The role of hsc-70 in very low density lipoprotein transport vesicle golgi fusion complex formation

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THE ROLE OF HSC-70 IN VERY LOW DENSITY LIPOPROTEIN TRANSPORT VESICLE
GOLGI FUSION COMPLEX FORMATION.

by

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ABSTRACT

Excess production and secretion of very low-density lipoprotein (VLDL) by the liver into the circulatory system is directly related to atherosclerosis, a chronic cardiovascular disease that threatens the lives of many worldwide and continues to be a leading cause of death in the United States. The rate-limiting step in VLDL secretion is its transport from the site of biogenesis, the hepatic endoplasmic reticulum to the cis-Golgi. This step is mediated by a specialized ER-derived vesicle, the VLDL transport vesicle (VTV). Upon exit of the ER the VTV targets, fuses and delivers VLDL into the lumen of the Golgi. The targeting and fusion of the VTV with the Golgi is facilitated by specific set of soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins that form a SNARE complex, which is required for the VTV-Golgi fusion and thus delivery to the Golgi. Data from our laboratory indicates that the formation of the SNARE complex requires cytosolic factors. Through the purification of liver cytosol, chromatographic steps, detailed mass spectrometry, immunodepletion and western blotting data it was identified that the protein necessary for SNARE complex formation is Hsc-70. Although Hsc-70’s identification is significant, the role it plays in SNARE complex formation for VTV-Golgi fusion is a predicament and yet to be unraveled. In this study we performed a series of co-immunoprecipitation reactions to identify its role in SNARE-complex assembly. Using western blot data we confirmed binding of Hsc-70 with Sec22b, the v-SNARE on the VTV. Moreover, we confirmed the interaction of Hsc-70 with t-SNAREs, (syn5, rBet1 and GOS28) on the Golgi membrane. Removal of Hsc-70 from the liver cytosol resulted in significant reduction of SNARE-complex formation. Ultimately, the identification proteins involved in the process of VLDL delivery to the Golgi would offer therapeutic targets to control VLDL secretion into the blood by the liver.
ACKNOWLEDGEMENTS

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“Ask, and it will be given to you. Seek, and you will find. Knock, and the door will be opened to you. For everyone who asks, receives; and he who seeks, finds; and to him who knocks, the door will be opened.” (Matthew 7:7-8)
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ABREVIATIONS

ApoB100- Apolipoprotein
APS- ammonium persulfate
BSA- Bovine serum albumin
COPII- Coat protein complex II
GTP- Guanine triphosphate
IDL- Intermediate density lipoprotein
LDL- Low-density lipoprotein
mRNA- Messenger Ribonucleic acid
MTP- Microsomal triglyceride transfer protein
NEFA- Non esterified free fatty acids
PBS- Phosphate buffered saline
PBS-T- PBS with 0.05% Tween 20
PTV- Protein transport vesicle
rER- Rough Endoplasmic Reticulum
SNARE- Soluble N-ethylmaleimide sensitive receptor protein
SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
TEMED- Tetramethyethyl enediamine
VLDL- Very low-density lipoprotein
VTV- VLDL transport vesicle
Atherosclerosis & Lipid Metabolism

Atherosclerosis is a disease that is characterized by the hardening of the arteries due to the formation of plaque from excess cholesterol, fat and other substances [1]. The risks to develop atherosclerosis are related to the over production and secretion of a lipoprotein known as very low-density lipoprotein (VLDL) into the blood by the liver. The biogenesis of VLDL is crucial for lipid metabolism thus modulating its synthesis and secretion from the liver would offer a gateway to develop better treatments for plaque formation in arterial wall.

The liver is responsible for controlling lipid metabolism in mammalian species. A large portion of lipids entering the liver is contributed from non-esterified free fatty acids (NEFA), which are the major components of triglycerides, the major supply of energy and fat in our tissues. The potential sources of free fatty acids that enter hepatocytes also liver cells include: peripheral fats stored in adipose tissue, dietary fatty acids which enter the liver through chylomicron remnants and through the intestine via the portal vein [2]. Once NEFAs are up taken by the liver, their fate is determined based on energy requirements of the body, consequently excess influx of free fatty acids leads to the repackaging of triglycerides to form a VLDL particle that will be excreted from the liver into the blood. Once in the blood the VLDL particle is further metabolized into IDL (intermediate density lipoprotein) and LDL (low density lipoprotein) [3]. LDL particles contain a large amount of cholesteryl ester, which undergo oxidation when taken up by endothelial cells of blood vessels and thereby accelerate the formation of plaque in arteries [4]. Subsequently, production of VLDL directly contributes to the plaque formation of atherosclerosis. Herein, research will describe the role of a 70 kDa protein recently identified as being involved in the biogenesis of VLDL.
Biogenesis of VLDL

Lipoproteins are spherical particles utilized to transport lipids and lipophilic substances into circulation [5]. The VLDL lipoprotein is composed of a core of neutral lipids, which is surrounded, by phospholipids, cholesterol, high percentage of triacylglycerides, and specific apolipoproteins [6]. An apolipoprotein is a protein that combines with lipids such as triglycerides and cholesterol [7]. Amongst the various types of apolipoproteins, apolipoprotein B100 (apoB100) is present in the liver and provides structural stability thus being an essential component in the formation of the VLDL and its circulating metabolites LDL and IDL particles [8]. Furthermore, Apolipoprotein B is found in two forms: the Apo B100 and its truncated version Apo B48. Although they come from the same gene, ApoB100 is the full version made in the liver with a molecular weight of 550 kDa and 4536 amino acids. While ApoB48 is mainly found in the intestines, and mostly serves as a structural protein for chylomicrons, it is composed of only 48 percent of ApoB100 due to a posttranscriptional editing of the apoB messenger ribonucleic acid (mRNA) resulting in the switching of a glutamine (CAA) to a stop codon (UAA) [9, 10].

The formation of VLDL takes place in the lumen of the rough endoplasmic reticulum (rER) in hepatocytes. However, the assembly of the VLDL lipoprotein is broken down into two steps, first is the synthesis of the primordial VLDL particle by the partial lipidation of ApoB100 in the lumen of the hepatic rough ER, a process facilitated by microsomal triglyceride transfer protein (MTP). Conversely, the growing polypeptide of ApoB100 acquires a proper folding conformation in which the hydrophobic side will associate with lipid and the hydrophilic side of the lumen will interface with the aqueous environment of the cytosol. In the second step, neutral lipids or triglycerides (TAG) are added to the nascent VLDL thus forming a rich-TAG VLDL particle [11-13]. Once the nascent VLDL is formed in the lumen of the rER it will travel to the hepatic cis-Golgi apparatus to ensue it’s processing for further maturation. Although the site of VLDL maturation in the hepatocyte is subject to debate, some studies reveal the ER as its site of maturation, yet others propose the cis-Golgi as the final site for VLDL maturation thus being congruent with several studies, which indicate that upon VLDL-ApoB100 particle delivery to the
cis-Golgi, it undergoes phosphorylation and glycosylation obtaining endoglycosidase H resistance [14].

**VLDL Transport Vesicle (VTV)**

The production of ER-derived vesicles and their movement to the cis-Golgi serves as the rate limiting step for the secretion of VLDL from the liver to the bloodstream [14]. According to Siddiqi et al [14], the VLDL particle can easily get lipid hydrolyzed in the cytosolic environment therefore, a specific mechanism must take place in order to protect the VLDL particle from the potent proteinases found in the cytosol. Furthermore, several features indicate that proteins and lipids exit the ER in distinct manners. The protein transport vesicle (PTV), a vesicle synthesized in the ER and responsible of transporting nascent proteins to the Golgi, is mediated by the recruitment of a group of proteins called coat protein complex II (COP-II) consisting of five subunits: Sar1, Sec23/24 and Sec13/Sec31 [15]. However, the 60-80nm size of PTVs denotes a limitation to properly accommodate the VLDL particle thus posing a predicament in its transport to the Golgi. As a result, data from our laboratory and Fisher’s group [16], suggested that the specialized vesicle (VTV), which is morphologically and biochemically different than any other ER derived vesicle, enables the intracellular transport of VLDL from the ER to the cis-Golgi [10, 12]. VTVs range in size of 100-120nm, apt to comfortably fit the 90nm VLDL cargo, data collected from Siddiqi et al [14] also show that in addition to VTVs concentrating ApoB100, a VLDL marker protein, it is also protected from proteolysis as seen on experiments with proteinase K treatment. Finally, VTVs also concentrate the COPII proteins, a required complex for vesicles having the Golgi as their destination [10, 14]. Ultimately, the identification of proteins essential for selecting and packing VLDL into the VTV remain to be identified.

**Coat Protein Complex II (COPII)**

Although VTVs are notably different than PTVs, they also possess COPII dependent mechanism to transport their cargo [16]. COPII coat is used to shape lipid membranes to
produce transport vesicles by the recruitment of cytosolic proteins [17]. COPII assembly is broken down into four steps, the first step begins by the recruitment and conversion of Sar 1-GDP to Sar 1-GTP by Sec12, a guanine nucleotide exchange factor for Sar 1 found in the ER membrane. The second step takes place when the heterodimeric complex Sec23/24 is recruited, this complex plays a role in cargo selection and forms the inner coat of COPII. Step three arises when Sar1- Sec23/24 attaches to the ER membrane thus forming a pre-complex which will in turn recruit both Sec 13 and Sec 31 as a heterodimer, Sec13/31 will form the outer layer of COPII and signal membrane deformation leading to vesicle formation. Finally, the fourth step takes place when GTP is hydrolyzed and as a result Sar1 detaches from the ER membrane initiating the release of the newly formed vesicle [10, 18].

**SNARE Complex formation**

The presence of intracellular compartments is a salient feature of eukaryotic cells. Trafficking amongst cellular compartments is mediated by transport vesicles, thus specificity for accurate content delivery is facilitated by proteins that regulate the budding, target selection and fusion of membranes. It has been widely agreed that specific soluble N-ethylmaleimide-sensitive attachment receptor proteins (SNARE proteins) direct transport vesicles to their target membranes. SNARE proteins are membrane proteins that contain motifs of 60 to 70 residues in length [19, 20]. These SNARE motifs form specific domains-heptad repeats of hydrophobic residues that mainly function to form a parallel α-helical structure necessary to bring the membranes in close proximity thereby allowing fusion to occur. Since their discovery SNARE proteins are generally known to participate in fusion and docking of membranes therefore being responsible for the specificity of intracellular transport [21, 22]. In VLDL transport the VTV buds off the ER membrane and travels to the Golgi where it will first dock and eventually fuse with the cis-Golgi membrane releasing its cargo- the VLDL-ApoB100 particle.

In intracellular membrane fusions the SNARE pin of four member-α-helix coiled is formed by the contribution of a single SNARE motif by each of the four SNAREs. Usually three
motifs are contributed by one bilayer and one motif is contributed by the opposing bilayer. [3, 23, 24]. Each SNARE complex contains a central polar layer where each of the SNARE motifs donates a hydrophilic residue consisting of one arginine (R) and three glutamine (Q) side chains [19]. It has been described by Fasshauer et al. [25] that vesicles localize the (R) residue whereas the target layer contains the three (Q) side chains and are termed Q-SNAREs. In addition, the Q-SNAREs are further divided into Qa-Qb and Qc SNAREs donating each one motif and the R-SNARE contributing another motif forming the SNARE pin or SNARE complex. Furthermore, the association of the vesicular membrane and target membrane by the Q-SNAREs and R-SNARE is mediated by the so called zippering hypothesis where the complex is formed in an orderly manner forming a zipper from N-terminus of the motifs to the C-terminus conversely the zippering is essential for membrane fusion to take place [21, 26].

Ultimately, data from our laboratory has identified Sec22b to be present on the surface of the VTV acting as the v-SNARE or R-SNARE, and GOS28, Rbet1, Syntaxin5 (Syn5) as the three cognate Q-SNAREs on the target membrane, which in this case is the cis-Golgi [3]. Once docking and fusion takes place, the mature VLDL particle is released into the lumen of the Golgi where it will be further glycosylated and phosphorylated to then travel to the plasma membrane where it will be expelled to the circulation. However, the mechanism post-Golgi VLDL transport remains to be identified.

**Hypothesis and Specific Aim**

The nascent VLDL is directed towards the cis-Golgi membrane by the VTV. A specific set of SNARE proteins are integrated in the membrane of the VTV upon budding of the endoplasmic reticulum and bind to t-SNAREs found in the cis-Golgi, forming a SNARE complex, which facilitate the targeting of transport vesicles and mediate the fusion/docking of VTV with the cis-Golgi membrane. The association of the SNAREs to bring the membranes in close proximity without the use of energy-ATP (adenosine-5′-triphosphate) is denoted as the docking whereas fusion is the physical interactions of the opposing membranes and the release of
the cargo. Once this specific step takes place VLDL is released into the Golgi lumen. As a result, we hypothesized that in order for SNARE complex to form between the VTV and cis-Golgi cytosol is required. Unpublished data from the laboratory indicate that this important step requires cytosolic proteins. In order to separate proteins in cytosol, Fast Liquid Chromatography was employed, cytosolic fractions were collected and tested until the putative proteins were determined amongst these, Hsc-70, (Siddiqi et al; unpublished data) a 70 kDa cytosolic protein that seems to plays an important role in VTV Golgi fusion complex. Hsc-70 is a member of the heat shock protein family that acts as an ATPase as well as a chaperone protein in secretory pathways in addition Hsc-70 has been known to take part in ER to Golgi transport [24, 27]. Conversely, its form of action in regulating the fusion of VTV’s with the Golgi’s lumen remains enigmatic. Thus, the aim of this study is to identify how Hsc-70 plays a role in SNARE complex formation for VTV Golgi-fusion. In sum, the identification of protein(s) involved in the process of VLDLs synthesis would offer potential targets for their inhibition and hence, serve as a potential control for VLDL secretion from the liver.
CHAPTER 2: EXPERIMENTAL METHODS

Materials

ECL (enhanced chemiluminescence) reagents were obtained from GE Healthcare. Mouse monoclonal antibodies anti-Sec22b and anti syntaxin5 anti-rabbit polyclonal were purchased from Santa Cruz Biotechnology. Mouse anti-GOS28 antibodies were obtained from StressGen Biotechnologies (Victoria, Canada) Goat anti-rabbit IgG agarose beads were purchased from Sigma Chemical Co. (St. Louis, MO) [12]. Western Blot, Electrophoresis and immunoblotting reagents were produced from Bio-Rad (Hercules, CA) [12]. [14C] Oleic acid, [3H] Oleic acid and [3H] Leucine were procured from PerkinElmer Life Sciences [3]. The liver used for experiments is from the Sprague-Dawley rats attained from Harlan (Indianapolis, IN).

Bradford Assay- Protein Quantification

Protein concentration for cytosol, ER and Golgi was calculated using the Bradford assay procedure. The general procedure is as follows: seven glass tubes are acquired and labeled. One tube is the blank used to calibrate spectrophotometer, three are labeled standard and the other three tubes contain different amount of the specific sample. The blank is filled with 100 µl of dH2O; the three standards are prepared by using 10µl, 20µl and 40µl of 1mg/mL of Bovine Serum Albumin (BSA) and 90µl, 80µl and 60µl of dH2O respectively. The sample tubes were made by using 1:10, 2:10 and 4:10 dilutions. Moreover, five milliliters of BIO-RAD dye is used however, the BIO-RAD dye is first prepared by employing a 1:4 dilution. Likewise, when the standard and sample tubes are prepared, five milliliter of BIO-RAD dye is added to each tube including the blank, each tube is vortexed and kept in incubation for five minutes. Finally, each tube is transferred to a cuvette and placed on the DU 800 spectrophotometer where it will be read at 590nm, the wavelength employed to read protein concentration. The blank is placed first to calibrate the machine following with the lowest to highest concentrated standard thus a graph is
acquired using a computer software system. Next the sample tubes are placed one at a time again from lowest to highest concentrated sample, once the results for each is determined the concentration is calculated.

(SDS-PAGE) Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis

The running buffer used for the experiments is 12% and the stacking gel used is 4%. The percentage of the gel is determined on the amount of acrylamide used as well as size of proteins of interest. The higher the percentage of acrylamide the smaller the pores on the gel thus acquiring a better separation of the proteins. The 12% running buffer is made first by using 3.4mL dH₂O, 2.5mL of running gel buffer, 4.0mL of acrylamide; before adding SDS the chemicals are mixed by employing a vortex to create a homogeneous mixture. Next, 0.1mL of SDS is added along with 50µl of APS and 5µl of TEMED to allow the polymerization reaction to occur. With a 5mL pipette the running gel is added to a glass cassette where it stays on an incubation time of 45 minutes thus 50µl of butanol is added to prevent the gel from drying upon polymerization. To make the 4% stacking gel 6.1mL of dH₂O, 1.3mL of acrylamide and 2.5mL of stacking gel buffer are placed together in 50mL conical tube and vortexed, immediately after mixing 0.1mL of SDS is added along with 50µl of APS (ammonium persulfate) and 10µl of TEMED (tetramethylethylenediamine) is added last so the polymerization reaction can begin. The conical tube is mixed eight times slowly to avoid the production of bubbles and then using a 5mL pipette, the mixture is placed on the same glass cassette as the running gel and a comb is used to create the wells where the samples will be added.

The samples are prepared using Laemmli 2x buffer which is made to solubilize the protein samples, likewise the 2X buffer is made by using 190µl of laemmli sample buffer (LB) and 10µl of B-mercaptoethanol, along with PBS (phosphate-buffered saline) and the desired sample concentration, the samples are boiled for five minutes to disrupt the bonding and linearize the proteins. After boiling the samples the Precision plus protein marker along with the samples added to their respective well on the stacking gel and the cassette is placed on tank
buffer and hooked to the Fisher Scientific FB300 machine where the current is programmed to run constantly at 21mA for approximately ninety minutes. The proteins travel through the pores of the gel and are separate based on their size. When the gel is finished running, it is taken out of the cassette and transferred to a nitrocellulose membrane where a “sandwich” is formed using two sponges and blotting paper. The assembly is placed in transfer buffer and kept at 4°C at constant voltage and 51mA over night.

Blocking Procedure

Once the transfer is complete, the nitrocellulose membrane is washed twice with PBS-T for five minutes. While washing is taking place, 10% milk is prepared; 5 grams of milk are weighed and 50mL of PBS-T is used to dissolve the milk. The membrane is labeled with pencil and incubated under the 10% milk for at least five hours. The blocking is essential since it prevents non-specific binding while the Western blot takes place.

Final step: Western Blot

Once the blocking is completed, the membrane is washed twice for five minutes with PBS-T (PBS with 0.05% Tween20) and the primary antibody is added. The primary antibody is made in 30mL PBS-T 0.3g 1% Bovine Serum Albumin (BSA) and 50µl (10µg) of desired antibody. The primary antibody is added to the membrane and incubated overnight. Next the membrane is washed with PBS-T and a secondary antibody is added to the membrane to add specificity. The secondary antibody is made by using 5% (w/v)- 0.5g of non-fat milk, 50 mL of PBS-T and 3µl of either (anti-rabbit, anti-goat, anti-mouse). Furthermore, the secondary is added and the membrane is placed on incubation for one hour. Upon incubation the membrane is washed with PBS-T three times for five minutes and two times with PBS to remove non-specific binding. Finally, the detection of the proteins on the membrane is acquired by exposing the membrane to ECL reagents displaying the results in a Biomax film.
**Stripping Buffer**

To utilize the nitrocellulose membrane more than once and test with different antibodies, the membrane needs to be “stripped” meaning the previous antibody binding to the membrane must be removed. First the membrane is washed twice with PBS-T and twice with PBS each wash in five minutes intervals. The stripping buffer is prepared, first weigh 0.38g of TRIS and 1g of SDS dissolve and in a 50mL conical tube filled with 45mL of dH$_2$O. Subsequently, using different concentration HCL and NAOH adjust the pH to 6.7, once the desired pH is reached fill, to 50mL and add 350µl of B-mercaptoethanol. Add the stripping buffer to the nitrocellulose membrane and incubate at 60°C for 40 minutes. Preceding incubation, wash the membrane four times in five minutes intervals with PBS-T. Once washings are finalized the membrane is ready to be blocked again.

**Procedure of Radiolabeled Hepatic Endoplasmic Reticulum**

The Endoplasmic Reticulum is doubly labeled with [H$^3$] and [C$^{14}$] or singly labeled with [C$^{14}$] [3, 14]. The 4-5g liver obtained from Sprague-Dawley rats attained from Harlan (Indianapolis, IN) is cut up into small pieces (3mm x 5mm x 0.5mm) and washed with ice cold 0.25M sucrose in 10mM Hepes buffer with pH of 7.2 and incubated with [H$^3$] Leucine and albumin-bound [C$^{14}$]oleate for 30 minutes at 37°C. To remove excess [H$^3$] Leucine and [C$^{14}$]oleate it is washed with PBS encompassing 2% (w/v) BSA [3]. The plasma membrane must be disrupted therefore the liver is placed in a buffer solution and homogenized in a Parr bomb at 6210 kPa for 40 minutes. The ER is then separated from the other cellular organelles using a step sucrose gradient that must be repeated to obtained a purified portion of ER. Finally, to prove the purity of the ER sample, the ER is tested for calnexin- a ER marker protein and also tested for GOS28 and r-BET1, proteins that serve as markers for the cis-Golgi and lysosomes respectively [3].
Hepatic Cytosol

The cytosol is prepared from hepatic cells obtained from rat liver. The cytosol is acquired from the same homogenate mixture explained previously. Following the centrifugation step, the PNS (post-nuclear supernatant) is further centrifuged at 100,000g for 90 minutes at 4°C subsequently the supernatant collected is the cytosol [14]. In the next step, the cytosol is dialyzed to remove ATP and GTP, conversely the cytosol is placed against an ice cold dialysis buffer solution for 6 hours at 4°C, and further concentrated with a Centricon filter [14].

Hepatic Golgi apparatus

The Golgi has two faces the cis and trans side hence in our laboratory both the cis and trans Golgi are non-radiolabeled and isolated from the rat liver that it is cut into pieces (3mm x 5mm x 0.5mm). The pieces are homogenized to disrupt the plasma membrane thus allowing the separation of cell organelles through a sucrose step gradient used to isolates organelles based on different densities by centrifugation. Finally, the purity of the Golgi is determined on the basis of resident proteins through Western blotting.

Co-Immunoprecipitation

For the Immunoprecipitation technique, we used 170µl of ER (1mg of ER protein), 50µl of PBS, added 10µl of TritonX to solubilize the membrane of the ER and wait until mixture turns clear. Next add 50µl of PBS, 5µl of 10% (w/v) SDS, 5µl of 10% TritonX, 20-30µl of desired antibody and leave sample in four hour incubation. Meanwhile, pipette 50µl of agarose beads and washed with PBS twice for five minutes. Subsequently, after the four hour incubation add the supernatant from previously made sample to the beads and let it spin in incubation at 4°C overnight. Preceding incubation the desired antibody is complexed to the beads; therefore the beads are washed with PBS to remove any unbound protein. The 2X Laemmli buffer was added to the beads and these were boiled for five minutes, vortexed and the supernatant was removed.
and loaded into the gel. Finally, immunoprecipitated proteins are separated through SDS-PAGE, transblotted into a nitrocellulose membrane and detected through the use of specific antibodies.

**VTV- Golgi Docking Assay**

The in-vitro docking of VTV with cis-Golgi was accomplished by incubating VTVs with cis-Golgi along with Adenosine triphosphate, EDTA (Ethylenediaminetetraacetic acid) and cytosol. Once incubation is complete the undocked and docked VTVs are separated based on their densities on sucrose step gradients explained in more detailed in Siddiqi et al [3, 14]. For my interest, I aspirated the VTVs associated with the cis-Golgi with the purpose of isolating the SNARE complex. The VTV-Golgi complexes are solubilized and incubated overnight with rabbit agarose beads. Upon completion of incubation, the beads are washed to remove unbound protein. Lastly, the unbound protein will be assessed using protein assay following Western blots.
CHAPTER THREE: RESULTS

The objective of this experiment is to show the significance of cytosol in the SNARE complex formation for VTV-Golgi association. To carry out these experiments an in vitro docking assay was developed. Docking is the event that takes place when there is an associating of the VTV with cis-Golgi, a step required for fusion. In the above results Bar 1 indicates that VTVs interact with the cis-Golgi upon incubation at 4°C in the presence of cytosol and in the absence of ATP-Mg²⁺. However, as seen on bar 2 the association of VTV with Golgi was reduced when incubation is done at 4°C with ATP-Mg²⁺ in the absence of cytosol, this indicates that cytosolic factors are needed for VTV-Golgi docking. In Bar 3 we observed the effect of ATP-Mg²⁺, which suggests that in the presence ATP-Mg²⁺ VTV-Golgi docking d.p.m count show a reduced docking activity. Finally bar 4 shows the incubation of VTVs with Golgi in presence of cytosol and ATP-Mg²⁺ at 37°C, the conditions favorable for the VTV-Golgi fusion event.

Figure 1. *In vitro* VTV-Golgi fusion-complex formation

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Figure 2 (A) Fusion-complex formation activity of various pure recombinant proteins identified by tandem mass spectrometry:

Formation of the SNARE complex requires cytosol however; the cytosolic factors remain to be identified. To identify cytosolic factors we performed a series of chromatographic steps on liver cytosol, and recognized a specific protein needed for SNARE complex formation. Using tandem mass spectroscopy the protein on the active fraction was identified as being a Heat shock protein. Each of the bars on figure 2A depicts the recombinant proteins (40g) of each identified protein which were assayed for Fusion-complex formation activity. Proteins were incubated with $[^{14}\text{C}]$TAG-loaded hepatic ER (500 ug of protein). (1); Glucose-regulated protein (GRP) /BIP (2); Heat shock cognate 70 (3); Profilin A (4); and Retinol binding protein 2 (5).

Figure 3 (B) Effect of Hsc70-depleted cytosol on fusion-complex formation:

After determining Hsc-70 as the protein needed for SNARE complex formation, liver cytosol was immunodepleted of Hsc70 using specific antibody and the immunodepletion was determined by Western blotting. The immunodepletion of Hsc-70 from the cytosol resulted in 80% reduction in fusion activity. However after adding the recombinant Hsc-70 to the depleted cytosol the fusion activity was completely restored. Native cytosol (1); Hsc70 depleted cytosol (2); and Hsc70-depleted cytosol + recombinant Hsc70 protein (3)
Figure 4. Co-Immunoprecipitation (Co-IP) with Sec22b antibodies using cytosolic proteins, membrane probed with Hsc-70

The objective of this experiment was to pull down the SNARE complex and probe the membrane with Hsc-70 to determine whether Hsc-70 binds to the v-SNARE Sec22b. In order to carry out this experiment we used 1mg of cytosolic proteins, Sec22b as primary antibody and agarose beads with anti-mouse. Furthermore, in the ER sample the band at 70Kda indicates the presence of Hsc-70, also in both the cytosol and supernatant samples but no presence Hsc-70 in the IP sample signifying no binding Sec22b and Hsc-70 using Cytosol. Finally, the IP sample indicates that after developing of 35 minutes, there is a no prominent band signifying no binding of Hsc-70 and Sec22b.
Figure 5 Co-Immunoprecipitation (Co-IP) with Sec22b antibodies using cytosolic proteins, membrane probed with Sec22b.

The main objective was to show the presence of Sec22b in the IP sample. First we stripped the membrane from the Hsc-70 antibody and proceeded to reprobe membrane with Sec22b. The results in the above figure depict that Sec22b is present at 22kDa in the ER since in functions as a v-SNARE for ER derived vesicles. In addition, Sec22b is also present in the IP sample at 22kDa however the band present is very light to confirm binding with Hsc-70, therefore to confirm the binding of Hsc-70 and Sec22b, an IP with Hsc-70 and testing with Sec22b antibody must be carried out.
The main objective was to prove that the IP of Hsc-70 with cytosol was successful. To carry out this experiment we stripped the membrane from Sec22b antibody and reprobed with Hsc-70 primary antibody. Furthermore, the results portray bands found at 70kDa for the ER, cytosol and IP samples. The ER bands shows that there could be binding at the level of the ER, the cytosol further confirm the presence of Hsc-70 in the cytosol since naturally it is a cytosolic protein. Likewise, the IP of Hsc-70 sample indicates the existence of Hsc-70 at 70kDa, which signifies the success of the IP.
The goal of this experiment was to determine which t-SNAREs bind to Hsc-70 in SNARE complex formation. For the immunoprecipitation we used cis-Golgi, Hsc-70 as the primary antibody and secondary antibody anti-mouse agarose beads. Furthermore, the above results depict that Gos28 binds to HSC-70 since there is a clear bold band that comes up in 28kDa.

The membrane was stripped from the Gos28 primary antibody and reprobed with Syn5 primary antibody. The results indicate that Hsc-70 does bind with Syn-5, the t-SNARE on the Golgi membrane.
R-Bet-1 is a protein that functions as one of the t-SNARE for the cis-Golgi and forms part of the SNARE complex with the VTV which is a necessary step for proper delivery of VLDL. In the above blot we used primary R-Bet-1 with an anti-mouse secondary antibody and found that there was no binding in the IP sample since there is no band around 18kDa, the molecular weight of R-Bet1. Finally, the results indicate that r-Bet1 does not bind to Hsc-70.

To further confirm binding of Sec22b and Hsc-70 we reprobed the membrane with Sec22b primary antibody and the results verify binding of Sec22b and Hsc-70 at the cis-Golgi level, the IP and ER sample indicate a clear band at 22kDa, the molecular weight of Sec22b. In addition, there is no band showing up in the cis-Golgi sample since Sec22b is present on the ER membrane and part of the VTV acting as a v-SNARE.
To confirm Hsc-70 binding with Sec22b, we co-immunoprecipitated ER with Hsc-70, we proved the membrane with Sec22b and the results confirm that Sec22b does bind to Hsc-70 at the ER level. The band is shown at the 22kDa mark in both the ER and the Co-IP with Hsc-70.

Syntaxin 5 is a t-SNARE proteins that functions to form the SNARE complex for VTV-Golgi fusion. However, Syn5 is also a protein that it is found in the ER since it has been found to take part in ER-Golgi transport of vesicles. Therefore, we tested the Co-IP with Hsc-70 using solubilized ER proteins with the Syn5 antibody. Conversely the above results indicate that although Syn5 is present in the ER it does not bind to Hsc70.
CHAPTER FOUR: DISCUSSION

The present study evaluates the role of the heat shock protein, Hsc70 in SNARE-complex formation required for docking and fusion of the VTV with the hepatic cis-Golgi. The slowest step in the transport of VLDL happens in its pathway from the hepatic ER to cis-Golgi, therefore unraveling the intricate details in which VLDL biogenesis and secretion takes place would put forth a solution towards the regulation of how VLDL is expelled into the circulatory system. Briefly, the biogenesis of the VLDL lipoprotein begins in the rough ER in response to the fatty acid influx, the liver uptakes fatty acids by: chylomicron remnants, through the portal vein, dietary fat and adipose tissue [2, 3]. The biogenesis of VLDL is broken down into two steps, first ApoB100- a structural protein necessary for the proper formation of the VLDL is translated in the rough ER and then translocated to the lumen of the ER where MTP enables the transfer of lipids to ApoB100, the lipidation of ApoB100 relies on the accessibility of triglycerides in the ER [10, 28]. The second step constitutes the adding of triglycerides to the primordial poorly lipidated ApoB100 particle. Although the site of VLDL maturation is subject to debate, data from Siddiqi et al [14] indicated that the VLDL particle is packed into a specific vesicle- the VTV which will travel in anterograde fashion and fuse with the cis-Golgi membranes where the VLDL-ApoB100 will undergo further glycosylation and phosphorylation.

As opposed to the PTV, an ER-derived vesicle with Golgi destination, the VTV comfortably fits the 100-120 nm VLDL particle and protects the apoB100 protein from degradation in the cytosol. Additionally, the surface of the VTV concentrates Sec22b that serves as a v-SNARE and will eventually form a SNAREpin or SNARE complex with GOS28, Syn5 and rBet1 the three t-SNAREs found on the cis-Golgi membrane. The SNAREpin will allow for the VTV and the cis-Golgi membrane to fuse permitting the release of VLDL into the lumen of the Golgi [3].

The fusogenic SNARE complex requires cytosolic proteins that will facilitate the SNARE pairing. After several chromatographic steps on liver cytosol and detailed mass spectrometry studies it was revealed that heat-shock, Hsc-70 to play a role in the formation of the SNARE complex [24]. In addition, (Siddiqi et al; unpublished data) have indicated that the
presence of Hsc-70 is vital for SNARE complex formation thus proper delivery of VLDL. Experiments have demonstrated that when using Hsc-70 depleted cytosol the fusion-complex formation is diminished by almost 80 percent, however, when Hsc-70 recombinant protein is added the complex formation activity is restored (see figure 2,3). Furthermore, in order to examine how Hsc70 plays a role in SNARE complex formation, we carried out immunoprecipitaion experiments to identify to which SNARE proteins Hsc-70 binds. Western blot data suggest that Hsc70 binds to Sec22b at the ER and cis-Golgi level, (see figures 5,10, 11). These data indicate that Hsc-70 could in fact act as a chaperone protein, supporting previous studies that suggest the involvement of Hsc-70 in ER to Golgi transport. It also supports Hsc-70 established role of serving as a chaperone in the cell [24, 27]. We predict that Hsc-70 binds to Sec22b, the v-SNARE on the VTV in the ER and brings the VTV into close proximity with the t-SNAREs allowing the SNARE complex to form. Interestingly, figure 12 shows the presence of Syn5 in the ER sample, studies done by Dasher and Rowe [29, 30] indicate that although Syn5 has been known to be a t-SNARE, it has an isoform that has been shown to possibly serve as a potential regulator for ER to Golgi transport [31]. In addition, (figure 7, 8 and 9) suggest that Hsc-70 binds to GOS28, Syn5 but not rBet1; its role here continues to be enigmatic. Some possibilities arise; one is that since the SNARE complex forms through the zippering action of the contributing SNARE motifs, the bound chaperone might act to promote the assembly of the SNAREs so that the α-helical coiled structure forms, studies from the Hay group show that this assembly does not require ATP, consistent with our data that docking, the step before fusion is ATP – independent. Other possibility is that Hsc-70 might act to provide the energy necessary so fusion can occur. In addition, the SNARE complex is specific for each transport step and is also thermodynamically and kinetically stable, therefore the dissociation of the complex requires ATP hydrolysis; we believe Hsc-70 can act as an ATPase an action seen by Hsc70 in other trafficking pathways with clathrin and synaptic vesicles [32, 33]. Ultimately, understanding the role Hsc-70 plays in the transport of VTVs from the ER to the Golgi could in fact offer further dissection of how VLDL is secreted into the bloodstream.
The assembly of VLDLs occurs in the lumen of the endoplasmic reticulum (ER). After their biogenesis in the ER lumen, VLDLs are packaged into specialized vesicles known as VLDL transport vesicles (VTVs). The average diameter of the VTVs is ~110 nm, which is sufficient to enclose VLDL-sized particles. VTVs bud off the ER membrane and move to and fuse with the cis-Golgi, delivering their VLDL cargo to the Golgi lumen. Nascent proteins are transported from the ER to the Golgi protein transport vesicles (PTVs). Their size ranges between ~55 and 70 nm. Biogenesis of both VTVs and PTVs from the ER membrane requires coat protein complex II (COPII) machinery. VTVs are, however, different from PTVs in their size, buoyant density, protein composition and require a unique set of SNARE proteins for fusion-complex formation.
REFERENCES


