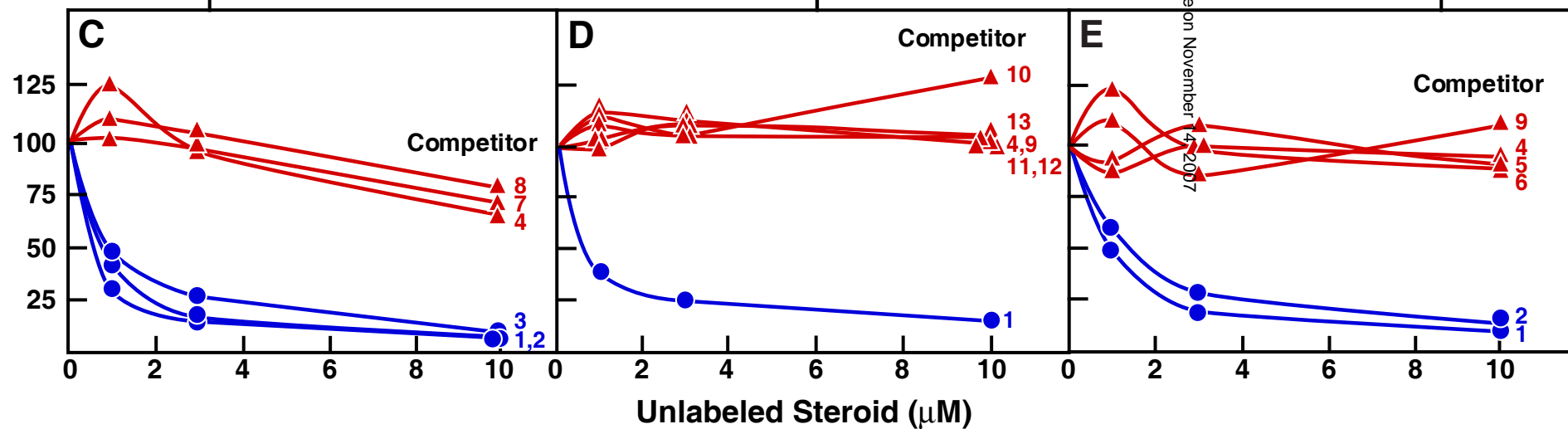
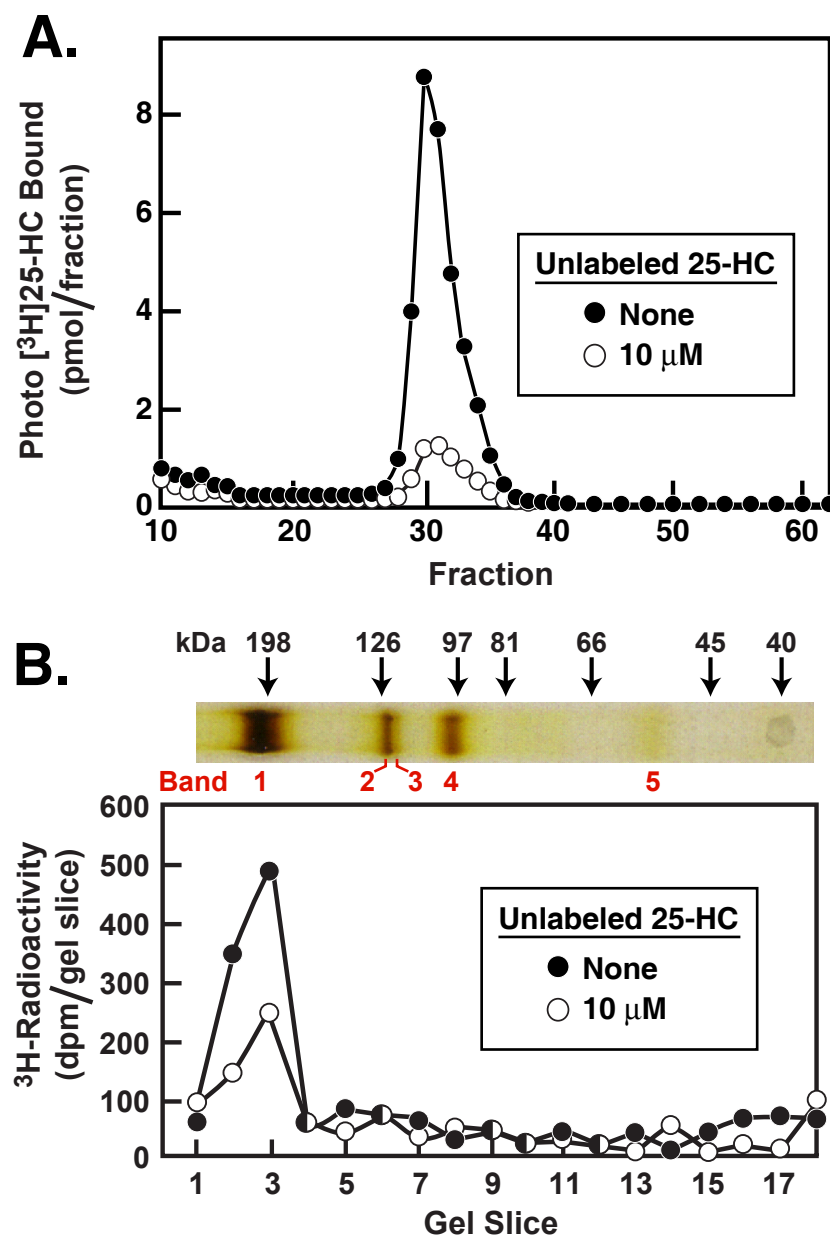


Fig. 3



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Fig. 4

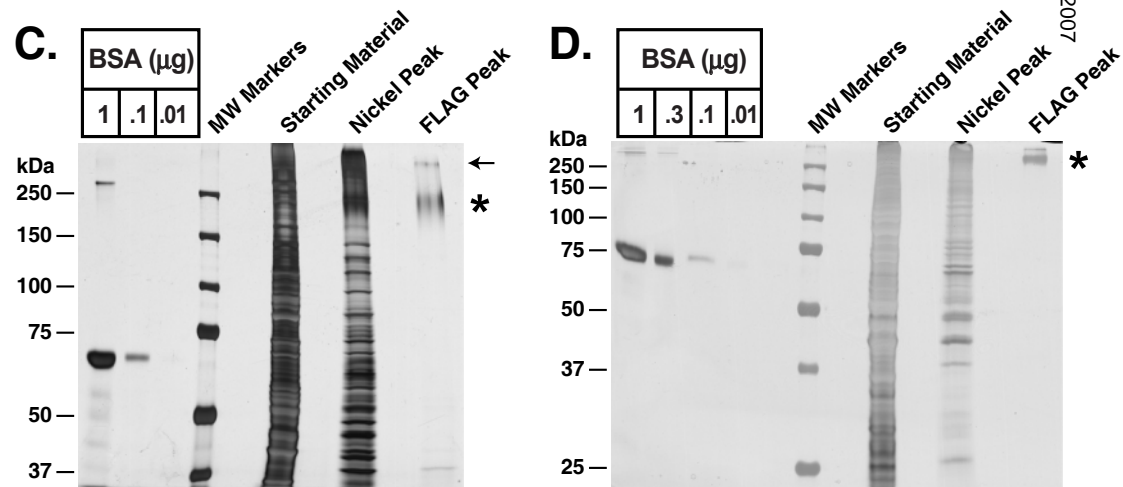
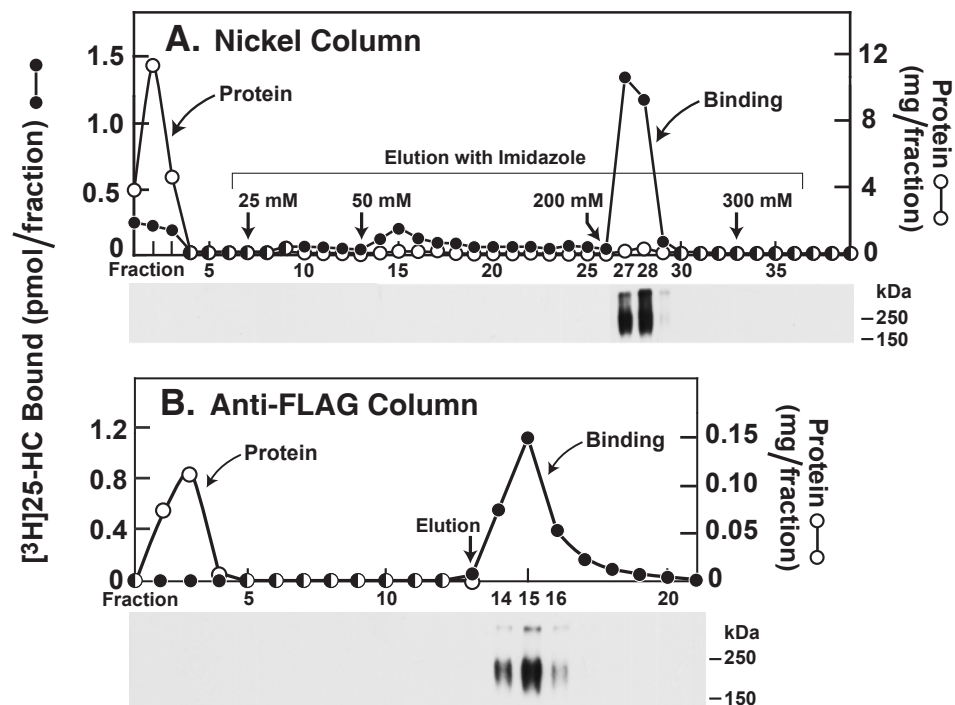


Fig. 5

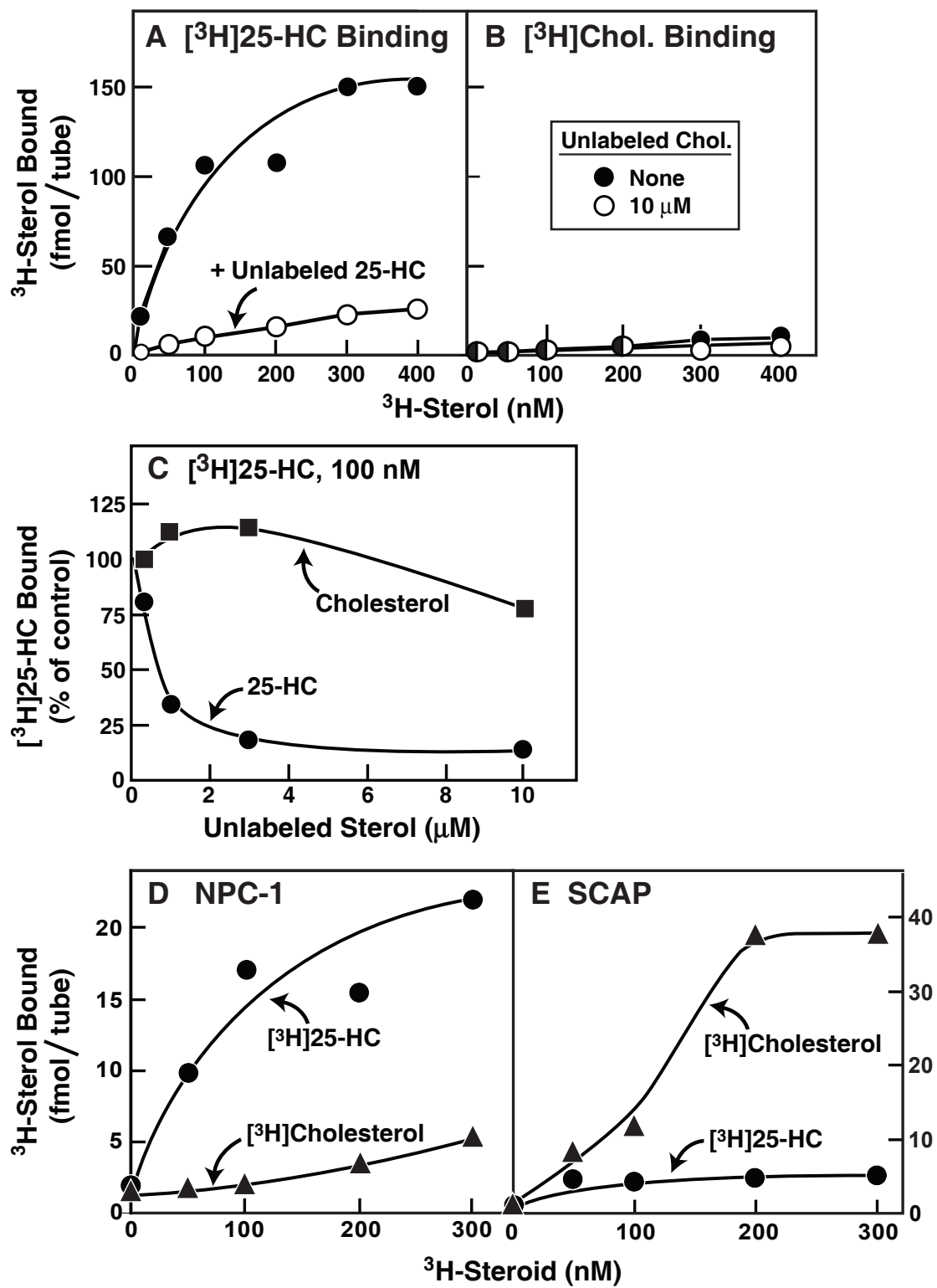




Fig. 6

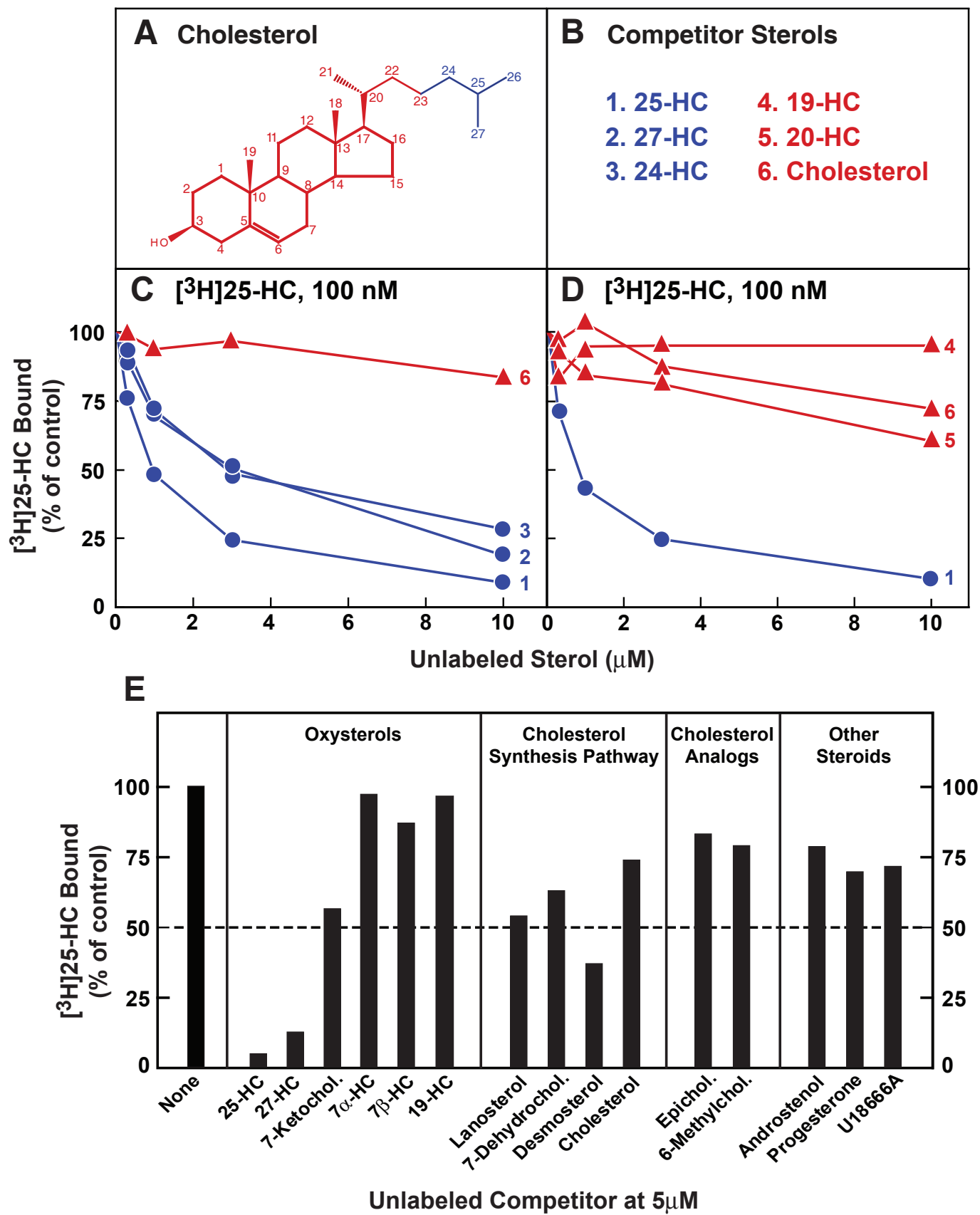


Fig. 7

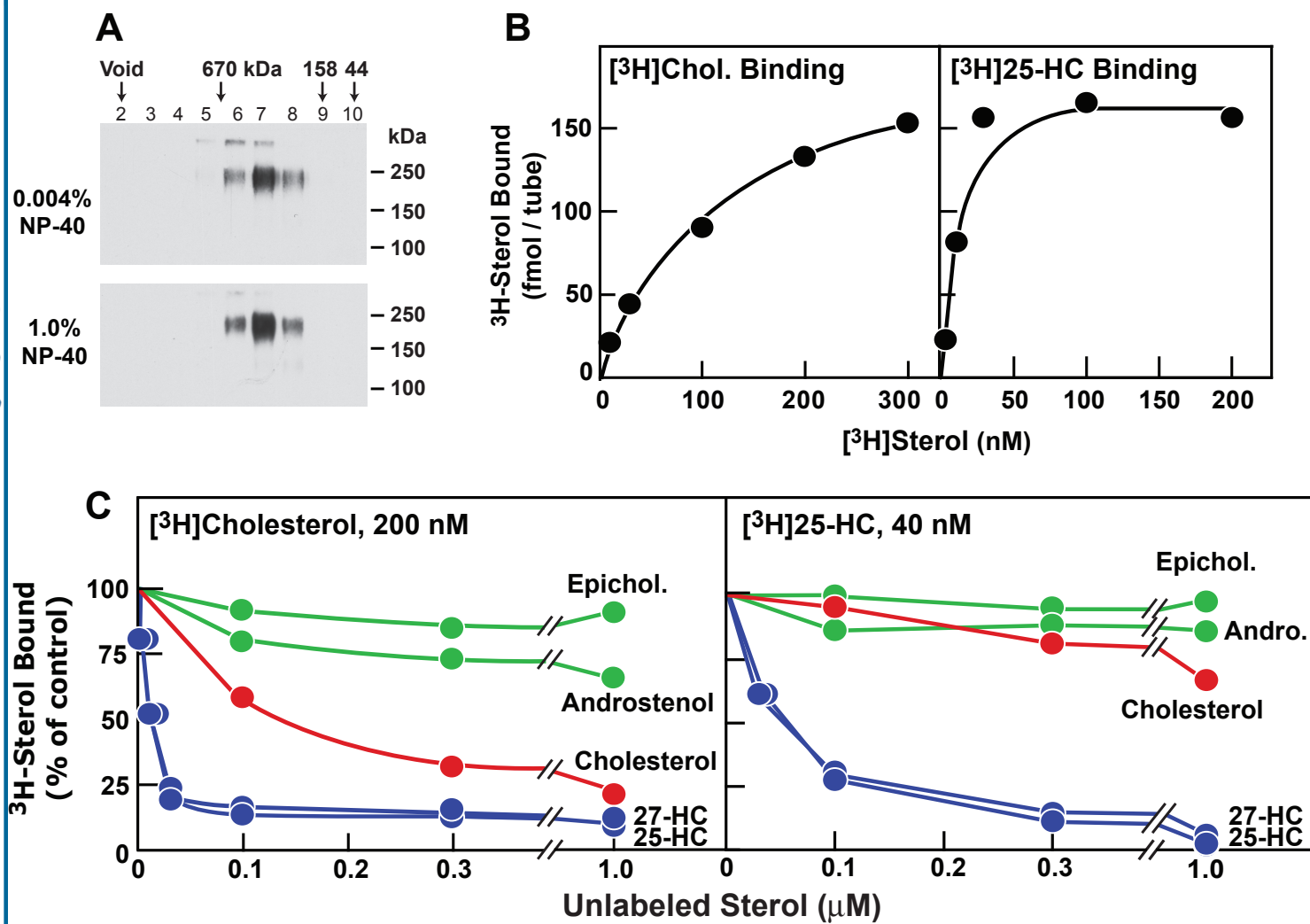


Fig. 8

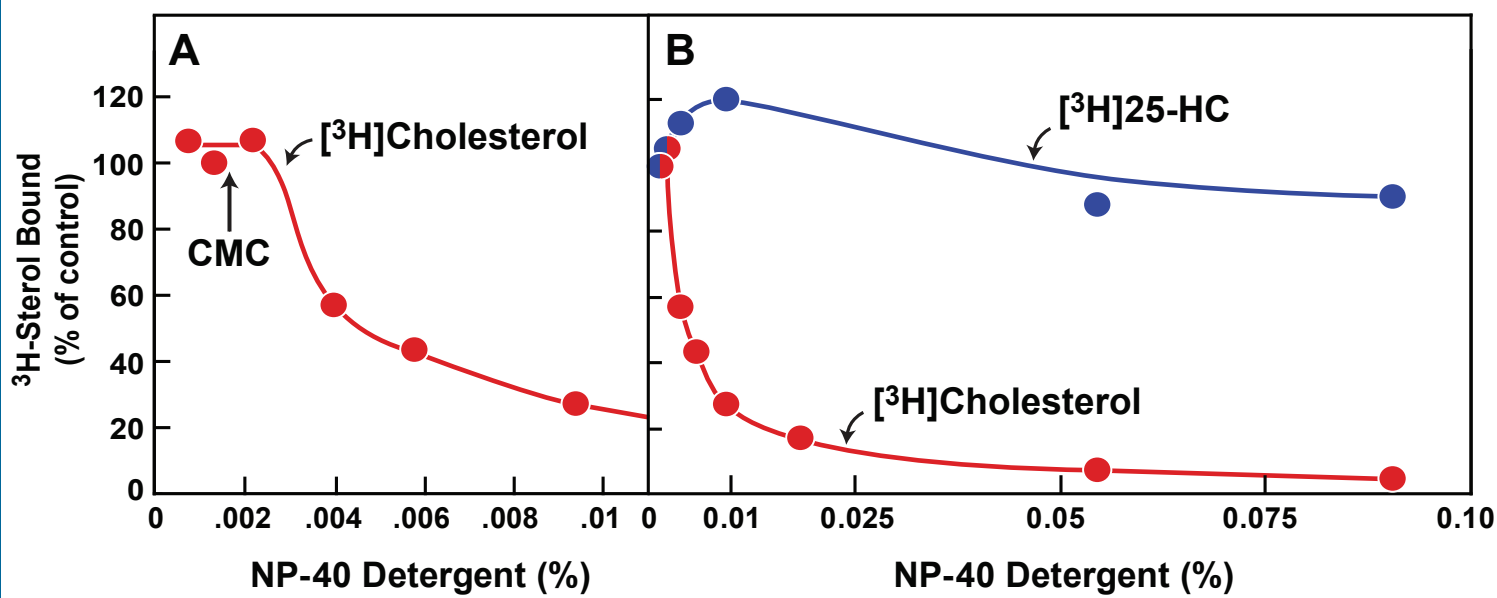


Fig. 9

