ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ

«ΚΥΤΤΑΡΙΚΗ ΚΑΙ ΓΕΝΕΤΙΚΗ ΑΙΤΙΟΛΟΓΙΑ ,ΔΙΑΓΝΩΣΤΙΚΗ ΚΑΙ ΘΕΡΑΠΕΥΤΙΚΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ»

ΑΝΑΦΟΡΑ ΠΡΟΟΔΟΥ ΜΕΤΑΠΤΥΧΙΑΚΟΥ ΤΙΤΛΟΥ ΕΙΔΙΚΕΥΣΗΣ

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ΠΡΟΛΟΓΟΣ

Η παρούσα μεταπτυχιακή εργασία εκπονήθηκε στα πλαίσια του μεταπτυχιακού προγράμματος «ΚΥΤΤΑΡΙΚΗ ΚΑΙ ΓΕΝΕΤΙΚΗ ΑΙΤΙΟΛΟΓΙΑ ,ΔΙΑΓΝΩΣΤΙΚΗ ΚΑΙ ΘΕΡΑΠΕΥΤΙΚΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ» του τμήματος Ιατρικής,του Πανεπιστημίου Κρήτης.Η ερευνητική εργασία και η συγγραφή της αναφοράς πραγματοποιήθηκαν στο Department of Molecular Genetics,University of Texas Southwestern Medical Center at Dallas.

Οφείλω να ευχαριστήσω τον επιβλέποντά μου Καθηγητή Βασίλη Ζαννή για την επίβλεψη και την καθοδήγησή του κατά τη διάρκεια ,όχι μόνο της ερευνητικής εργασίας,αλλά των δύο χρόνων του μεταπτυχιακού προγράμματος.Ο κυριότερος λόγος για να τον ευχαριστήσω είναι ότι εκτός από τη συνεχή βοήθεια,οι συμβουλές του και το παράδειγμά του αυτά τα χρόνια ,επηρέασαν καθοριστικά τις επιλογές μου.Επίσης, ευχαριστώ πολύ τον αναπληρωτή Καθηγητή Βιοχημείας Δημήτρη Καρδάση για τη βοήθεια και τις χρήσιμες και ουσιαστικές συζητήσεις.

Επιπλέον,θέλω να ευχαριστήσω την Dr.Helen H.Hobbs και τον Dr. Jonathan C.Cohen για την αποδοχή μου στο εργαστήριο τους, την καθοδήγηση, τις συμβουλές τους και τη βοήθεια τους στη συγγραφή της εργασίας.Όλο το προσωπικό του εργαστηρίου στάθηκε πολύτιμος αρωγός στην εκπόνηση της εργασίας,με παρατηρήσεις,σχόλιακαι βοήθεια στην εκτέλεση ορισμένων πειραμάτων.

Μεγάλο στήριγμα κατά τη διάρκεια των μεταπτυχιακών μου σπουδών ήταν οι φίλοι μου,που πάντα με στηρίζουν και μου δίνουν κουράγιο και δύναμη να συνεχίσω την προσπάθειά μου.Ένα μεγάλο ευχαριστώ,λοιπόν,στο

Δημήτρη,το Θοδωρή,τον Ιωσήφ,τον Κώστα,το Νίκο και στις φίλες μου Αριστέα,Ρίτα και Σταυρούλα.
Κλείνοντας,θέλω να εκφράσω την ευγνωμοσύνη στην οικογένεια μου,τους γονείς μου Γιώργο και Χαρίκλεια και τις θείες μου Ρίτσα και Ιάσμη.Η ηθική,οικονομική και ψυχολογική στήριξη που μου παρέχουν απλόχερα όλα αυτά τα χρόνια είναι πραγματικά συγκινητική και σε αυτήν οφείλονται όλα όσα έχω πραγματοποιήσει μέχρι τώρα.Πάνω από όλα τους ευχαριστώ, επειδή μου έμαθαν ότι τα πτυχία δεν έχουν αξία αν δε συνοδεύονται από μόρφωση και ηθικές αξίες.Ως ελάχιστο δείγμα ευγνωμοσύνης και αγάπης, τους αφιερώνω αυτή τη διατριβή.

CONTENTS

ABSTRACT

Intestinal cholesterol absorption plays an important role in determining the levels of circulating cholesterol. Recently a new pharmacological agent, ezetimibe, was released that reduces cholesterol absorption and plasma cholesterol levels. The molecular target of ezetimibe is likely Niemann-Pick C1 Like 1 protein (NPC1L1), which is expressed predominantly in the small intestine . Inactivatıon of NPC1L1 in mice results in a 70% reduction in the absorption of orally administered radiolabeled cholesterol, which is similar to the reduction seen in wild-type mice treated with ezetimibe. The molecular mechanism by which NPC1L1 transports cholesterol is unknown. Previously, a post-doctoral fellow in the Hobbs/Cohen laboratory observed a cholesterol-associated change in the migration of NPC1L1 to a higher apparent molecular mass on a native gel. The first aim of my project is to determine if this higher molecular weight band represents a dimer of NPC1L1. Initial experiments have been performed by expressing differentially-tagged versions of NPCC1L1 in Chinese Hamster Ovarian(CHO-K1)cells and determining if the two forms of NPC1L1 co-immunoprecipitate (Co-IP).

ΠΕΡΙΛΗΨΗ

Η απορρόφηση χοληστερόλης από το έντερο έχει σημαντικό ρόλο στον καθορισμό των επιπέδων χοληστερόλης στην κυκλοφορία. Πρόσφατα,κυκλοφόρησε ένας νέος θεραπευτικός παράγοντας η εζετιμίδη(ezetimibe)που μειώνει την απορρόφηση χοληστερόλης και τα επίπεδα χοληστερόλης του πλάσματος.

Ο στόχος του φαρμάκου αυτού σε μοριακό επίπεδο είναι πιθανότατα η πρωτεινη Niemann Pick C1 L1 (NPC1L1), που εκφράζεται κυρίως στο λεπτό έντερο. Ποντίκια που δεν εκφράζουν την NPC1L1 έχουν κατά 70% μειωμένη απορρόφηση χορηγούμενης από το στόμα ραδιοσημασμένης χοληστέρολης.Την ίδια μείωση στην απορρόφηση χοληστερόλης έχουν ποντίκια στα οποία χορηγήθηκε το φάρμακο.

Ο μοριακός μηχανισμός με τον οποίο η NPC1L1 μεταφέρει τη χοληστερόλη είναι άγνωστος.Στο παρελθόν,έχει παρατηρηθεί στο εργαστήριο των Hobbs/Cohen ότι υπάρχει μια ρυθμιζόμενη από τη χοληστερόλη αλλαγή στην εντόπιση της μπάντας της NPC1L1(εμφάνισης μιας ακόμη μπάντας της NPC1L1 σε μεγαλύτερο μοριακό βάρος).Ο πρώτος στόχος της εργασίας μου είναι να διερευνήσω αν η υψηλότερου μοριακού βάρους μπάντα της NPC1L1 αντιστοιχεί σε ένα διμερές της NPC1L1.Τα αρχικά πειράματα πραγματοποιήθηκαν με την έκφραση NPC1L1 που έχουν διαφορετικούς επιτόπους σε κύτταρα Chinese Hamster Ovarian (CHO-K1)και τη μελέτη της συν-ανοσοκατακρήμνισης των δύο μορφών της NPC1L1 που έχουν διαφορετικούς επιτόπους.

INTRODUCTION

Clinical and epidemiological studies have established that elevated plasma cholesterol levels (especially LDL-cholesterol) promote atherosclerosis. Circulating cholesterol levels depend on the secretion and catabolism of plasma lipoproteins. There are two sources of cholesterol: endogenous synthesis (from acetate) and the diet.

Intestinal absorption of dietary cholesterol

Intestinal absorption of cholesterol is a multi-step process that involves multiple proteins. Dietary cholesterol and plant-derived sterols (predominantly sitosterol and campesterol) are solubilized in mixed micelles and taken up into the enterocytes in the proximal small intestine. The fraction of cholesterol absorbed from the diet varies remarkably between individuals (from ~25-85%) (1). A much smaller fraction of plant-derived sterols are absorbed (<5%) due to these sterols being preferentially secreted from the enterocytes back into the gut lumen by the action of ABCG5 and ABCG8. The bulk of the cholesterol is delivered to the endoplasmic reticulum (ER) where it is esterified by acyl CoA:cholesterol acyltransferase isoform 2 (ACAT2) and incorporated into chylomicrons. In contrast, only a small fraction of plant-derived sterols are in chylomicrons.

Inhibition of cholesterol absorption is an effective strategy for lowering plasma cholesterol levels and reducing coronary artery disease. Less dietary cholesterol is delivered to the liver, resulting in an increase in LDL receptor activity and accelerated clearance of circulating LDL. Several agents have been developed that target this pathway (2). The most

exciting such agent is ezetimibe (Zetia, Merck-Shering Plough), which was released four years ago. Daily administration of ezetimibe is association with a 15-20% reduction in plasma levels of LDL-cholesterol. Combination of ezetimibe with statins increase the reduction of LDL levels further (3).

NPC1L1 – the target of ezetimibe

Ezetimibe was shown to act directly at the level of enterocytes and not to interfere with micellar solubilization of cholesterol (4). Ezetimibe undergoes glucuronidation in the enterocytes and then is transported to the liver and secreted into the bile. The molecular target of ezetimibe was revealed in 2004 with the discovery by Altmann and co-workers of NPC1L1. They used a genomics-bioinformatics approach to find the gene. First, they prepared two expressed sequence tags (ESTs) cDNA libraries: one from the mucosa of the rat jejunum and the other from jejunal enterocytes obtained by laser capture microdissection. They looked for transcripts predicted to encode proteins with the expected characteristics of a cholesterol transporter, such as a plasma membrane secretion signal sequence, a transmembrane domains and a cholesterol-sensing domain. Only one EST fulfilled all these criteria, which was the rat homolog of NPC1L1 (5). In mice, NPC1L1 was predominantly expressed in small intestine (peak expression in proximal jejunum) and at low levels in the liver, stomach, gallbladder and testis (5).

Altmann and his colleagues inactivated NPC1L1 in mice and showed that the mice had a 70% reduction in the absorption of orally administered radiolabeled cholesterol, despite having a macroscopically and histologically normal intestinal tract (5). The magnitude of the reduction in cholesterol

absorption was similar to that seen in wild-type mice treated with ezetimibe, and no further reduction in cholesterol absorption was seen when the NPC1L1 null mice were treated with ezetimibe. Localization studies show that the labeled ezetimibe glucuronide appears to bind the apical intestinal brush border membrane of wild-type mice but not *NPC1L1*$^{-/-}$ mice. Ezetimibe also binds membranes prepared from human embryonic kidney cells (HEK 293) expressing NPC1L1, but not to cells expressing the vector alone (6). The NPC1L1 null mice were also found to have increased levels of the 3-hydroxy 3-methylglutaryl (HMG) CoA reductase mRNA, presumably due to the reduced delivery of dietary cholesterol from the intestine to the liver. These data are consistent with NPC1L1 being either directly or indirectly the target of ezetimibe.

NPC1L1 is also required for the absorption of plant-derived sterols(7,8). Individuals with inactivating mutations in *ABCG5* or *ABCG8* (sitosterolemia) accumulate plant-derived sterols in tissues. Inactivation or inhibition of NPC1L1 using ezetimibe prevents the accumulation of plant sterols as well as cholesterol (9) in patients with this autosomal recessive disorder.

A proposed model of cholesterol absorption that incorporates the action of ezetimibe is shown in **Figure 1**.

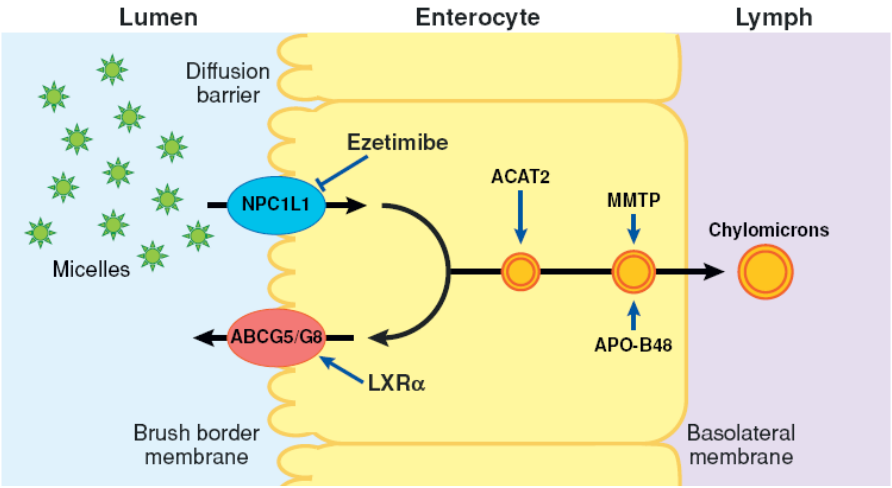

Figure 1. (From D.Wang, Annu.Rev.Physiol 69:11.1-11.28)(10). NPC1L1 on the apical membrane of enterocytes facilitates sterol uptake. Ezetimibe inhibits NPC1L1 and reduces sterol absorption. ABCG5 and ABCG8 promote sterol efflux from enterocytes to the intestinal lumen; the genes upregulated by the nuclear hormone receptor LXRα in response to diet enriched in cholesterol. Other proteins, like ACAT2,apolipoprotein B-48 (apo B-48) and microsomal triglyceride transfer protein (MTP) are required for the esterification of cholesterol and the formation of chylomicrons that are secreted from the basolateral surface into the lymph.

Human genetics of NPC1L1

To determine the role of NPC1L1 in humans, Cohen *et al.* sequenced the coding regions of NPC1L1 in individuals predicted to have either a high or a low fractional absorption of cholesterol. An excess of sequence variations that changed the amino acid (nonsynonymous sequence variations) at highly conserved residues were found in the low absorber group. Moreover, when the variations found in the lower group were examined in a larger population, they were associated with a lower cholesterol absorption and a 9% reduction in plasma LDL-cholesterol levels (11). These data provide evidence that NPC1L1 participates in cholesterol absorption in humans as well as rodents.

Structure of NPC1L1

NPC1L1 shares 42% amino acid identity with NPC1 (Niemann Pick type C1), which is involved in the intracellular transport of cholesterol and is defective in Niemann-Pick disease type C1 (12). NPC1L1 is predicted to have a signal peptide, a conserved N-terminal NPC1 domain and 13 putative transmembrane domains that include a sterol sensing domain (SSD). The sequence of the SSD resembles a similar sequence not only in NPC1 but also in SREBP-sterol regulatory element binding protein-cleavage activating protein (SCAP) (13), HMG CoA reductase, and Patched, a membrane protein that serves as a receptor for Hedgehog protein. The SSD consists of five putative transmembrane domains. Selected mutations in the SSD of SCAP (Y298C and D443N) interfere with the cholesterol regulatory complex moving from the ER to the Golgi.

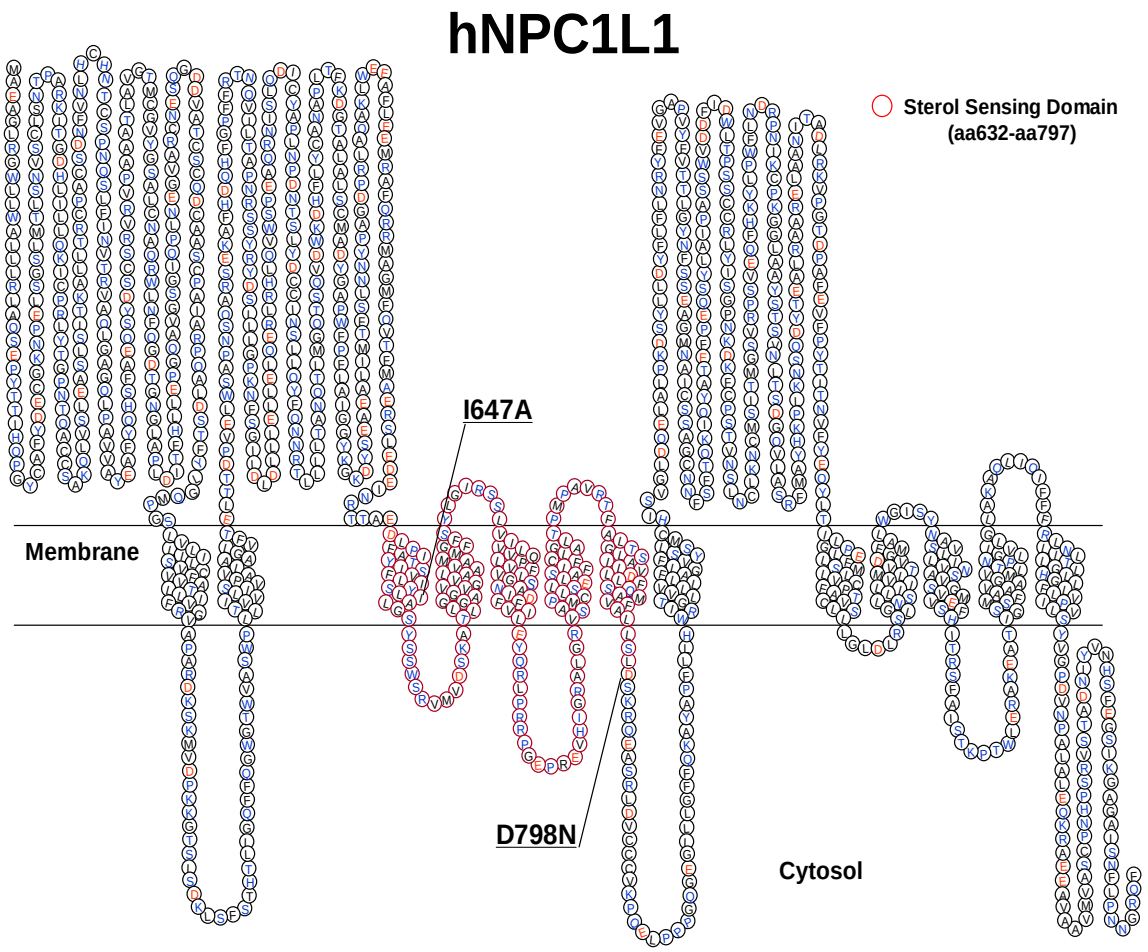

Figure 2. A schematic representation of NPC1L1. NPC1L1 has 13 putative transmembrane domains.The sterol sensing domain is between amino acids 632 and 797. The I647A (substituting isoleucine with alanine at position 647) and D798N (substitution of aspartic acid with asparagine at position 798) are mutant forms of human NPC1L1 that will be used in future experiments.

Regulation of NPC1L1

When wild-type mice are fed a cholesterol/cholate rich diet, *NPC1L1* expression is down-regulated in the intestine, presumably to prevent absorption of excess dietary cholesterol (7). Activation of the nuclear hormone receptor LXR using a potent synthetic LXR agonist also results in down-regulation of NPC1L1 (14).

Subcellular localization of NPC1L1

The subcellular localization of NPC1L1 is not entirely clear. Immunohistochemical studies suggest NPC1L1 is localized in the apical membrane of enterocytes (5), although these data remain controversial. Other investigators performing studies in human hepatoma HepG2 cells and in a colon cancer cell line (Caco-2) cells have reported the protein to be co-localized with Rab5, an early endosomal protein (8). Yu *et al* . (15) proposed that the cellular itinerary of NPC1L1 was cholesterol-regulated, based on studies using immunofluorescence and biotinylation of cell surface proteins. He observed that cholesterol depletion using cyclodextrin caused the translocation of NPC1L1 to an "apical-like" subdomain of the plasma membrane, whereas cholesterol accumulation was associated with the protein being located in an intracellular compartment.

Mechanism of action of NPC1L1

The molecular mechanism by which NPC1L1 transports cholesterol is not known. It may directly transport cholesterol from the intestinal lumen into cells. Alternatively, it may shuttle the cholesterol from the cell surface to the ER. The protein contained SSD, so may act as a cholesterol biosensor in the cell, and thus regulate the absorption process. It may work as an analog of NPC1 and affect cholesterol transport as a consequence of its effects on sphingolipid transport. My project focuses on if NPC1L1 forms a larger functional complex with itself or other proteins.

Only a few studies have been performed suggesting that NPC1L1 interacts with other proteins. Ezetimibe was show to disrupt the

interaction between caveolin-1 and annexin-2 in zebrafish and mouse intestine (16). These authors suggest that this complex may partner with NPC1L1 in the cholesterol absorption pathway. However, more recently, other investigators demonstrated that ezetimibe blocks absorption of cholesterol in caveolin-1 null mice to the same degree as wild type mice. Thus the caveolin 1:annexin-2 complex must not have a crucial function in cholesterol absorption (17).

PRELIMINARY RESULTS

Chendong Yang, a post-doctoral fellow in the Hobbs/Cohen laboratory, has examined the effect of cholesterol on the migration on a native gel of NPC1L1 from membranes from Chinese Hamster Ovarian (CHO) K1 cells. In these experiments a total of 2 μg/ml of cholesterol (complexed to cyclodextrin) was added to the medium. Immunoblot analysis of the native gel with an anti-human NPC1L1 antibody revealed a band of the expected size (175 kDa) cells not receiving cholesterol. In the cholesterol-treated cells, two bands were seen. One of 175 kDA and one at a higher apparent molecular mass (230-400 kDa). No slower migrating band was visible when cells were treated with 25-hydroxycholesterol.

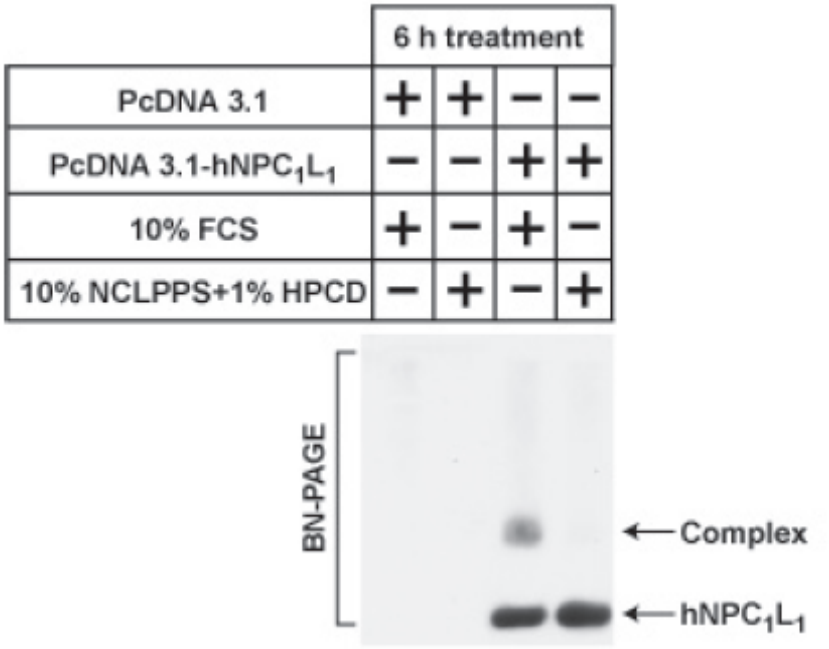

Figure 3.Blue Native-PAGE of lysates from cells treated with or without cholesterol. Cells were transfected with hNPC1L1 or empty vector as a control. 36 h after transfection, cells were treated with or without cholesterol for 6h. Cell lysates were then subjected to BN-PAGE

The first aim of my project is to determine if this slower migrating band represents a dimer of NPC1L1 or an association of NPC1L1 with other proteins. Initial experiments have been performed by expressing differentially-tagged versions of NPCC1L1 in CHO-K1 and determining if the two proteins co-immunoprecipitate.

MATERIALS AND METHODS

Materials

All reagents for cell culture (DMEM-Dulbecco's Modified Eagle's Medium, PBS-Phosphate Buffer Saline, fetal calf serum) were purchased by Cellogro-Mediatech, Inc. Fugene6 transfection kit and the protease inhibitors tablets were purchased from Roche. Anti-FLAG monoclonal (M2) antibody (F-3165) and the anti-FLAG polyclonal antibody (F-7425) were purchased from Sigma. The anti-Myc polyclonal antibody was purchased by Upstate, NY (06-549). The anti-Myc monoclonal antibody (9E10) and the anti-human NPC1L1 rabbit polyclonal antibody were prepared by Cristina Zhao and Liangcai Nie in the Hobbs/Cohen laboratory. Immobilized protein A beads were purchased from Repligen, MA. Super Signal West Pico Enhancer Solution and Super Signal West Pico Stable Peroxide were purchased from Pierce.

Plasmids

The expression vector used for the experiments was pcDNA3.1 (-) expression vector (Invitrogen). Expression plasmids with the following inserts were made: 1) hNPC1L1 cDNA with a FLAG tag at the C-terminus, 2) hNPC1L1 cDNA with 3 myc tags at the C-terminus, 3) hNPC1L1 containing the I647A mutation and 4) the D798N mutation hNPC1L1 cDNA with three Myc tags at the C-terminus.

Cell culture

CHO-K1 cells were cultured in Dulbecco's Modified of Eagle's Medium/Ham's F-12 50/50 Mix (DMEM/F-12 50/50,1X)with L-glutamine, supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin and Streptomycin at 37^{o}C in a 8% CO_2. The day before transfection, cells were seeded in 100 mm dishes at a density of one million cells per dish. Transient transfections or co-transfections were performed the next day, when the cell were 50-80% confluent. Transfections were performed using Fugene6. The Fugene6 reagent:DNA ratio used in these experiments was 3:1(µl/µg) and the total amount of plasmid DNA added to the dish was 2 µg. Fugene6 was diluted in serum free medium and then DNA was added to the complex. After a 15 min, the complex was added to cells in a drop by drop manner. A total of 36 h after transfection, cells were treated with or without cholesterol. Cholesterol was diluted in 5% MβCD (methyl- β cyclodextrin) and then added to the medium at a final concentration of 2 µg/ml.

For the cells treated under cholesterol depletion conditions, the medium was removed from the dish and the cells were washed with PBS. The medium was then replaced with DMEM F-12 50/50 supplemented with 5% NCLPPS-newborn calf lipoprotein-deficient serum (NCLPPS), 10 µM compactin and 1% cyclodextrin. Compactin inhibits HMG-CoA reductase and cyclodextrin removes cholesterol from the plasma membrane (18). The treatment time for these experiments was 6 h.

Immunoprecipitation and co-immunoprecipitation

For IP and Co-IP experiments, CHO-K1 cells were washed once with ice-cold PBS and collected in PBS. Cells were pelleted by centrifugation at 1000 g for 10 min at 4°C. The cell pellets were solubilized with 1 ml of NP-40 lysis buffer (50 mM Hepes-HCL, pH 7.4, 100 mM NaCl,1.5 mM $MgCl_2$, 1%(v/v) NP-40, 1 mM DTT and one tablet of protease inhibitor cocktail (per 7-10 ml of buffer). Cells were lysed by pass through a 23-gauge needle 15 times and then the lysate was rotated for 1 h at 4°C. Cell lysates were collected by centrifugation at 13,000 rpm for 2.5 min at 4°C. The lysates were pre-cleared by incubation with 50 μl of pre-equilibrated (washed 3 times with NP-40 lysis buffer) Protein-A beads, rotating for overnight at 4°C. After pre-clearing, the supernatants were collected by centrifugation at 13,000 rpm for 2.5 min at 4°C and transferred to a new tube containing 10 μl of the first antibody (anti-FLAG or anti-Myc monoclonal antibody),rotating on a rotary platform at 4°C for 4 h to overnight. The lysates were then incubated with 50 μl of protein-A beads for a 4 h to overnight. After centrifugation at 6,000 rpm for 5 min at 4°C, the beads were washed 3X with 1 ml of NP-40 lysis buffer. Finally, the beads were precipitated and resuspended in 60 μl of 1X 3% SDS lysis buffer (for 20ml of buffer:4ml 10%SDS,11ml H_2O,5ml 4X loading buffer) containing 1.5 μl of β -mercaptoethanol (2.5%). Samples were boiled at 95°C for 5-10 min and analyzed by 8% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Hybond-C Extra membrane, Amersham) and immunoblotting were performed using the primary antibody (1:1000 for the anti-FLAG, anti-Myc and anti-NPC1L1 polyclonal

antibodies) for 2 h, followed by incubation with an anti-rabbit IgG secondary antibody (1:10,000 dilution) for 45 min. The blots were exposed to Blue X-ray film (Phenix) after treatment with enhanced chemiluminescence substrate.

RESULTS

Immunoprecipitation of hNPC1L1-FLAG with anti-FLAG antibody.

To determine if the hNPC1L1-FLAG could be efficiently immunoprecipitated with an anti-FLAG antibody, CHO-K1 cells were transfected with wt-hNPC1L1-FLAG and pcDNA3.1(-) empty vector as a control. In the same experiment, we examined the effect of cholesterol on immunoprecipitation of the protein. The cell lysates were immunoprecipitated with anti-FLAG monoclonal and immunoblotted with anti-FLAG polyclonal antibodies

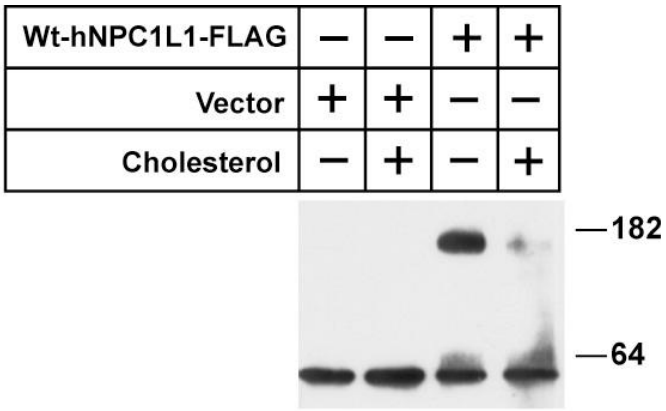

Figure 4. Immunoprecipitation of hNPC1L1-FLAG with anti-FLAG antibody in presence or absence of the addition of cholesterol-cyclodextrin. CHO-K1 were transfected with hNPC1L1-FLAG (2 μg) and pcDNA3.1(-)vector (2 μg) using Fugene6 reagent. 36 h after transfection, cells treated with or without cholesterol-cyclodextrin. Half of the dishes were treated with 2 μg/ml cholesterol. The other half were washed in PBS and the culture medium was replaced by 5%NCLPPS, containing 10 μM compactin and 1% MβCD. After 6 h, cells were collected in PBS and harvested by centrifugation at 1000 g. Cell pellets were resuspend in 1% NP-40 lysis buffer. Cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody and incubated with 50 μl of protein A beads. After IP, the proteins from the cell lysates were subjected to SDS-PAGE and immunoblotting with an anti-FLAG monoclonal antibody (see Methods). A band at ~60 kDa is a nonspecific band.

No band was present in the cells transfected with empty vector, whereas a band of the expected size was seen in the cells expressing the FLAG-tagged NPC1L1. Intensity of the band was significantly higher in the cells that were in cholesterol-depleted medium. The observation that there were similar amount of a nonspecific band (~60 Kda) in the cells lines suggest this different was not due to recovery. The difference in intensity may be due to a difference in expression level (due to post-translational degradation) or to differences in the efficiency of immunoprecipitation.

In cholesterol abundant conditions, NPC1L1may undergo a conformational change (possibly due to interactions with other proteins) so is not recognized well by the anti-FLAG antibody. Alternatively, NPC1L1 may be localized in a cellular compartment that cannot be solubilized by NP-40 lysis buffer. In this case, increased cholesterol levels may lead to translocation of NPC1L1 in cellular structures like lipid rafts.

To address these possibilities, we will repeat the experiments and perform the following analyses:

1) Assess the efficiency of the immunoprecipitation by determining the amount of protein in the supernatant as well as in the pellet.

2) Alter the concentration of the detergent used to solubilize the cell lysate.

3) Determine if the same result is obtained when another tagged form of NPC1L1 is used.

<u>Immunoprecipitation of hNPC1L1-Myc with anti-Myc antibody</u>

Next we tested if the anti-Myc antibody could IP of Myc-tagged hNPC1L1.

Cells where transfected with hNPC1L1-Myc, I647A-Myc and empty pcDNA3.1(-) vector and then treated with cholesterol as described in the Methods. The lysates and the beads alone were subjected to immunoprecipitation using the anti-Myc antibody. Beads alone were included in the experiment to confirm that NPC1L1 is immunoprecipitated specifically by the antibody and does not attach nonspecifically to the beads. Cell lysates were immunoprecipitated with anti-Myc monoclonal antibody and immunoblotted with anti-Myc polyclonal antibody.

Wt hNPC1L1-Myc	**+**	**-**	**-**	**-**
I647A-Myc	**-**	**+**	**-**	**-**
Vector	**-**	**-**	**+**	**-**
Beads	**-**	**-**	**-**	**+**

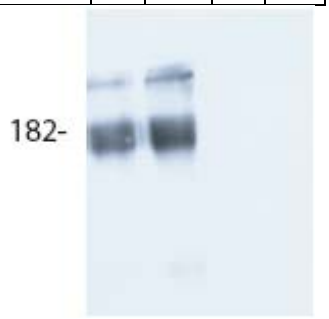

Figure 5. Immunoprecipitation of wt hNPC1L1 and hNPC1L1-I647A with anti-Myc antibody.CHO-K1 cells were transfected with hNPC1L1-Myc(2μg),I647A-Myc(2μg) and pcDNA3.1(-) empty vector(2μg)as a negative control. 36h after transfection,cholesterol added to cell medium in a final concentration of 2μg/ml.Cells were treated with cholesterol for 6h. Then, cells were collected in PBS, harvested by centrifugation at 1000g and cell pellets were resuspend in 1% NP-40 lysis buffer.Cell lysates were immunoprecipitated with anti-Myc monoclonal antibody.Cell lysates were also incubates with protein A beads only,without the presence of antibody in order to test the specificity of IP. Immunoprecipitated cell lysates were subsequently immunoblotted with anti-Myc polyclonal antibody.

A major band of the expected size (~180 kDa) was seen in the cells expressing both wt hNPC1L1 and hNPC1L1-I647A. The immunoprecipitation was specific since no bands were seen in the absence of the antibody or in the cell lysates transfected with the empty vector.

Co-Immunoprecipitation of different tagged hNPC1L1 proteins

Previously, Yang had shown that addition of cholesterol to cells was associated with the presence of a slower migrating band on native gel electrophoresis that was detected with a NPC1L1-specific antibody (Figure 3). The size of the higher molecular weight band (230-400 kDa) was estimated to be similar to the size of a dimer of NPC1L1 (~350 kDa). To determine if addition of cholesterol is associated with dimerization of NPC1L1, we performed a co-immunoprecipitation experiment after expressing hNPC1L1 containing two different epitope tags (FLAG and Myc) in the same cells in the presence of cholesterol. CHO-K1 cells were transfected with the following plasmids: empty vector, hNPC1L1-FLAG alone, hNPC1L1-Myc alone, and hNPC1L1-FLAG plus hNPC1L1-Myc. Cells were treated with cholesterol at a final concentration 2 μg/ml.

Cell lysates were placed in duplicate tubes and immunoprecipitated with either the anti-FLAG or the anti-myc monoclonal antibody. The immunoprecipitates were then subjects to immunoblotting using the complementary antibody. The filters were striped and allowed to incubate with an anti-hNPC1L1 antibody.

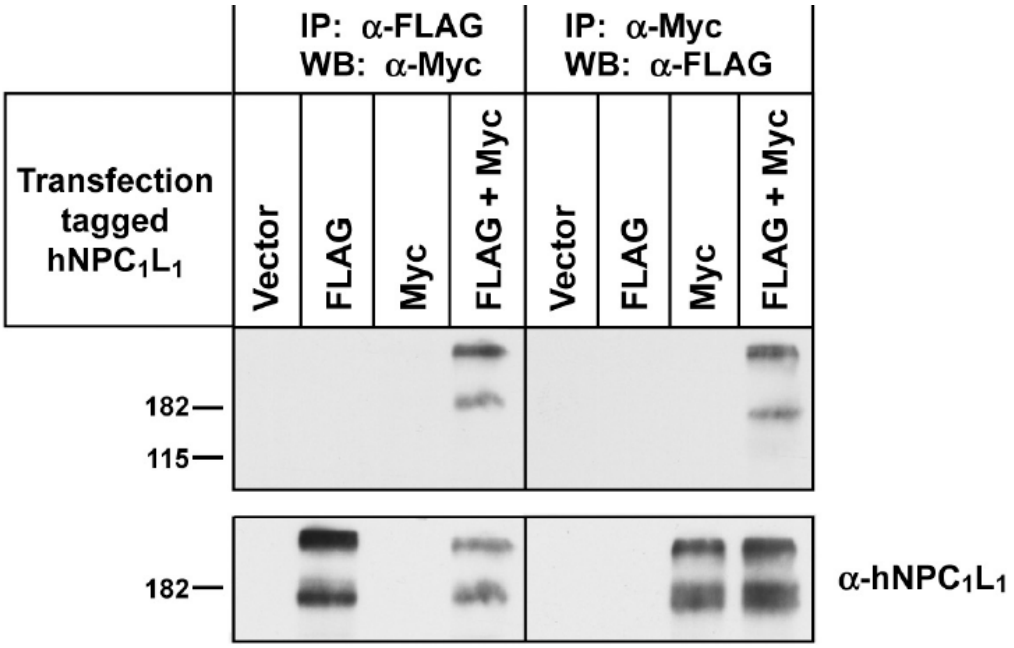

Figure 6. Co-immunoprecipitation of hNPC1L1-FLAG and hNPC1L1-Myc. Immunoprecipitation of vector,hNPC1L1-FLAG and hNPC1L1-Myc are the controls of IP efficiency and specificity.CHO-K1 cells were transfected with pcDNA 3.1(-)vector,hNPC1L1-FLAG,hNPC1L1-Myc or co-transfected with hNPC1-FLAG and hNPC1L1-Myc. 2µg of each plasmid were used for transfection. 36h after transfection, 2µg /ml of cholesterol were added in each dish. Cells were collected after 6h ,harvested by centrifugation at 1000g and cell pellets were resuspend in 1ml of 1% NP-40 lysis buffer. Cell lysates were placed in duplicated tubes and immunoprecipitated with 10µl of either the anti-FLAG or the anti-Myc antibody. Then, the immunoprecipitated samples were immunoblotted with the complementary antibody.Finally, the same nitrocellulose membrane were striped and re-immunoblotted with anti-hNPC1L1 antibody.

No bands were present under these conditions in lysates from cells transfected with only a single plasmid. Immunoblotting with anti-hNPC1L1 verifies that the cells expressed the proteins and that the tagged versions of NPC1L1 were IP'd. Only when hNPC1L1-FLAG and hNPC1L1-Myc are co-expressed in cells are bands of the expected size found. Vector, FLAG-tagged protein and Myc tagged protein represent a control and failed to pull down any protein.

In this experiment a slower migrating band was found that has an apparent molecular mass of ~300-400 kDa. This band may represent a dimer of NPC1L1, or alternatively, may be due to aggregation of protein.

DISCUSSION

These results, when taken together, are consistent with NPC1L1 forming a dimer, and that dimerization is promoted by the addition of cholesterol to cells. Many questions remain regarding this result. When and where in the cell do the NPC1L1 dimers form? Does NPC1L1 for even larger molecular complexes with itself or with other proteins? How does interference with dimer formation impact on the function of the protein? We will address these questions by pursuing the following experiments.

1) Further characterize the relationship between dimerization and the cholesterol content of cells. Does the addition of cholesterol to cells promote dimerization? CHO-K1 cells will be co-transfected with hNPC1L1-FLAG and hNPC1L1-Myc in the presence or absence of cholesterol and assayed for co-immunoprecipitation. In these experiments we will also assess the effect of cholesterol on the amount of NPC1L1 as reviewed previously.

2) We will determine if NPC1L1 complexes with other cellular proteins under high-cholesterol conditions. For these studies we will perform cellular fractionation studies using gel filtration with standards to estimate the size of the higher molecular weight complex. We will

immupoprecipitate NPC1L1 and analyze co-immunoprecipitated proteins using sliver stain gels and mass spectrometry.

3) We will determine if the SSD of NPC1L1 behaves in a similar fashion to the SSD of SCAP and interacts with another protein. Previously, Brown and Goldstein found that two mutations in SCAP result in a failure to downregulation SREBP cleavage with cholesterol repletion by interfering with the association of SCAP with another protein (INSIG). To test whether hNPC1L1 behaves in a similar fashion, we have made one of the mutations in the corresponding residues of hNPC1L1 (D798N). We also will examine the effect of another mutation in the SSD that was found in a person with very low levels of cholesterol absorption (I647N) (11).

Cells will be co-transfected with wt FLAG-NPC1L1 and Myc-tagged mutants or with Flag-tagged and Myc-tagged mutants to examine if mutant NPC1L1 has the ability to form the higher molecular weight complex.

4) NPC1L1 contains a SSD so it may act as a cholesterol sensor. We will determine if NPC1L1 interacts with either Insig-1 and/or Insig-2. We will perform co-immunoprecipitation experiment to determine if hNPC1L1 and Insig-1 or Insig-2 interact when co-transfected in cells.

5) Determine how changes in cellular cholesterol content affect the subcellular localization of NPC1L1. For these experiments we will use both biochemical methods and immunocytochemistry. Cells transfected with hNPC1L1 (and vector alone) and will be treated with and without

cholesterol. Then the cell surface proteins will be biotinylated and the cell lysates will be immunoprecipitated with avidin beads. After IP, cell lysates will be analyzed by SDS-PAGE and immunoblotting with anti-hNPC1L1 antibody. This experiment will address whether NPC1L1 moves to the plasma membrane in response to changes in the cellular cholesterol content. We will also use our NPC1L1 antibodies to examine the cellular distribution of NPC1L1 in response to cholesterol treatment.

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