PURIFICATION OF NATIVE AND RECOMBINANT NPC1

APPROVED BY SUPERVISORY COMMITTEE

Committee Chairperson _____
Joseph L. Goldstein

Committee Member _____
Michael S. Brown

Committee Member _____
Russel DeBose-Boyd

Committee Member _____
Jin Ye

Committee Member _____
Rodney E. Infante

PURIFICATION OF NATIVE AND RECOMBINANT NPC1

by

JARROD DONALD DALE

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ABSTRACT

PURIFICATION OF NATIVE AND RECOMBINANT NPC1

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Jarrod Donald Dale

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Supervising Professor: Joseph L. Goldstein, M.D.

The Niemann-Pick, Type C1 protein (NPC1) is required for the transport of lipoprotein-derived cholesterol from lysosomes to endoplasmic reticulum. The 1278-amino acid, polytopic membrane protein has not been purified, and its mechanism of action is unknown. We encountered NPC1 in a search for a membrane protein that binds 25-hydroxycholesterol (25-HC) and other oxysterols. Described here is the initial purification of rabbit NPC1 using a classical biochemical approach and an analysis of the sterol binding properties of native and recombinant NPC1. Our purification yielded a membrane-bound 25-HC-binding protein which was purified more than 14,000-fold from rabbit liver membranes. This protein was 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. Initial characterization of the sterol binding properties showed specific binding for 25-HC; however, we were unable to demonstrate significant binding of NPC1 to cholesterol using our current experimental conditions. The availability of assays to measure NPC1 sterol binding *in vitro* may further the understanding of intracellular sterol transport.

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PRIOR PUBLICATIONS AND PRESENTATIONS AND ABTRACTS

2008 **"Fluency Covered Stent Treatment of PTA- Related Extravasation during Hemodialysis Access Intervention: Technical and 180 Day Patency Results."** (2008) **Dale, J.D.,** Winder, R., Duch, J.M., and Dolmatch, B.L. Journal of Vascular and Interventional Radiology. *In process*

"Purtscher's Retinopathy In A Postpartum Patient with Hypertensive Encephalopathy" Dani, M.D., Lubahn, J.G., **Dale, J.D.** Rosenburg, R.N., Stuve, O., and Warnack, W.R. 2008 American Academy of Neurology, Chicago, IL. April 12-18th 2008

2007 **"Purified NPC1 Protein: I Binding of Oxysterols and Cholesterol to a 1278-Amino Acid Membrane Protein."** *Currently unpublished, expected publication date 2007*

"Pre-Training on Southwestern Stations Decreases Training Time and Cost for Proficiency-Based Fundamentals of Laparoscopic Surgery (FLS) Training." Goova M, Hollett L, **Dale J**, Scott D. ACS Clinical Congress, New Orleans, LA. 2007

2006 **"Purification and Characterization of Membrane-Bound Oxysterol Binding Protein (MOBP)":** 1st Author Abstract and Presentation at the 44th Annual UTSW Medical Student Research Forum, Dallas Texas. 2006

2005 **"Characterization of Improved Galactose-Regulated Promoters with Reduced Basal Level Expression":** 1st author Abstract and Poster presented at the American Society of Biochemistry and Molecular Biology Annual Meeting, San Diego, CA. 2005

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LIST OF ABBREVIATIONS

ACAT - acyl-CoA; cholesteryl acyltransferase

ALLN - *N*-acetyl-leucinal-leucinal-norleucinal

CHO - Chinease Hampster Ovary

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

KCL - Potassium Chloride

MOBP - membrane-bound oxysterol binding protein

OSBP - oxysterol binding protein

ORP - OSBP-related protein

NP-40 - Nonidet P40

NPC1 - Niemann-Pick, Type C1

SREBP - sterol regulatory element binding protein

CHAPTER 1

Introduction

Cholesterol is a vital component of mammalian cells. Cholesterol is a precursor molecule for many hormones and is fundamental for cellular membrane structure. Disturbances of cholesterol concentrations are toxic to the cell; therefore, the cell has numerous levels of regulation for controlling cholesterol homeostasis.

Many of the details of cholesterol regulation have been elucidated, but relatively little is known about how unesterified cholesterol is transported within cells (Liscum and Dahl, 1992; Liscum and Underwood, 1995; Lange and Steck, 1996). The uptake of exogenous cholesterol occurs through receptor-mediated endocytosis of lipoprotein-derived cholesterol, and this cholesterol is transferred from endosomal vesicles to the endoplasmic reticulum (ER) where it can carry out regulatory functions or be stored as cholesteryl esters (Brown and Goldstein, 1986). The mechanism of how this lipoprotein-

derived cholesterol is transported from the endosomal vesicles to the other membranes is poorly defined.

Studies of clinical patients with disorders in cholesterol metabolism serve as the foundation for the study of subcellular cholesterol transport. Pentchev et al. (1995) described patients with Niemann-Pick Type C1 (NPC1) disease who developed large accumulations of sphingolipids and cholesterol within many tissues, including the cerebellum, spleen, and liver, resulting in severe neurological deterioration and hepatic failure. NPC1 patients' cells were shown to have retention of low density lipoprotein-derived cholesterol in their late endosomes and lysosomal systems (Pentchev et al., 1985; Liscum and Faust, 1987; Pentchev et al., 1987). It was discovered that most patients with this phenotype had a defect in the NPC1 protein (Loftus et al., 1997). NPC1 is a 1278 amino acid protein which mainly resides in late endosomes and lysosomes. Topology analysis indicates NPC1 contains 13 putative transmembrane domains (TMDs), three large hydrophilic luminal loops, and a five TMD putative "sterol-sensing domain" (SSD) (Davis, 2000; Ioannou, 2000). This SSD motif is also observed in other proteins involved in cholesterol homeostasis, Srebp Cleavage Activating Protein (SCAP), a regulatory protein responsible for transporting SREBPs to the golgi, and 3-hydroxyl-3-methylglutaryl CoA Reductase (HMG CoA reductase), the rate limiting enzyme in cholesterol biosynthesis (Nohturfft et al., 1998, Millard et al., 2005). It is known that SCAP directly binds cholesterol using this SSD domain (Radhakrishnan et al., 2004), but the role of the SSD in NPC1 is currently unknown.

There is no direct evidence explaining the function of the NPC1 protein, and the mechanism of how NPC1 is involved in cholesterol transport is unclear. However, the

observed defective transport of cholesterol in mammalian cells with mutant NPC1 indicates NPC1 plays a compulsory role in subcellular trafficking of cholesterol (Pentchev et al., 1987; Liscum and Faust, 1987; Pentchev et al., 1994; Pentchev et al., 1995). The NPC1 protein in its native membrane-bound form has never been purified, and the characterization of recombinant forms of the protein is limited.

Previous studies in our lab have demonstrated that elevated levels of both cholesterol and its hydroxylated derivative 25-Hydroxycholesterol (25-HC), an oxysterol, regulate several key steps in cholesterol homeostasis. Both cholesterol and oxysterols decrease the processing of sterol regulatory element-binding proteins (SREBPs) by causing their retention in the ER (Sun et al., 2007); increase the rate of HMG CoA reductase degradation (Gil et al., 1985); and activate the esterification and storage of cholesterol through the ER protein acyl-CoA; cholesteryl acyltransferase (ACAT) (Cheng, et al., 1995) The mechanism of oxysterol regulation of the various cholesterol homeostasis pathways is unknown. Our first attempt to identify a protein that bound to oxysterols identified a soluble protein called Oxysterol Binding Protein (OSBP-1) (Dawson et al., 1989a; Dawson et al., 1989b). Through overexpression and knockdown studies, this protein was never linked to the oxysterol regulation of SREBP, HMG CoA reductase, or ACAT. Other data suggests that some of these oxysterol effects were being transmitted through a membrane protein. Therefore, we set out to purify and identify a novel membrane-bound oxysterol binding protein (MOBP). Using rabbit liver membranes, we purified a MOBP with an overall enrichment of 14,000 fold. Mass spectroscopy identified the candidate gene, Niemann-Pick Type C1 (NPC1) disease protein from our most purified sample. To identify NPC1 as a MOBP, we purified

epitope-tagged recombinant NPC1 and confirmed its ability to bind oxysterols. This work will serve as the starting point for future studies to determine the mechanisms of intracellular cholesterol transport.

CHAPTER II

Experimental Procedures

Materials and Methods - We obtained [26,27-^{3}H]25-hydroxycholesterol (75-80 Ci/mmol), [1,2,6,7-^{3}H]cholesterol (60 Ci/mmol), [3β-^{3}H]7,7-azocholestan-3β,25-diol (20 Ci/mmol) (photo[^{3}H]25-HC), and [1-^{14}C]oleic acid (54.6 Ci/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 P40 (NP-40) from Roche Applied Sciences; and Hybond C nitrocellulose filters and all chromatography products (unless otherwise mentioned) from GE Healthcare Biosciences. Solutions of compactin and sodium mevalonate were prepared as previously described (Brown et al., 1978; Kita et al., 1980).

Buffers - Buffer A contained 50 mM Tris-cholride at pH 7.4, 50mM KCL, 10% (v/v) glycerol, 5mM dithiothreitol (DTT), 1mM sodium EDTA, and protease inhibitor mixture (1μg/ml pepstatin A, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 200 μM phenylmethysulfonyl floride). Buffer B contained 50 mM Tris-chloride a pH 7.4, 10% (w/v) NP-40, 150 KCL, 1 mM EDTA, and 0.1 mM DTT. Buffer C contained 50 mM

Tris-chloride at pH 7.4, 1% NP-40, 1mM EDTA, and 0.1 mM DTT. Buffer D contained 50 mM Tris-chloride at pH 5.5, 1% NP-40, 1mM EDTA, and 0.1 mM DTT. Buffer E contained 10mM sodium phosphate at pH 7.4, 1% NP-40, 150 mM KCL, 1 mM EDTA, and 0.1 mM DTT. Buffer F contained 50 mM Tris-chloride at pH 7.4, 1% NP-40, and 150 mM KCL. Buffer G contained 50 mM Tris-chloride at pH 7.4, 1% NP-40, and 100 mM KCL. Buffer H contained 50 mM Tris-chloride at pH 7.4, 0.1% (w/v) Fos-choline 13, and 100 mM NaCl. Buffer I contained 50 mM Tris-chloride at pH 7.4, 0.004% NP-40, and 100 mM NaCl.

Plasmid Construction - pCMV-NPC1-His_8-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_8-Flag was sequenced to ensure integrity of the construct.

Solubilization of Sterols in Detergent of Ethanol - For solubilization of sterols in detergent, an ethanol solution containing 4-5 nmol of [^{3}H]25-hydroxycholesterol ([^{3}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 (0.5ml) was added to the tube, after which it was agitated for 4 h on a vortex mixer and centrifuged at 20,000*g* for 30 min at room temperature. As determined by scintillation counting, the concentration of solubilized [^{3}H]25-HC in the supernatant ranged from 0.4-1.5 μM. Stock solutions of [^{3}H]cholesterol at 60 Ci/mmol were prepared in a similar manner. Saturated solutions of unlabeled sterols in buffer G of buffer H were prepared by

evaporating an ethanolic solution of each sterol (50 μg) 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 [^{3}H]25-HC or [^{3}H]cholesterol, which allowed calculation of the concentrations of various sterol solutions. The choice of the labelled tracer was based on structural similarity to the bulk sterol. All 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 [^{3}H]25-HC Binding - Each reaction, in a final volume of 120 μl of buffer C, contained 50 nM [^{3}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 1.5 to 2 h at 4°C, the mixture was loaded onto a pre-treated glass-fibre filter (G4, Fisher) placed on a vacuum apparatus that contained 1 ml of buffer F. The filter was pre-treated by soaking in 0.3% (w/v) polyethaleneimine for at least 1 h. After application of the vacuum, the free [^{3}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 [^{3}H]25-HC was measured by scintillation counting. Non-specific binding was determined by incubating a duplication reaction with 10 μM unlabeled 25-HC delivered in ethanol.

Solubilization of NOBP from Rabbit Liver Membranes - All operations were carried out on ice or at 4°C. Frozen rabbit liver (200-300 grams, Pel-Freeze Biologicals) was thawed in 300 ml of cold buffer A supplemented with 25 μg/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 100,000*g* for 1 h. The resulting 100,000*g* pellet was washed in three sequential steps, each followed by centrifugation at 100,000*g* for 30 min. 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 Na_2CO_3 (pH 11.3) and protease inhibitors. The final washed membrane pellet was resuspended in 300ml buffer C containing the protease inhibitor mixture. Protein concentration was measured using a BCA kit, and the final detergent (NP-40) to protein ration was adjusted to a 4.5 (w/w) with buffer B. This mixture was rotated overnight at 4°C and centrifuged at 100,000*g* 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 as described above was used to follow [^{3}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*). The soluble extract was loaded onto a 20-ml Q Sepharose ion-exchange column (Hi Prep 16/10 Q FF, Ph 7.4) as described in Fig 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 FF) pre-equilibrated with buffer D (*Step 3*). The flow through was collected and loaded onto a 5-ml Q Seqparose ion-exchange column (HiTrap Q HP, pH 5.5) column 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 into 5 to 10 2-ml RCA Lectin columns (EY Laboratories) pre-equilibrated with buffer E (*Step 5*). Each column was washed with 3 column columes of buffer E, and proteins were eluted with buffer E plus 250 mM α-Lactose. The eluted fractions containing binding activity were pooled, dialyzed into buffer C, and concentrated by loading onto a 1-ml MonoQ ion-exchange column pre-equilibrated in buffer C. The column was washed with 8 column columes of buffer C and eluted with a continious 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 concentrations. The diluted fractions (containing ~50 mM KCL) were concentrated over a 1-ml MonoQ 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.

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_2 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 and $6x10^5$ cells/100-mm dish. On day 2, each dish was transfected with 4-5 μg of pCMV-NPC1-His_8-Flag in medium A with 5% FCS, using FuGene 6 reagent as described (Rawson et al., 1999). After incubation at 37°C for 24h, 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 100,000*g* centrifugation for 30 min at 4°C. The membrane pellet was resuspended by Dounce homogenization in buffer C containing protease inhibitor mixture and 25 μg/ml ALLN (4 dishes of cells per 1 ml of buffer), rotated overnight at 4°C to solubilize membrane proteins, and centrifuged at 100,000*g* for 30 min. The resulting 100,000*g* supernatant (containing detergent solubilized membranes) was dialyzed against buffer G for 6-12h at 4°C, after which inidazole was added at a final concentration of 20 mM. This material was then loaded onto a 1-,l 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 inidazole, 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 10 column volumues of buffer G (1% NP-40). Bound protein was eluted with 0.1 mg/ml of Flag peptide in 9 column

volumes of buffer G. Protein concentration of purified NPC1 was estimated by silver staining estimating and densitometric scanning of an 8% SDS-PAGE gel in which known amounts (50 to 500 ng) of bovine serum albumin (Pierce) were used as a reference.

Immunoblot Analysis - Column fractions from liver purification of MOBP and membrane fractions from cultured cells were subjected to 8% and 12% SDS-PAGE, after which the proteins were transfered 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 fractions; Sigma); and 4 μg/ml of monoclonal 1gG-11H9 directed against rabbit OSBP (IgG fraction) (Dawson et al., 1989a). 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.

CHAPTER III

Results

Purification of MOBP from Rabbit Liver – We carried out a biochemical purification to isolate and identify MOBP from rabbit liver membranes as described in "Experimental Procedures." MOBP's 25-HC binding activity was followed during each step in the purification by a filter assay for [^{3}H]25-HC binding as described in "Experimental Procedures." The overall enrichment of MOBP through this 7-step purification is detailed in Table 1.

Step 2 of the purification scheme involved running the detergent-solubilized rabbit liver membranes over a Q-Sepharose column, and the elution/binding profile is shown in Fig. 1A. The bound protein was eluted between fractions 25 and 60 (*open circles).* In contrast, [^{3}H]25-HC binding activity was organized into two distinct peaks (*filled circles).* OSBP-1, a soluble protein which binds 25-HC with nanomolar affinity is known to interact tightly with membranes through its plexihomology domain (Dawson et

al., 1989b). We hypothesized that the bimodal distribution of the [^{3}H]25-HC binding peak was due to the presence of membrane associated forms of OSBP-1. Therefore, an immunoblot (*inset*) was performed and all detectable OSBP-1 co-eluted with the second binding peak (fractions 39-51). The first [^{3}H]25-HC binding peak (fractions 30-35) contained no detectable OSBP-1 immunoreactivity, and these fractions became the material for the remaining steps in the purification as we hypothesized this peak contained the novel 25-HC binding protein MOBP (See Table 1 for complete purification scheme[1]). Molecular mass of MOBP•detergent complex was estimated to be ~440 kDa using gel filtration chromatography (Fig. 1B).

Fractions from multiple Q-Sepharose columns were combined and [^{3}H]25-HC binding activity was followed through the purification scheme as described in "Experimental Procedures." Overall outcome of the purification resulted in an approximate 14,000-fold purification and an 8% recovery of activity relative to the starting solubilized membrane fraction (see Table 1).

Sterol-Binding Characteristics of MOBP - Purified material from *Steps 4* and *5,* (see Table 1), was used to determine the specificity of MOBP sterol binding. Cholesterol (Fig. 2A) has a steroid nucleus and a iso-octyl side chain. 13 different sterols (Fig. 2B) that represent modification to the cholesterol molecule were assayed to determine their ability to compete for binding of 50 nM [^{3}H]25-HC to purified MOBP. All assays were completed in 1% NP-40 detergent as explained in "Experimental Procedures." As shown in Figs. *2C-2E,* addition of sterols 1-3, which included 25-HC, 27-HC, and 24(S)-HC respectively, resulted in significant competition at the concentration of 10 μM. In

contrast, sterols 4-13 showed little to no competition at 10 μM, indicating, that under these conditions, a hydroxyl group on the iso-octyl side chain is required for binding to MOBP.

Identification of MOBP as NPC1 - Purified MOBP material from *Step 7* of the purification scheme was visualized on silver-stained SDS-PAGE gels and 5 protein bands were identified (Fig. 3). We were able to crosslink a photoactivatable form of 25-HC to Band 1, identifying it as the novel MOBP. Through mass spectrometry analysis, 17 of 19 peptides were identified to be NPC1. The other 2 remaining bands corresponded to sialoadhesion. We chose to further investigate NPC1 in future studies because of its abundance, its proposed sterol sensing domain, (Nohturfft et al., 1998, Millard et al., 2005), and because of the abnormalities observed in patients with mutations in this protein.

Purification of Recombinant NPC1 and Demonstration of Oxysterol Binding in Presence of 1% NP-40 Detergent - Using a plasmid containing recombinant human NPC1 with a His_8-tag and Flag-tag fused to the COOH terminus, we transfected CHO-K1 cells. The 100,000*g* pellet of the transfected CHO-K1 membranes was solubilized in 1% NP-40 and purified in a two step process including Ni-chromotography (Fig. 4A), followed by anti-Flag affinity chromatography (Fig. 4B). Filter assays (see "Experimental Procedures") were performed on all elution fractions to determine oxysterol binding activity (*closed circles)*, and a standard BCA assay kit was used to determine protein concentration (*open circles*). When elution fractions were immunoblotted for the C-terminal Flag-tag, all

[^{3}H]25-HC binding activity co-eluted with the NPC1 recombinant protein (Fig. 4A and 4B). The recombinant NPC1-His$_8$-Flag protein was purified to near homogeneity as judged by samples subjected to SDS-PAGE followed by silver staining (Fig. 4C and 4D). The most significant band (marked by *asterisks*) migrated to approximately 250 kDa in both gels, and reacted with anti-Flag antibody when immunoblotted (data not shown). Sequence analysis with mass spectrometry confirmed the major protein band to be NPC1. Two other faint bands were visible in the 8% gel, and mass spectrometry confirmed that the faint band which migrated above the 250 kDa marker (marked by an *arrow* in Fig. 4C) was also NPC1, most likely representing a NPC1 oligimer resistant to SDS denaturation. The smaller band at approximately 37 kDa was confirmed to be non-muscle γ-actin by mass spectrometry, a likely contaminant (data not shown).

Binding of 25-HC but not Cholesterol to Recombinant NPC1 in Presence of 1% NP-40 - Using a modified assay developed during earlier experimentation involving SCAP (Radhakrishnan et al., 2004), we investigated the binding activity of NPC1-His$_8$-Flag for ^{3}H-labeled sterols. 1% NP-40 detergent was used to solubilize the ^{3}H-sterols, the same detergent concentrations used during the solubilization and purification of NPC1-His$_8$-Flag. NPC1-His$_8$-Flag was bound to a nickel column, and the ^{3}H-sterol binding activity of the construct was assayed by separating free ^{3}H-sterol and NPC1-bound forms. A saturation curve for the binding of [^{3}H]25-HC to NPC1-His$_8$-Flag is shown in Fig. 5A. Saturation kinetics was observed with a half maximum of approximately 50-100 nM. This binding was reduced when unlabeled 25-HC was added to the reaction. When [^{3}H]Cholesterol binding was examined under similar detergent conditions (Fig. 5B),

binding was not detectable. Accordingly, Fig. 5C shows that unlabeled 25-HC, but not cholesterol, competed for binding of 100 nM [^{3}H]25-HC to the NPC1-His$_8$-Flag construct.

Table 1

Purification of MOBP from Rabbit Liver Membranes

Step	Fraction	Protein[a] (mg)	Specfic Actiivty (pmol/mg)	Total Activity (pmol)	Purification (fold)	Recovery (%)
1	Solubilized Membranes[b]	29,008	0.01	300	----------	100
2	Q Sepharose (pH 7.4)	1,727	0.19	332	19 (74)[c]	110
3	SP Sepharose	846	0.26	295	34 (135)	98
4	Q Sepharose (pH 5.5)	127	1.3	144	150 (601)	48
5	RCA Lectin to MonoQ	9	3.6	31	347 (1388)	10
6	S-300 Gel Filtration to MonoQ	9	4.5	29	309 (1237)	10
7	Reactive Blue	0.7	37.4	25	3618 (14471)	8

[^{3}H]25-HC binding activity was assayed under standard conditions in the presence of 50 nM [^{3}H]25-HC. Incubations were carried our for 90 min at 4°C as described under "Experimental Procedures."

[a]Protein concentration of the various fractions was determined as described under "Experimental Procedures."

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

[c]Numbers in parenthesis denote the *fold*-purification of MOBP when the contribution of OSBP-1 binding in the starting fraction is subtracted.

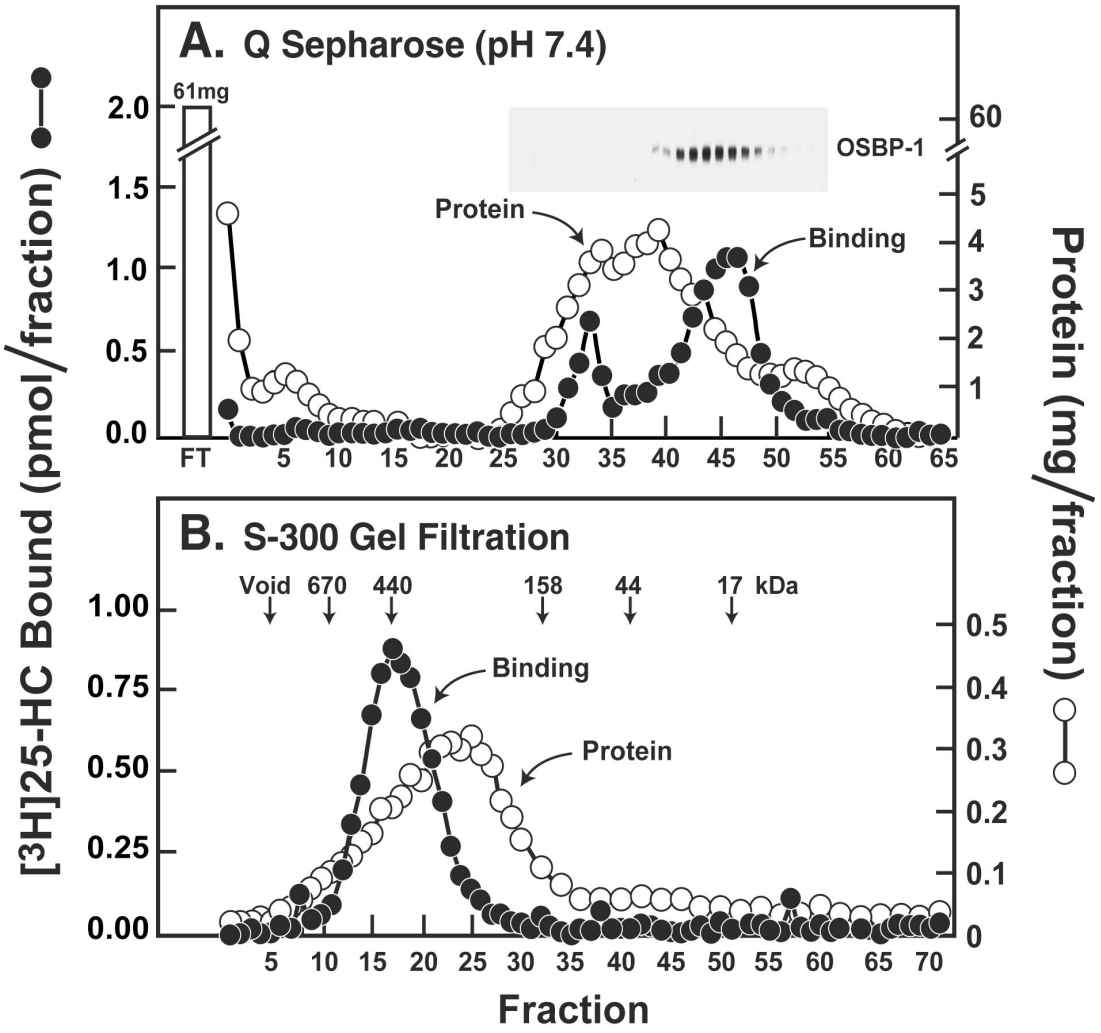

Figure 1 - Representative steps in purification of rabbit MOBP

A, Separation of MOBP from OSBP-1 by Q-Sepharose Chromatography. The 100,000*g* 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 (5ml) were collected and assayed for [^{3}H]25-HC binding activity (*closed circles*) and protein concentration (*open circles*) using 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 filtrations. 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 [^{3}H]25-HC binding activity (*closed circles*) and protein concentration (*open circles*) as described in (A). Standard molecular weight markers (thyroglobulin, 670 kDa; ϒ-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.

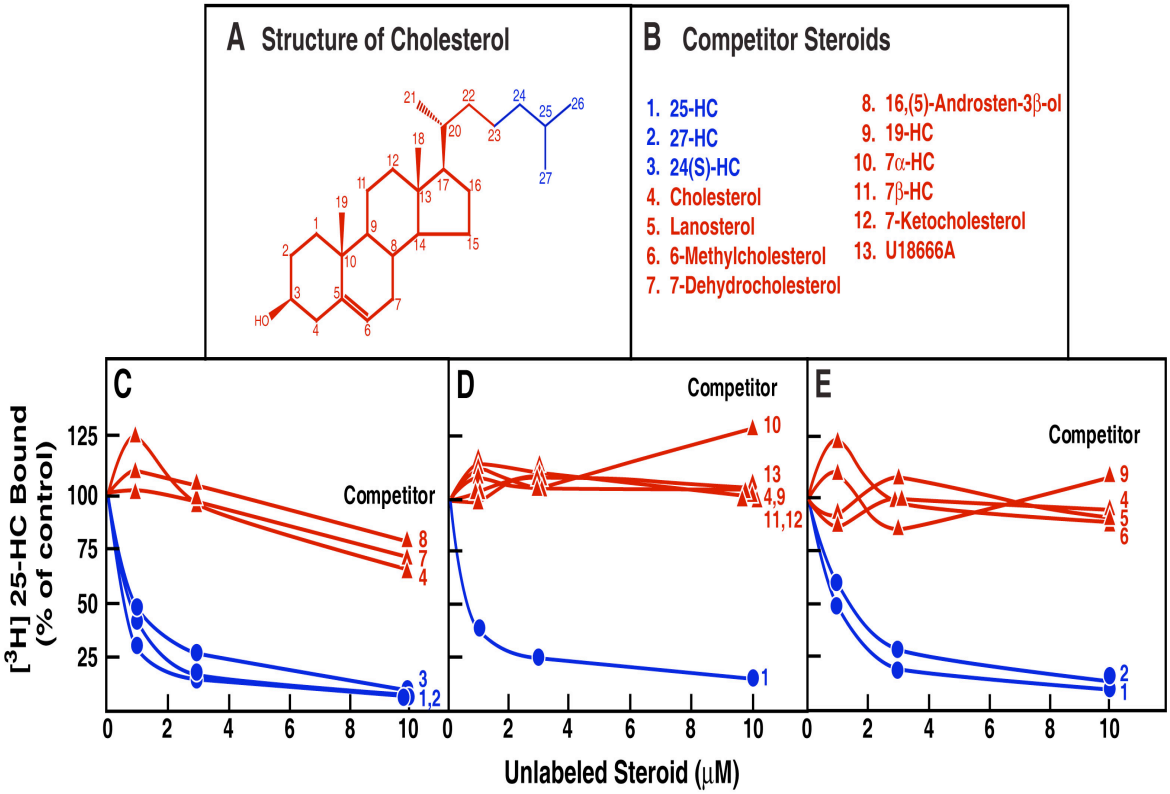

Figure 2 - Specificity of Sterol Binding to Partially Purified Rabbit MOBP
A, chemical structure of cholesterol. **B**, list of unlabeled sterols tested for their ability to compete with binding of [^{3}H]25-HC to MOBP. Blue and red colored sterols denote those that compete and that that do not compete for [^{3}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 [^{3}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 [^{3}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**, easy 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.

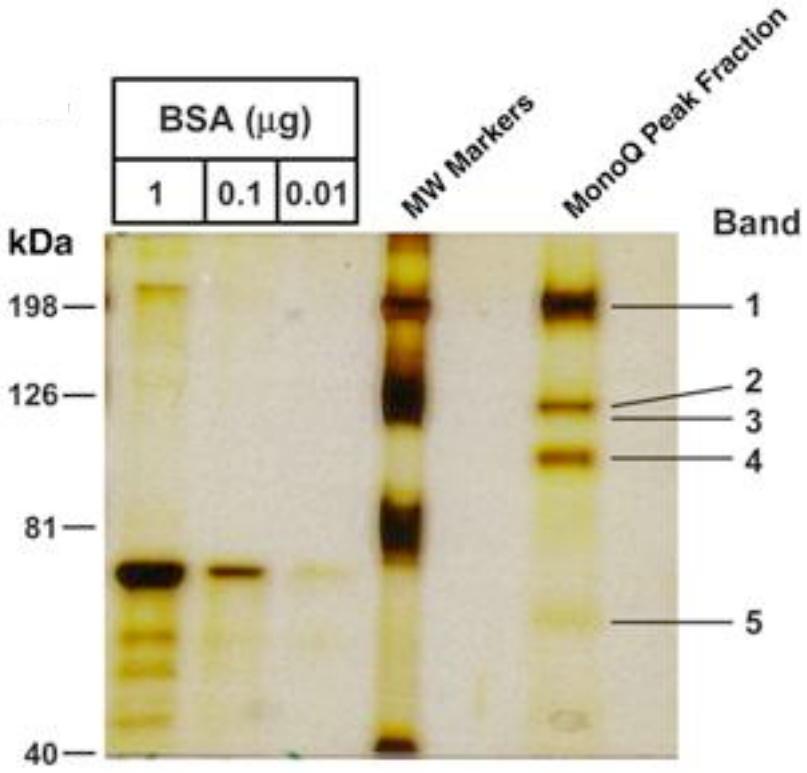

Figure 3 - Silver-Stained SDS-PAGE Gel of Purified MOBP from Blue Dye Column

Purified MOBP material from blue dye column subjected to 8% SDS-PAGE and silver staining. BSA standards in left three lanes were loaded to demonstrate sensitivity of silver staining. Molecular weight markers are noted on left edge. Purified samples contained 5 bands, and through experiments utilizing a crosslinking photocholesterol (data not shown) the 25-HC binding activity was localized to Band 1. Mass spectrometry identified Band 1 to be NPC1.

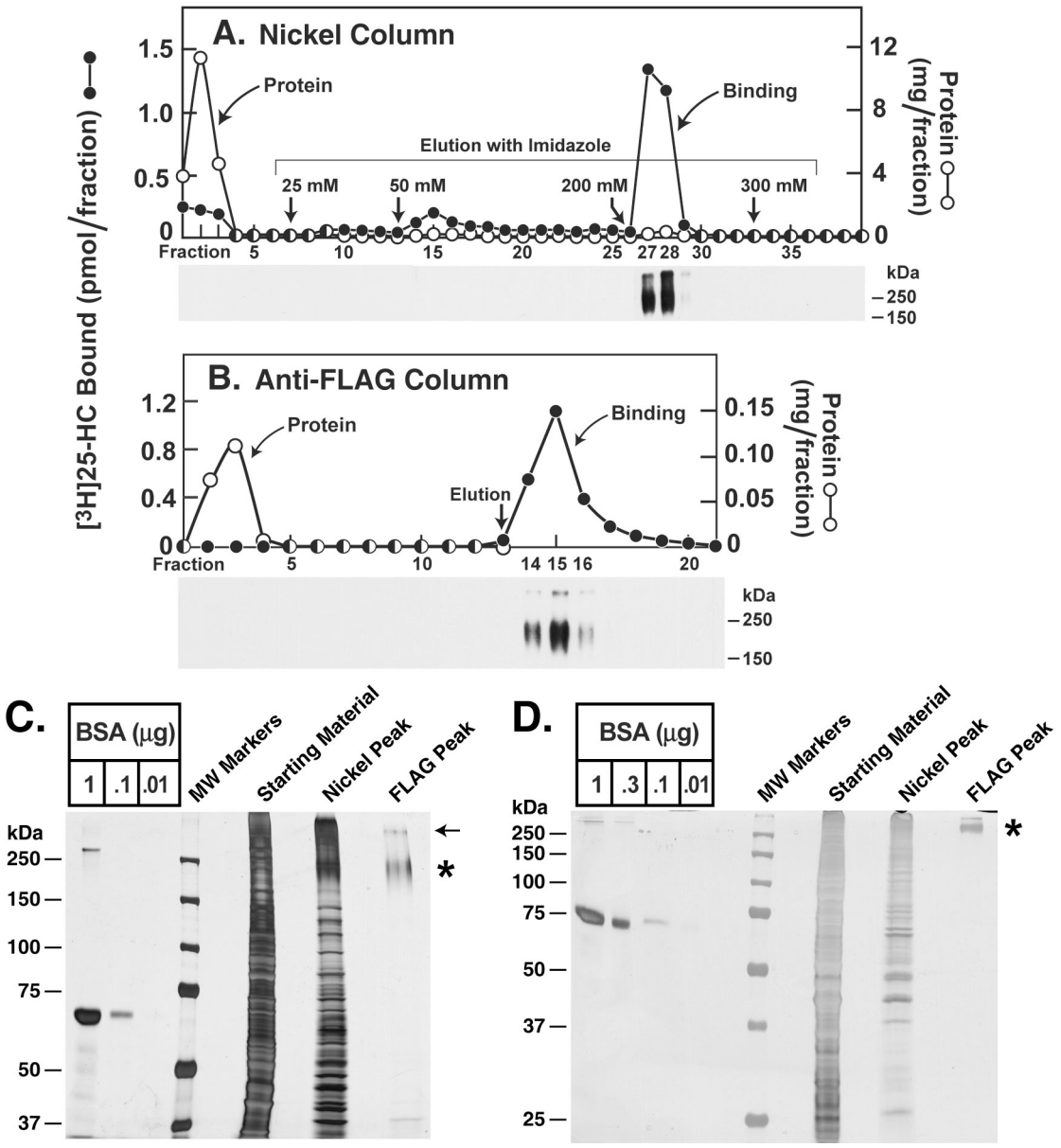

Figure 4 - Purification of Recombinant Human NPC1 form Transfected CHO-K1 cells.
A, nickel column chromatography. CHO-K1 cells (50 100-mm dishes) were transfected with 5 μg/dish of human NPC1-His_8-Flag as described under "Experimental Procedures." Detergent-solubilized membranes (100,000*g* fraction, 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 imadazole washes (25, 50, 200, 300 mM) as indicated. Fractions (1.5 ml) were tested for protein concentration (*open circles*), [^{3}H]25-HC binding activity (*closed circles*), and immunoblot analysis with monoclonal Flag antibody (shown below fraction numbers). ***B***, anti-Flag choromatography. Fractions 27 and 28 from (*A*) were pooled, loaded onto a 1-ml 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 (*open circles*), [^{3}H]25-HC binding activity (*closed circles*), and immuno blot analysis with monoclonal anti-Flag antibody. Protein measurements for fractions 14-21 are not shown since they contain eluted Flag peptide. ***C*** and ***D***, SDS-PAGE of purified recombinant NPC1. An aliquot of fraction 15 forn 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 bans 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.

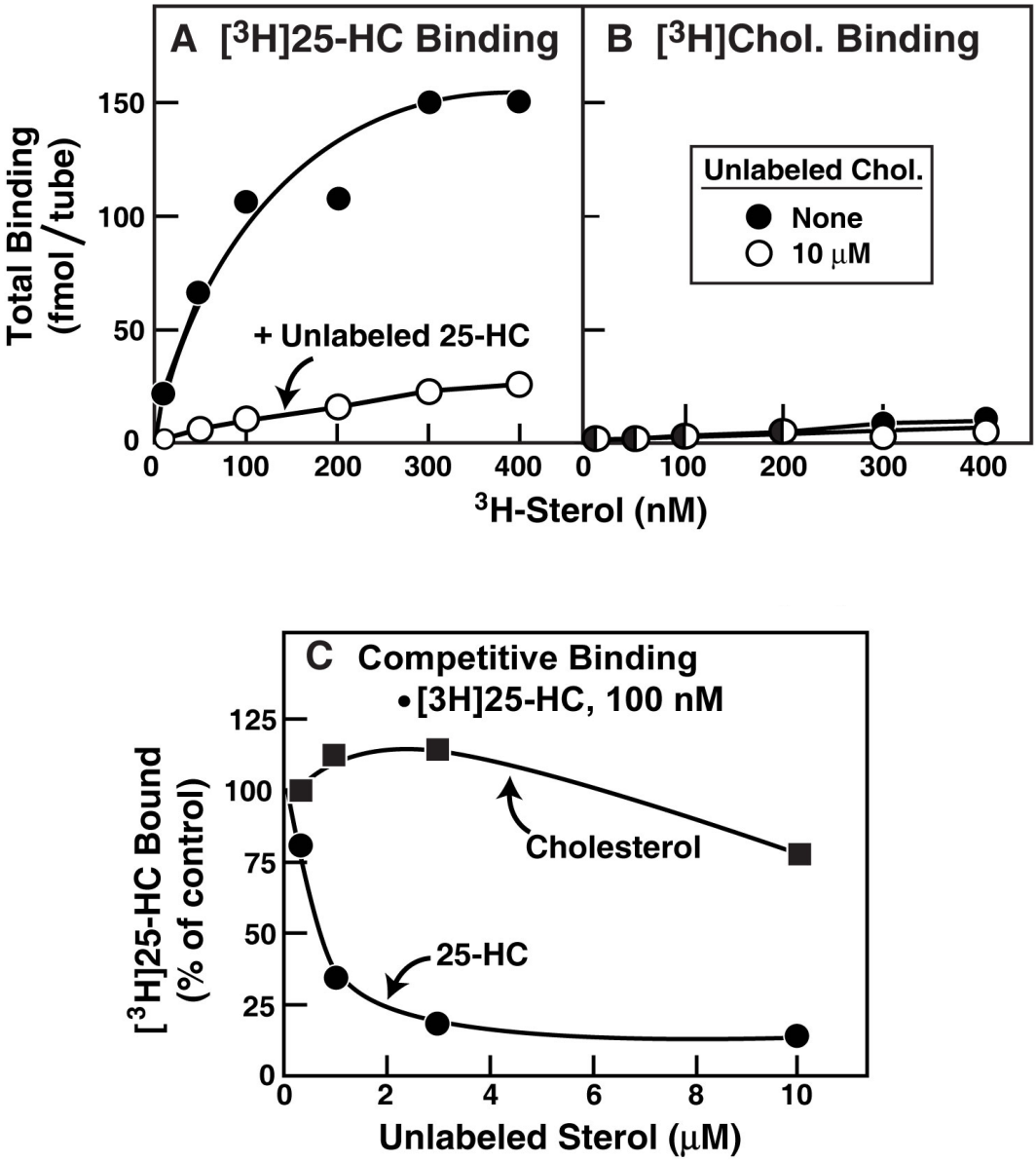

Figure 5 - NPC1 Binds 25-Hydroxycholesterol, but Not Cholesterol in 1% NP-40 detergent.

A and **B**, saturation curves for NPC1 binding of [^{3}H]25-HC and [^{3}H]Cholesterol. Each reaction, in a final volume of 120 μl of buffer G, contained ~250 ng of purified human NPC1-His$_8$-Flag and 10-400 nM of either [^{3}H]25-HC (*A*) or [^{3}H]Cholesterol (*B*) (both solubilized in NP-40) in the absence (*closed circles*) or presence (*open circles*) of 10 μM unlabeled 25-HC (*A*) or cholesterol (*B*) was measured using the Ni-ATA Agarose binding assay as described under "Experimental Procedures." Each data point represents total binding without subtraction of blank values. **C,** competitive binding of [^{3}H]25-HC to NPC1 in presence of unlabeled sterols. Each reaction, in a total volume of 140 μl of buffer G, contained ~150 ng of purified NPC1-His$_8$-Flag, 100 nM of [^{3}H]25-HC, and the indicated concentration of unlabeled 25-HC (*closed circles*) or cholesterol (*closed squares*). After incubation for 3 h at 4°C, bound [^{3}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.

CHAPTER IV

Discussion

The goal of this study was to discover a membrane-bound receptor of oxysterols which regulates cholesterol homeostasis. It was hoped that finding such a protein would help explain why oxysterols, specifically 25-Hydroxycholesterol, are such potent repressors of SREBP processing. Elevated levels of both cholesterol and 25-HC cholesterol inhibit the migration of the SCAP-SREBP complex from the ER to the golgi (Adams et al., 2004; Radhakrishnan et al., 2007). We know that cholesterol binds directly to SCAP causing a conformational change which facilitates binding to anchoring protein INSIG, but the receptor and mechanism of SREBP repression due to 25-HC was unknown at the onset of this study. Distinguishing a novel membrane-bound oxysterol binding protein (MOBP) from the soluble oxysterol binding proteins (OSBP-1 and ORPs) was accomplished using whole rabbit livers because of the availability of monoclonal antibodies against rabbit OSBP-1.

A seven step purification sequence was developed using various chromatographaphy (Table 1), which lead to the identification and purification of a membrane-bound protein which bound 25-HC with high affinity and specificity. An approximately 14,000 fold purification was achieved. Gel filtration studies were consistent with a molecular mass of ~440 kDa for the hypothesized MOBP•detergent complex. Mass spectrometry analysis was utilized to identify MOBP, revealing a protein with a known role in cholesterol homeostasis, Niemann-Pick Type C1 (NPC1). NPC1 is known to play an important role in intracellular cholesterol transport (Pentchev et al., 1985; Liscum and Faust, 1987; Pentchev et al., 1997), but its ability to bind oxysterols and possible involvement in the regulation of cholesterol biosynthesis and esterification has never been determined.

To prove that NPC1 was the MOBP in our rabbit purification, we purified a recombinant version of human NPC1 with epitope tags (Fig 4). Recombinant NPC1 bound 25-HC with nanomolar affinity (Fig 5 A and B). Through competition studies we showed that oxysterols with hydroxyl groups located at positions 24, 25, or 27 bound with high specificity, while oxysterols with hydroxyl groups attached to the steroid nucleus or those attached proximally on the side chain failed to demonstrate significant binding in our conditions of 1% NP-40.

In our current experiments and conditions, we were unable to show that NPC1 bound cholesterol, the very molecule it is predicted to transport (Pentchev et al., 1987; Pentchev et al., 1995; Pentchev, 2004; Liscum and Sturley, 2004). Further studies are required to investigate the effects that detergent concentrations in our binding assay have on the binding activity of NPC1 to cholesterol. Additionally, characterization of NPC1's

binding site for oxysterols is needed. Topology analysis indicates NPC1 contains a putative sterol sensing domain which is seen in other membrane proteins such as SCAP, and HMG CoA Reductase. SCAP is known to directly bind cholesterol using its SSD, and investigations directed at NPC1's SSD will hopefully provide insight as to its function. If NPC1's SSD is not involved in directly binding sterols, is it possible the SSD plays a different and still unknown role in cholesterol homeostasis?

NPC1's role in the SREBP pathway is still undetermined. Additional questions arise as to whether NPC1 deficient cells exhibit the same inhibitory response in the SREBP pathway upon exposure to oxysterols, and whether SREBP processing plays a role in regulating NPC1 expression? NPC1's main known function is to transport LDL-derived cholesterol from recently endocytosed endosome and lysosomes to the ER and plasma membrane. The question of whether this activity of NPC1 is regulated by oxysterols is still unanswered. Now that the sterol binding activity of NPC1 has been partially characterized, these questions can be answered[#].

(# - See Addendum for additional project information)

CHAPTER V

Addendum

The amount of true scientific knowledge discovered in the Brown and Goldstein laboratory is overwhelming. My contribution and time spent working on this project is relatively minute when you think back and remember all that has been accomplished since that fateful day in 1972 when Drs. Michael Brown and Joseph Goldstein encountered the family with FH. This lab has been a juggernaut, and true to form, between my departure at the end of the summer of 2006 and my recent time in 2008, many questions regarding NPC1 have been answered.

This project has been spearheaded by a MSTP student, Rodney E. Infante, and the completed manuscripts can be found below:

Infante, R.E., Abi-Mosleh, L., Radhakrishnan, A., Dale, J. D., Brown, M. S., and Goldstein, J. L. (2008) *J. Biol. Chem*. **283**, 1052-1063.

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.

Infante, R.E., Wang, M.L., Radhakrishan, A., Kwon, H. J., Brown, M.S., and Goldstein, J. L. (2008) *PNAS. In process*

A short summary of our progress is listed below:

In the first publication, we have been able to demonstrate the NPC1 binds both 25-HC and cholesterol, however cholesterol binding requires sub-micellar concentrations of detergent. Binding of 25-HC took place in detergent concentrations well exceeding the critical micellar concentration (CMC). The questions regarding NPC1's role in oxysterol regulation of SREBP-2 processing has also been addressed, and we have failed to show any requirement for NPC1 to be involved. In the second paper, a detailed analysis and localization of NPC1's oxysterol binding site was performed. Oxysterol binding was determined to take place within the first luminal loop of NPC1, a region of approximately 240 amino acids with a highly conserved sequence and a large number (18) of cysteines. Mutational analysis and binding characterization was also performed. This Luminal domain of NPC1 appears to play a role in the transfer of cholesterol with the other protein involved in this disease, NPC2.

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Drs. Brown and Goldstein for allowing me the privilege of learning how science should be conducted. You trusted me, provided fantastic guidance, and inspiring mentorship.

Rodney Infante – for training me with daily patience, and for his major role in the publication of this work. A great teacher and a better friend.

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Dr. Michael McPhaul whose dedication to student research has sculpted the careers of many academic clinicians.

UTSW Student Research Program

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VITAE

Jarrod Donald Dale was born in Nelson, British Columbia, Canada on February 8th, 1983 as the first son to Jill Ann and Randall Monroe Dale. After graduation from L.V. Rogers High School of Nelson, BC in 2001, he moved to Texas to pursue a baseball and academic scholarship at Texas State University in San Marcos, Texas. He first discovered the excitement of basic science research while working in the Biochemistry Laboratory of Dr. Kevin Lewis, Ph.D studying the interplay between telomerase and DNA repair complexes. Jarrod graduated Summa Cum Laude with a Bachelor of Science in Biochemistry and a minor in biology in 2005. It was at this time that Jarrod decided to pursue an academic career, instead of an athletic career in baseball, and he joined the class of 2009 at The University of Texas Southwestern Medical School. During the summer of 2005, he joined the Department of Molecular Biology as a summer student researcher under the mentorship of Dr. Michael Brown and Dr. Joseph Goldstein. He has worked closely with MSTP student Rodney Infante over the summers of 2005, 2006, and during a research elective in 2008 to elucidate the function and role of Membrane-bound Oxysterol Binding Protein (MOBP) in cholesterol homeostasis. During his time in the lab, they have made significant gains in identifying and characterizing MOBP, which has been identified as Niemann-Pick disease, type C1 protein (NPC1). Jarrod will complete his Doctorate of Medicine, with a Distinction in Research in June of 2009.

Permanent Address: 1116 Hidden Ridge Drive #2089 Irving, Texas 75038