JBC Papers in Press. Published on October 22, 2002 as Manuscript M207976200

Nascent lipidated apoB is transported to the Golgi as an incompletely folded intermediate as probed by its association with network of ER molecular chaperones, GRP94, ERp72, BiP, calreticulin and cyclophilin B

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Running title: ApoB-chaperone interactions

Summary

We have previously demonstrated that ER-resident molecular chaperones interact with apolipoprotein B-100 (apoB) during its maturation. The initial stages of apoB folding occur while it is bound to the ER membrane where it becomes partially lipidated to form a primordial intermediate. We determined whether this intermediate is dependent on the assistance of molecular chaperones for its subsequent folding steps. To that end, microsomes were prepared from HepG2 cells and lumenal contents were subjected to density gradient centrifugation. Immunoprecipitation of apoB followed by Western blotting showed that the lumenal pool floated at density 1.12 g/ml, and like the membrane-bound pool was associated with GRP94, ERp72, BiP, calreticulin and CyPB. Except for calreticulin, chaperone/apoB ratio in the lumen was several-fold higher than that in the membrane suggesting a role for these chaperones in both facilitating the release of the primordial intermediate into the ER lumen and in providing stability. Subcellular fractionation on sucrose gradients showed that apoB in the Golgi was associated with the same array of chaperones as the pool of apoB recovered from heavy microsomes containing the ER, except that chaperone/apoB ratio was lower. Density gradient fractionation showed that the major pool of lumenal apoB in the Golgi was recovered from $1.02 < d < 1.08$ g/ml, while apoB in ER was recovered primarily from $1.08 < d < 1.2$ g/ml. Both fractions were associated with the same spectrum of chaperones. Together with the finding that GRP94 was found associated with sialylated apoB, we conclude that correct folding of apoB is dependent on molecular chaperone assistance which play multiple roles in its maturation throughout the secretory pathway including distal compartments such as the trans-Golgi network (TGN).

INTRODUCTION

The correct folding of apolipoprotein B (apoB) into its mature, secretion-competent form is a complex process that leads to the formation and secretion of triacylglycerol (TAG)-rich lipoproteins, chylomicrons, and the atherogenic lipoproteins, very low density lipoproteins (VLDL) (1,2). A two-step model for the formation of VLDL, was originally proposed on the basis of electron microscopical studies in rat liver (3). This model was further supported by latter studies in rat liver (4), rat hepatoma cells, McA-RH7777 (5-8), and in transgenic mice lacking the gene for apoB in the intestine (9). According to this model, the first step involves partial lipidation of apoB to form a primordial particle with HDL/LDL-like density. In the second step this intermediate fuses with a large apoB-free lipid droplet composed primarily of TAG to form nascent VLDL or chylomicrons. In HepG2 cells however, these distinct steps were not clearly demonstrated. Nonetheless, the initial steps of lipidation are thought to be similar to other systems in that they are mediated by microsomal triglyceride transfer protein (MTP) (reviewed in (10-12)) and occur co-translationally, while apoB is bound to the ER membrane (13). Partial lipidation of apoB leads to formation of an intermediate with HDL/LDL-like densities. This intermediate is then released into the ER lumen. Subsequent recruitment of lipids by this intermediate seems to occur continuously (13) to ultimately form and secrete larger TAG-rich particles with primarily LDL-like density and to a lesser extent particles with VLDL and IDL-like densities.

In the absence of sufficient lipids, primarily TAG and cholesterol esters (1,14,15), or when MTP activity is diminished, either due to mutations (11,16) or due to specific inhibitors (7,17), apoB is unable to form lipoproteins and is targeted to proteasome-dependent

degradation both co- and post-translationally (18,19) in an hsp70- and hsp90-dependent mechanism (20-22).

In addition to MTP, the folding of apoB like that of other secretory proteins seems to require the assistance of ER-resident molecular chaperones including BiP, calnexin, calreticulin, ERp72 and GRP94 (18,23-28). Molecular chaperones bind transiently to nascent polypeptides to prevent their aggregation, thereby maintain them in conformations competent for efficient folding. Once correct folding is attained, chaperones dissociate, and folded proteins exit the ER. However, if nascent polypeptides fail to attain their native form, they are retained in the ER bound to chaperones which ultimately target them for proteasome-dependent degradation (29) to prevent their transport to their target destination (30-34).

Given the complexity of apoB folding which involves lipidated membrane-bound and lumenal intermediates, it was of interest to characterize in great detail the involvement of chaperones in various stages of its biogenesis.

We hypothesized that the membrane-bound pool of apoB will interact with molecular chaperones. However, it was unclear whether partially lipidated apoB that is translocated into the ER lumen as a primordial intermediate is still dependent on the assistance of molecular chaperones for its subsequent folding steps and if so, whether they dissociate from apoB-containing particles before their transport to the Golgi. This study was designed to address these questions.

We demonstrate that the same array of molecular chaperones, interact with both the membrane-bound and the lumenal pools of apoB. However, the relative level of BiP, GRP94, ERp72, and cyclophilin B (CyPB) associated with the lumenal pool of apoB is several-fold higher than that associated with the membrane-bound pool, suggesting a role for these

chaperones in facilitating the release of apoB from the microsomal membrane. We further demonstrate that GRP94, ERp72, BiP, calreticulin and CyPB remain bound to apoB that is transported to the Golgi albeit the chaperone/apoB ratio was lower than that in the ER. This is consistent with the idea that these chaperones play multiple roles both in early and in late folding events occurring in the ER and the Golgi, respectively. Thus, apoB is recognized as an incompletely folded protein throughout most of the secretory pathway.

Experimental Procedures

Materials

Antibodies: Goat polyclonal antibodies to apoB were from Biodesign, and Calbiochem. Mouse monoclonal antibodies to apoB 2D8, was kindly provided by Dr. E. Marcel and Dr. Milne, B4 was a generous gift from Dr. J. Fruchard (Université de Lille, France) with permission from Dr. H. Bazin (University of Louvain, Belgium). Polyclonal antibodies to GRP94 were kindly provided by Dr. S. Cala (Wayne State University). Rat monoclonal antibodies to GRP94, mouse monoclonal antibodies to BiP, rabbit antibodies to ERp72, and rabbit polyclonal antibodies to calreticulin, were from Stressgen Biotechnologies-Corp. (Victoria BC, Canada), rabbit antibodies to CyPB, mouse monoclonal antibodies to Golgin 97 were from Molecular Probes. Monoclonal antibodies to GPP130 were kindly provided by Dr. H. Hauri (University of Basel, Switzerland). Mouse monoclonal antibodies to p58 and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Sigma.

Other materials: Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Life Technologies Inc. Cycloheximide, leupeptin and aprotinin and essentially fatty acid-free albumin were from Sigma. N-Acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) was from Roche Molecular Biochemicals, 4-[2-AminoEthyl]-Benzyl Sulfonyl Flouride (AEBSF) was from Calbiochem. Oleic acid was from Neucheck Inc. Gelatin- and Protein G-Sepharose were from Amersham Pharmacia Biotech. PVDF membranes were from Millipore and centricon microconcentrators were from Amicon. Reagents for the ECL system and [^{35}S]methionine/cysteine, specific activity > 1,000 Ci/mmol were purchased from NEN Life Science Products. Biotinylated lectin Sambucus nigra agglutinin (SNA-I) was from EY Labs. HRP-conjugated extravidin was from Sigma.

Dithiobis(succinimidyl propionate) (DSP) was from Boehringer Mannheim. BMS-197636 was a generous gift from Dr. D. Gordon (Bristol Myers Squib).

Methods

Cell cultures, pulse-chase experiments and KBr density gradient fractionation

HepG2 cells were pulse-labeled with [^{35}S]methionine/cysteine and chased as described (23). Cell monolayers were subjected to crosslinking, then solubilized and apoB immunoprecipitated (26). Media were collected and spun at 800 X g for 5 min, concentrated using micro-concentrators, adjusted to 1.25 g/ml with solid KBr, over-laid by 1.006 g/ml solution and spun in an SW41 rotor at 40 k rpm for 20-24h. Density fractions were collected from the top and their density determined by an Abbe Refractometer (American Optical Corporation). Fractions were washed in micro-concentrators, adjusted to 2% CHAPS and subjected to immunoprecipitation.

Preparation and extraction of microsomes

HepG2 cells were incubated for 2 h in media supplemented with 0.8 mM oleic acid complexed to 0.2 mM albumin and 25 μg/ml ALLN to increase lipid availability and inhibit degradation, respectively. Cells were either subjected to crosslinking and then scraped with a rubber policeman (23) or were scraped without prior crosslinking. Washed cells were suspended in homogenization buffer (HB) (0.25M sucrose; 10 mM HEPES pH7.5 and protease inhibitors: 10 μg/ml each, aprotinin and leupeptin, 0.1mM AEBSF and 5 μg/ml ALLN). Cells were then homogenized with a tight fitting Dounce homogenizer with 20 strokes. In some experiments, homogenates were also passed through a 25-gauge needle.

Homogenates were spun for 5 min at 6,000 x g and post-nuclear supernatants were spun for 40 min at 200,000 x g at 3ºC to pellet the microsomes.

Microsomes were extracted either with carbonate alone (0.1M Na_2CO_3, pH 7.4) (35), or with carbonate supplemented with 0.025% sodium deoxycholate with or without 1.2M KCl (carbonate/DOC or carbonate/DOC/K, respectively) (36). Samples were then spun as described before, to separate the soluble contents (lumenal) from the membranes. Soluble contents were neutralized, and salts removed by ultrafiltration. Membranes and lumenal contents were then adjusted to 2% CHAPS containing protease inhibitors as described above, and apoB immunoprecipitated. In some experiments lumenal contents and membranes were adjusted to 1% SDS in PBS, heated to 65ºC for 15 min then diluted to 0.1% SDS with 1% triton X-100; PBS and subjected to immunoprecipitation.

Subcellular fractionation on sucrose gradients

HepG2 cells were incubated for 2 h in media supplemented with oleic acid and ALLN as described before. Cells were either subjected to cross-linking and then scraped with a rubber policeman (23), or were scraped without cross-linking as indicated in the figures. Washed cells were suspended in HB, homogenized and centrifuged as described above. Post nuclear supernatants were adjusted to 1.3 M sucrose and overlaid with 1.2, 1.15 0.86 and 0.25M sucrose; 10 mM HEPES (37). Gradients were spun for 18 h at 24 k rpm at 4ºC in SW41 rotor and eight fractions collected from the top as follows: top 1 ml, light Golgi (inter-phase 0.25/ 0.86M sucrose), Heavy Golgi (interface 0.86/1.15M sucrose), (1.15M sucrose), 1.2M sucrose, inter-phase (1.2/1.3M), and heavy microsomes, (1.3M and pellet). The concentration of sucrose in each fraction was determined by measuring their refractive index using a refractomer as described before. Fractions were then washed in 0.25M sucrose by

centrifugation in a Ti 50.3 rotor (Beckman) at 40 k rpm for 1 h at 4ºC. Pellets were extracted either with carbonate or carbonate/DOC/K as described above. Fractions were then centrifuged for 30 min at 100,000 X g to separate membranes from lumenal contents. Membranes were washed in PBS and then solubilized in 2% CHAPS. Lumenal contents were adjusted to pH 7.4, concentrated using microconcentrators and adjusted to either PBS pH 7.4 or to 2% CHAPS; 50 mM HEPES pH 7.4 both containing protease inhibitors as described above. Membranes and lumenal contents were then processed for immunoprecipitation. In some experiments, lumenal contents were subjected to KBr density gradient centrifugation as described above and apoB in density fractions immunoprecipitated.

Immunoprecipitation and Western Blotting

Samples in either PBS, 0.1 % SDS/1% TritonX-100; PBS, or 2% CHAPS/50 mM Hepes pH 7.4 containing protease inhibitors were pre-cleared by incubation with gelatin- and protein G-Sepharose and non-immune goat or rabbit serum, mouse or rat IgGs for 1 h at 4°C. Beads were removed by centrifugation, and supernatants were incubated at 4°C for 18-20 h with antibodies to apoB, GRP94 or ERp72 along with appropriate controls as indicated in the figures. Immunocomplexes were captured with protein G-Sepharose as described (26). For native immunoprecipitation, beads were washed in PBS followed by phosphate buffer containing 0.5M NaCl, and finally, in PBS.

Immunocomplexes were solubilized by heating the beads to 95°C in Lammeli sample buffer (38) containing 10% BME and 6 M Urea, then resolved by 4-15% SDS-PAGE followed by electrotransfer onto PVDF membranes (26). ApoB, chaperones, ER and Golgi marker proteins were probed by the appropriate antibodies, visualized by the ECL system

(NEN), and quantified by densitometry, using Duoscan T1200 Agfa Densitometer and Zero-Dscan image analysis system.

Lectin Blotting

Microsomes were prepared from uncrosslinked HepG2 cells as described above, then extracted with carbonate and lumenal contents subjected to immunoprecipitation with either rabbit antiserum to GRP94 or non-immune rabbit serum. Immunocomplexes were resolved on 4-15% SDS-PAGE and transferred onto PVDF membranes. Non-specific sites were blocked by incubating the membranes in 5% albumin; PBS for 1 h. Membranes were then incubated in either buffer alone (phosphate buffer pH 6.8; 1 mM $CaCl_2$; 0.01% albumin) or buffer containing 20 mU/ml Neuraminidase for 20-24 h at 37°C. Membranes were then washed and incubated with SNA-1, which was visualized using the ECL system following incubation with HRP-Avidin.

Results and Discussion

Chaperones interact both with the membrane-associated pool of apoB and with the pool released into the microsomal lumen

Unlike other secretory proteins, the newly-translated pool of apoB remains associated with the ER membranes during early stages of folding which involve MTP-dependent lipidation to form a primordial lipoprotein intermediate that is ultimately released into the ER lumen (39). We hypothesized that it is during these early stages of folding that molecular chaperones would be most critical for its successful folding and maturation. The partially lipidated intermediate that is translocated into the lumen is conceivably more stable than the membrane-bound pool and therefore, may not require the assistance of molecular chaperones, so that it could proceed to acquire the bulk of lipids to form VLDL/IDL independently of chaperones. Alternatively, this intermediate may still depend on chaperone assistance for its subsequent folding steps. In that case, two scenarios may be envisioned: a) it remains associated with the same array of molecular chaperone as those interacting with the membrane-bound pool or b) it remain associated with a subset of chaperones that may be necessary to keep it in a conformation competent for binding the bulk lipids. To test these possibilities, we determined first whether chaperones are associated with the lumenal pool of apoB. To that end, crude microsomes were prepared from HepG2 cells that were pre-incubated with 0.8 mM oleate and the calpain inhibitor ALLN, which also effectively inhibits proteasomes (40) to increase folding efficiency. Microsomes were subjected to 3 extraction protocols to release the lumenal contents: carbonate (0.1M pH 11.5) alone or carbonate supplemented either with deoxycholate (carbonate/DOC) or deoxycholate and KCl (carbonate/DOC/K). The latter 2 protocols were demonstrated to increase the efficiency of

extraction of lipidated apoB from microsomal membranes prepared from McA-RH7777 cells (36). Immunoblotting showed that the inclusion of 0.025% DOC, which is well below its critical micellar concentration, did not solubilize the microsomal membranes as evidenced by the fact that the integral membrane protein, calnexin, was not extracted (data not shown). Therefore, these protocols can be applied to HepG2-derived microsomes. Quantification of apoB in membranes and lumen showed that the amount of apoB extracted by carbonate-supplemented DOC with or without KCl was similar (data not shown), and both protocols typically led to higher level of extracted apoB compared to carbonate alone albeit the difference between these protocols was not statistically significant. Thus, an average of about 48 ±12 (range 30 to 60%) and 62±10 % (range 47 to 72%) (n=5) of the total pool of apoB was extracted by carbonate and carbonate/DOC, respectively. Therefore, either carbonate alone, or carbonate/DOC with or without KCl can be used interchangeably with sufficient recovery of the lumenal apoB pool for further characterization.

Fig. 1A shows that apoB released either by carbonate or carbonate/DOC was associated with the major ER-resident molecular chaperones including GRP94, ERp72, calreticulin (CRT), CyPB and BiP (not shown) (lanes 1,3). Importantly, the level of co-immunoprecipitated chaperones was substantially higher than the corresponding controls (compare lanes 1, 3 to lanes 2, 4, respectively). Therefore, co-immunoprecipitated chaperones reflect apoB-chaperone interactions existing in living cells. Furthermore, following quantification of apoB and chaperone bands we calculated the chaperone/apoB ratio and found that it was similar whether microsomes were extracted with carbonate or carbonate/DOC/K.

Next, it was of interest to determine the chaperone/apoB ratio in the lumen compared to the membrane-bound pool. In these series of experiments however, we could not determine with certainty whether the spectrum and the level of molecular chaperones associated with these pools of apoB are comparable, since the efficiency of immunoprecipitated membrane-bound apoB was very low following solubilization of microsomal membranes by CHAPS. To substantially increase the efficiency of solubilization, membranes were heated to 65°C in the presence of 1% SDS prior to immunoprecipitation with antibodies to apoB. Fig. 1B shows that the same array of chaperones is associated with both the membrane-bound and the lumenal pools of apoB (lanes 5 and 7, respectively), namely GRP94, ERp72, calreticulin and CyPB and BiP (not shown). When antiserum to apoB was replaced with non-immune serum, chaperone protein bands were diminished (lanes 6, 8) strongly suggesting that the observed interactions of these chaperones with apoB exist in living cells. Notably, the presence of SDS in the immunoprecipitation buffer led to a substantial reduction in background, consistent with reduction of non-specific ionic interactions between chaperone proteins and the protein G-Sepharose beads (lanes 6, 8). Quantification of apoB and chaperone bands revealed that chaperone/apoB ratio in the lumen was at least 3-4-fold higher than that in the membranes. This was consistently observed for GRP94, ERp72 and CyPB albeit the fold increase varied between experiments. In contrast, calreticulin/apoB ratio in the lumen was essentially similar to that in the membranes. These findings therefore clearly demonstrate different roles for these chaperones in the folding and maturation of apoB. Thus, GRP94, ERp72, BiP and CyPB presumably play a role not only in early folding events occurring cotranslationally while apoB is membrane-bound, but they also seem to facilitate the release of the lipidated intermediate from the membrane. One possibility

is that they replace hydrophobic interactions between apoB and the ER membrane thereby promoting its detachment from the membrane and providing stability to the primordial intermediate once translocated into the lumen. Calreticulin on the other hand, being a lectin-like chaperone, may be involved in other aspects of apoB folding, so that no additional molecules are recruited to interact with the primordial intermediate prior to or after its release into the lumen. Nonetheless, calreticulin seems to play a role both during early and late folding events, as it also remains associated with the primordial intermediate after its translocation into the lumen.

What is the nature of the membrane-bound pool of apparently full-length apoB? Since these experiments were carried out under conditions that support optimal lipoprotein assembly (e.g. by providing oleic acid and inhibiting degradation by ALLN), this pool of apoB does not represent misfolded molecules. Rather, this pool is likely to represent the precursor of the primordial intermediate, which ultimately matures into secretion-competent lipoproteins such as VLDL, IDL and LDL-like particles. Being membrane-bound and having an apparent size of full-length apoB, this pool is presumably not fully-translated, although very close to it (41). It follows that these molecules are almost fully lipidated as translation is closely coupled to lipidation in HepG2 cells (41). This pool being in the process of translation interacts both with the translocon and the ribosome (41). This could be partly responsible for a fraction of the pool to be non-extractable by either carbonate or carbonate/DOC in protocols involving crosslinking of proteins. In addition, it is conceivable that, domains enriched in β–strands predicted to form amphipathic β-sheets (42-44) interact with the lumenal leaflet of the ER membrane until sufficient core lipids are recruited to form the primordial intermediate.

These experiments also demonstrated for the first time a direct interaction between CyPB and apoB. This suggests that CyPB is involved in the folding of apoB and may even be critical for its successful folding. This is based on studies showing that the immunosuppressant, cyclosporine A, prevents the secretion of apoB (45), concurrently with diminished interaction of CyPB with apoB (Zhang and Herscovitz, unpublished observations). Although cyclosporine A also binds to cyclophilin A, its cytosolic residence makes it an unlikely candidate chaperone to promote folding of apoB which takes place in the ER. In fact, it was demonstrated that unlike the CFTR receptor (46), interaction of apoB with the cytosolic chaperones Hsp70 and Hsp90 leads to its targeting for degradation (20,22). CyPB is both a folding catalyst belonging to peptidyl prolyl cis-trans isomerases (PPIases) and a chaperone (47-49). Either activity or both may be important for the correct folding of apoB, since a) apoB has a number of prolines that may be dependent on the PPIase activity of CyPB for efficient folding, and b) CyPB like GRP94 and ERp72 seems to play a role in mediating the detachment of the primordial intermediate from the membrane. Further studies will be necessary to characterize the role of CyPB in apoB maturation in more detail.

Chaperones interact with lipidated apoB with HDL-like density released into the microsomal lumen

The previous series of experiments (Fig. 1) demonstrated that molecular chaperones are associated with the lumenal pool of apoB. However, these experiments did not provide direct evidence for the interaction between these chaperones and the lipidated pool of apoB. To directly demonstrate such interactions, microsomes prepared from cells following a 30-min pulse-labeling period, were extracted with carbonate, and the soluble contents were

subjected to KBr density gradient fractionation. This extraction protocol was chosen for two reasons: first, to allow for a direct comparison with previous studies involving careful characterization of apoB-100-containing particles in HepG2 cells (13), second, we determined that there was no qualitative difference in the density distribution of particles released by carbonate or carbonate/DOC (data not shown) consistent with other studies (36). Fig. 2A shows that about 50% of the newly synthesized pool of apoB was found in fractions having HDL-like density (e.g. 1.12 g/ml). This pool of apoB was associated with GRP94, ERp72 (Panel C) as well as BiP, calreticulin and CyPB (data not shown).

Since the association of chaperones with lipidated apoB is likely to increase the overall density of apoB-containing particles, it was necessary to ensure that the observed density distribution of apoB does not result from excessive crosslinking of proteins that are not otherwise associated with apoB in living cells. To that end, we performed another series of experiments in which cells were not subjected to crosslinking prior to microsomal preparation. These experiments showed that the density distribution of apoB present in the lumen was very similar to that obtained following crosslinking of cellular proteins (data not shown).

The size distribution of intracellular apoB-containing particles in HepG2 cells was analyzed by Bostrom et al (13) who demonstrated that particles isolated from HDL-like densities (e.g. 1.065-1.170 g/ml) had a mean diameter of about 25 and 20 nm following pre-incubation of cells with or without oleate, respectively (13). This size is typical of plasma LDL particles, which contain apoB-100 as their only protein constituent. The fact that the mean density of these particles is in the HDL range is consistent with these particles having higher protein to lipid ratio compared to plasma LDL that is contributed by the associated

chaperones. Indeed, these investigators suggested among other possibilities that perhaps the discrepancy between the size and the density may be due to presence of proteins (unknown at the time), other than apoB on the lipoproteins isolated from the microsomal lumen (13). However, since molecular chaperones have a slow turn-over rate (e.g. several days), no additional proteins were detected following a short-term radiolabeling protocols (13).

These series of experiments clearly demonstrated that apoB is released into the lumen as a lipidated intermediate with an HDL-like density that is still dependent on the assistance of molecular chaperones for its subsequent folding steps.

The density distribution of secreted apoB-containing particles is very different from that of the intracellular pool, as a significant fraction, about 44 % and 34% is found in lower densities, e.g. $d < 1.02$, and $d \leq 1.03$ g/ml, respectively (Fig. 2B), clearly reflecting a higher lipid to protein ratio presumably due to both higher lipid content and lower protein content (due to absence of chaperones). None is found in HDL-like densities consistent with the finding that the particles are secreted free of chaperones (not shown) and with the hypothesis that apoB-100 cannot attain a stable conformation when associated with particles having a lipid-poor core. This can be attributed to those domains starting at apoB-21 enriched in β-sheets (42,43) which presumably "prefer" to be bound to TAG since this is a more energetically favorable state (43,44). Therefore, it is conceivable that these domains are also most likely to be engaged in interaction with molecular chaperones, which stabilize them until enough lipids are recruited.

Chaperones are associated with advanced folding intermediates of apoB that are transported to the Golgi

The major folding steps of newly translated proteins that enter the secretory pathway take place in the ER, where resident chaperones and folding catalysts assist in their folding and assembly by direct physical interaction. Such interactions persist until proper folding is attained, whereby the affinity between chaperones and folding intermediates declines, ultimately leading to the release of chaperones (31,32). Correctly folded proteins are then transported to the Golgi for further modifications and for sorting to their target destination.

Since the folding of apoB is much more complex than most other secretory proteins and we have demonstrated that molecular chaperones remain associated with apoB following its translocation into the lumen as a lipidated primordial intermediate (Fig. 2), it was of interest to determine at what stage of maturation chaperones dissociate from apoB. To that end, cell homogenates were fractionated on sucrose density gradients, apoB in subcellular fractions immunoprecipitated and associated chaperones identified by Western Blotting. Fig. 3A shows the distribution of marker proteins. Both Golgi markers, e.g., Golgin 97 (50), GPP130 (51) and p58 (52), and the ER marker calnexin (53), are shown. As evidenced from this figure, all Golgi markers are present in fraction 3. GPP130 demonstrated to reside in the cis/medial Golgi (51) is also present to a certain extent in fraction 2. On the basis of this analysis, fraction 3 represents cis/medial Golgi. This fraction also contains the trans-Golgi network (TGN) as it also contains the entire pool of sialylated apoB (not shown).

The distribution of calnexin, an integral membrane protein of the ER is quite different, as it is found predominantly in heavy microsomes. Thus, the ER fractionates with heavy microsomes. From hereon heavy microsomes and ER will be used interchangeably.

The distribution of lumenal apoB is shown in panel B. As expected, a substantial fraction, about 40 % is found in heavy microsomes, which include the ER. A smaller but sizable fraction, about 20 %, was found in cis/medial Golgi (fraction 3). Such distribution suggests that during its folding, apoB resides primarily in the ER demonstrated to be the site where its major folding steps take place (23,54). The presence of a sizable fraction of the intracellular pool of apoB in the Golgi is consistent with other reports demonstrating the presence of apoB in Golgi from HepG2 cells (55). Similarly, VLDL particles were also detected in Golgi from rat (56) and mouse liver (57). The presence of apoB in this compartment under steady-state conditions is consistent with modifications of apoB such as glycosylation and phosphorylation (58-62) as well as possible remodeling by exchange of phospholipids between the Golgi membranes and the nascent particles (63,64).

Most interesting is the finding that apoB present in the Golgi compartment is associated with the same spectrum of molecular chaperones as those bound to apoB in the ER. These include GRP94, ERp72, calreticulin, BiP and CyPB (panel C). These findings therefore demonstrate that apoB exits the ER in complexes containing the same chaperones assisting in its early folding stages. It is possible that other molecular chaperones such as PDI are also present in these complexes. Chaperone/apoB ratio within the subcellular compartments is shown in Fig. 3D. It appears that within the early secretory pathway (e.g. ER) the chaperone/apoB ratio is substantially higher. Thus, chaperones dissociate from the particles before transport to the Golgi. The relative amount is dependent on the specific chaperones. Thus, while calreticulin/apoB in Golgi is about half that in the ER, GRP94/ and ERp72/ is about 30%, and 20%, respectively, and CyPB/and BiP/apoB decline to less than 10% (Fig. 3D). Thus, it appears that a substantial amount of chaperone molecules dissociate from apoB-

containing lipoproteins prior to their arrival at the Golgi compartment consistent with apoB being in a more advanced folding stage. Nevertheless, the fact that a sizable fraction remains bound to apoB during and after its transport to the Golgi is consistent with different roles for these chaperones folding of apoB occurring in post-ER compartments.

To ensure that the chaperones co-precipitated with the pool of apoB that is present in the Golgi interact with apoB in living cells, we performed 2 series of experiments: 1) Cells were exposed to BMS-197636, a specific inhibitor of MTP which blocks the lipid transfer activity of MTP *in vitro* (65). As a result, apoB is unable to form lipoproteins and is rapidly degraded in a proteasome-dependent mechanism (17). Under these conditions it is expected that the major pool of apoB would be largely unfolded/misfolded and therefore primarily localized to the ER. Therefore, only very low levels of chaperones are expected to co-precipitate with antibodies to apoB in the Golgi. We found that this inhibitor reduced the amount of secreted apoB by greater than 80% (data not shown) concomitantly with a substantial reduction in the level of apoB in the Golgi compared to control levels (compare lane 1 to 3). Consistent with the diminished level of apoB in the Golgi, the level of GRP94 and ERp72 co-immunoprecipitated with antibodies to apoB is also greatly reduced (compare lane 1 to 3). The reduced level of apoB in the ER lumen of cells treated with the inhibitor (lane 4) is consistent with diminished lipidation of apoB thus, preventing its translocation into the lumen.

Together these findings demonstrate that co-precipitation of ER-resident molecular chaperones with apoB that is present in the Golgi is dependent on the presence of apoB.

2) In the next series of experiments, crosslinking of cellular proteins was eliminated to minimize manipulations prior to fractionation and immunoprecipitation. To that end,

uncrosslinked cells were homogenized and subjected to subcellular fractionation. Immunoprecipitation of lumenal contents was carried out under native conditions, so that apoB-chaperone interactions were minimally disrupted. We used polyclonal antibodies to GRP94 and 2 monoclonal antibodies to apoB, 2D8 and B4 which bind to a domain encompassing residues 1438-1480 (66) and 1854-1878 (67) respectively. As shown in Fig. 5C antibodies to GRP94 co-immunoprecipitated apoB both in the Golgi (lane 9) and in the ER (lane 11), while control samples that were incubated with non-immune serum co-precipitated substantially less apoB (lanes 10 and 12, respectively). Similarly, antibodies to apoB, B4, specifically co-immunoprecipitated GRP94 from Golgi and ER (lanes 5 and 7, respectively) while control samples precipitated only low levels of GRP94 (lanes 6 and 8, respectively). In a similar series of experiments shown in Panel A, 2D8 and B4 antibodies co-immunoprecipitated calreticulin (not shown), GRP94 and ERp72 from the Golgi (lanes 1 and 3, respectively) and the ER (lanes 2 and 4, respectively). Thus, the observed apoB- chaperone interactions following crosslinking of cellular proteins does not result from excessive crosslinking of cellular proteins which are not otherwise in close contact with apoB, but rather reflect interactions that exist in living cells, and as long as the immunoprecipitation protocol does not require detergents, these interactions are not disrupted.

Thus, from the experiments shown in Figures 4 and 5 we conclude that ER-resident molecular chaperones interact with apoB both in the ER and in the Golgi. We calculated the relative amount of apoB associated with GRP94 in the ER and Golgi from the amount of apoB immunoprecipitated with antibodies to GRP94 and the efficiency of the immunoprecipitation of GRP94. We found that 40-60% and 25-35 % of the total pool of apoB in the ER and the Golgi, respectively, is associated with GRP94 under steady-state conditions.

Interestingly, the pool of apoB that was immunoprecipitated from the Golgi (Fig. 5) (lanes 1 and 3) migrated slightly slower than its ER-derived counterpart (lanes 2, 4) consistent with modifications of apoB occurring in a post-ER compartment, conceivably, the Golgi itself. These include modifications of the glycan residues, phosphorylation (62) or both. These differences between the pools of apoB were observed in a number of experiments and provide further support for efficient separation between the early secretory compartment, the ER, and the Golgi, and for the presence of distinct pools of apoB within these compartments. These findings therefore, strongly suggest that apoB-molecular chaperone complexes exist within the Golgi compartment in living cells, excluding the possibility that such complexes are derived from vesicles originating in the ER.

Overall these studies clearly demonstrate that apoB is transported to the Golgi as part of complexes containing ER-resident molecular chaperones. The presence of different levels of chaperones compared to the ER suggests distinct roles for these chaperones in late folding events occurring in the Golgi.

The major pool of apoB present in the Golgi and in heavy microsomes is lipidated and is associated with the same array of molecular chaperones

The next series of experiments was designed to determine whether the chaperone-associated pool of apoB present in the Golgi is lipidated. Fig. 6A shows that the majority of the apoB pool in the ER and the Golgi is found in $1.08 < d < 1.2$ g/ml, while that in the Golgi is found at lower densities, $1.02 < d < 1.08$ g/ml. Nevertheless, apoB present in this lower density range is still associated with the same array of chaperones associated with the pool of

apoB recovered from the ER, including GRP94, ERp72, BiP, calreticulin and CyPB (Fig. 6B). The lower density of apoB in the Golgi reflects a higher lipid to protein ratio. This could be due to either an increase in the relative content of lipid, decrease in relative content of protein without change in lipid content, or both. Increase in lipid content can occur by recruitment of neutral lipids in post ER pre-Golgi compartments, or in the Golgi itself. Such a model is consistent with continuous recruitment of lipids in these cells rather than a two-step model demonstrated in liver and McARH777 cells (3-8). The release of a substantial fraction of chaperones from apoB-containing particles prior to exit of particles from the ER (Fig. 3D) may partially account for the shift in the density of the particles in the Golgi with no substantial addition of lipid. To test this possibility, we estimated the content of lipid in the ER and Golgi.

The relative amount of lipid and protein was calculated from the density of the particle which equals to the sum of protein and lipid density using densities of 1.33 and 0.94 g/ml for protein and lipid, respectively.

$d_{particle} = 1.33(f_{protein}) + 0.94 \times (1-f_{protein})$.

Using average densities of 1.16 and 1.05 g/ml for particles in the ER and Golgi, respectively, we calculated that the fraction of protein and lipid in particles in the ER is 0.56 and 0.44, and in the Golgi, 0.28 and 0.72, respectively.

The molecular weight (MW) of protein is the sum of the MW of apoB and associated chaperones. To calculate that, we made two alternative assumptions: Particles in the Golgi have at least one molecule of each of the identified chaperones (e.g. GRP94 as a homodimer, ERp72, BiP, calreticulin and CyPB) per particle (or per apoB molecule). Alternatively, there are 2 chaperone molecules per particle. According to the first assumption, the MW of protein

in Golgi-derived particles is about 0.95 x 10^6 Daltons. The MW of protein in ER-derived particles where the chaperone/apoB ratio is several-fold higher, was calculated from the data presented Fig. 3D and found to be about 2.52 x 10^6 Daltons. From the lipid to protein ratio, the MW of lipid in particles derived from Golgi and ER is 2.44 and 1.98 x 10^6 Daltons, respectively. Thus, according to the first assumption, lipid content in Golgi-derived particles is about 23 % higher than that in ER-derived particles.

If calculations are made assuming 2 chaperones per particle, the MW of protein in Golgi and ER-derived particles is 1.36 and 4.52 x 10^6 Daltons, respectively, and that of lipid is 3.50 and 3.55 x 10^6 Daltons, respectively. According to the second assumption, the content of lipid in Golgi-derived particles is similar to that of ER-derived particles. Thus, it is possible that changes in the amount of chaperones per particle can have a noticeable effect on the density of the particle. Although it is conceivable that the number of individual chaperone molecules per particle varies, the calculations shown above give a reasonable estimate of the actual number.

The size of the particles in the ER and the Golgi was calculated from the anhydrous volume (V) of the particle:

$$V_{particle} = 1 \times Mass_{particle} \times 10^{24} / d \times N(\text{Avogadro's number})$$

Assuming a spherical particle, the radius (r) can be calculated from the following equation: $V = 4/3 \times \pi (r)^3$ (r; radius in Å)

The calculated size of particles in the ER and Golgi is 230 and 210 Å diameter, respectively, assuming 1 chaperone molecule/apoB in Golgi or 280 and 244 Å, respectively, assuming 2 chaperone molecules/apoB in Golgi. Since these calculations are based on the volume of an anhydrous sphere, the actual size of these particles is about 20% larger.

Clearly, the size and the density of the particles reflect the fact that there are proteins other than apoB on these particles. According to these calculations, particles in the Golgi seem slightly smaller than those in the ER. This may be due to the fact that the shape of the particles is not a perfect sphere and therefore, the calculations based on a spherical shape may deviate to a certain extent from the actual size. This is probably more pronounced for particles in the ER, which appear to contain several-fold more chaperone proteins compared to Golgi-derived particles. Nonetheless, these calculations give a good estimate of the true size of the hydrated particles particularly, as they are in agreement with those measured by negative-stain electron microscopy (15). Thus, under steady-state conditions, particles of at least LDL or IDL/VLDL size are present both in the ER and in the Golgi. This may indicate that in HepG2 cells the major recruitment of lipids occurs in the ER.

Notably, the density of apoB-containing particles in the Golgi is still substantially higher than that of the secreted particles 40% of which are found in $d < 1.02$ g/ml (Fig. 2B). This is presumably, due to associated chaperones in the Golgi. In search for proteins that might be involved in the second step of VLDL assembly Stillmark et al found that PDI, GRP94, ERp72, BiP and calreticulin co-fractionated with apoB-48-VLDL from rat liver (28). However, the subcelular localization of these particles was not determined.

GRP94 is associated with apoB that had been transported to the TGN

The previous series of experiments clearly demonstrated that ER-resident molecular chaperones are associated with apoB that had been transported to the Golgi complex. However, it was unclear in which compartment within the Golgi these chaperones dissociate from apoB-containing particles. We were interested in determining whether molecular

chaperones interact with apoB that had been transported to distal compartments of the secretory pathway, such as the TGN. To do that, we took advantage of the fact that the enzyme sialyl transferase resides in the TGN, and used lectin blotting to localize apoB. In the experiment depicted in Fig. 7, lumenal contents were subjected to immunoprecipitation with antibodies to GRP94 followed by lectin and immunoblotting. Membranes were incubated either in buffer alone (lanes 1, 2) or buffer containing neuraminidase (lanes 3, 4). Membranes were then probed with the lectin SNA-I that preferentially binds to sialic acid attached to terminal galactose in α–2,6 linkage. Bound SNA-I was visualized following binding of HRP-avidin. A clear band of SNA-I was observed in the sample that was immunoprecipitated with antibodies to GRP94 and was incubated in buffer alone (lane 1), while only a faint band was seen in the control sample in which antibodies were replaced by non-immune serum (lane 2). As expected, incubation of samples with neuraminidase prevented the binding of SNA-I (lane 3), consistent with removal of terminal sialic acid residues by this enzyme. Thus, the binding of SNA-I reflects the presence of sialic acids. Probing of these membranes with antibodies to apoB confirmed that the bands identified with SNA-I represent apoB-100. These findings therefore, are consistent with the notion that at least a fraction of the pool of apoB that was modified by enzymes residing in the TGN was associated with GRP94. Similar results were obtained using antibodies to ERp72 (data not shown). From the relative amount of the sialylated pool of apoB that was co-precipitated with antibodies to GRP94 and from the efficiency of GRP94 immunoprecipitated with these antibodies we estimated that about 15-25% of the sialylated pool of apoB is associated with GRP94. Thus, at least GRP94 and possibly ERp72 remain associated with apoB even after it had been transported to the TGN.

Given the role of ER-resident molecular chaperones in primary quality control in the ER (31,34), the question remains as to what is the role of their prolonged interaction with apoB well beyond the ER? It is generally thought that these chaperones assist in the folding of nascent polypeptides in the ER. The progression of the folding process is very closely monitored by these proteins so that successful folding results in release of the nascent proteins which then exit from the ER and move to the Golgi compartments free of chaperones. However, if correct folding is not achieved, misfolded proteins are retained in the ER bound to these chaperones, which prevent their transport to their target destination. This mechanism is very efficient in mammalian cells, so that only a very small fraction of misfolded proteins escapes this primary quality control (31,34). Backup mechanisms beyond the ER (provided by a different set of proteins (31) ensure that those that escaped the ER are prevented from delivery to their destination. Consistent with such a tight quality control mechanism, it was demonstrated that apoB can be targeted for degradation at different stages of its maturation beyond the ER (15,40,55,68,69). This however, is an unlikely scenario occurring in HepG2 cells under the conditions used in this study, since the cells were incubated under conditions that support optimal folding of apoB resulting in a very efficient secretion. Therefore, a more likely explanation for the prolonged interaction between ER-resident proteins and apoB is that the maturation of IDL/VLDL is an ongoing process that presumably begins in the ER but continues throughout the secretory pathway including distal compartments of the Golgi as demonstrated by a number of investigators (56,57,63,64,70). This includes modifications of apoB such as glycosylation and phosphorylation, remodeling of the phospholipid on the surface of the particles and possibly continued lipidation (71,72). All of these may tag apoB as an incompletely folded (e.g. it is on a path for successful folding), rather than a misfolded

protein, therefore requiring assistance of ER-resident chaperones for its stabilization. Furthermore, molecular chaperones could also facilitate the transport of apoB-containing particles through the secretory pathway, consistent with a proposal that chaperones are also involved in protein trafficking, as demonstrated for Drosophila whereby the cyclophilin homologue, ninaA, appears to be required for rhodopsin Rh1 as it travels through the distal compartments of the secretory pathway (73), while CyPB and HSP47 were suggested to play a role in export of procollagen from the ER as they were found associated with procollagen in the intermediate compartment (74).

ApoB-associated chaperones could dissociate just before apoB VLDL/IDL particles are packaged into secretory vesicles consistent with the finding that the pool of apoB recovered in the light Golgi fraction (Fig.3, Fraction 2) is not associated with chaperones. This fraction presumably contains secretory vesicles (37).

If however, apoB fails to undergo complete maturation either in the early or in distal compartments of the Golgi, these chaperones may then target it for degradation.

In Summary, we demonstrated that a) CyPB directly interacts with apoB and therefore, plays a role in folding and maturation of apoB, b) All 5 major ER-resident molecular chaperones and folding catalysts, GRP94, ERp72, BiP, CRT, and CyPB interact with the membrane-bound pool of apoB to conceivably keep it in a conformation competent for MTP-mediate lipidation. Except for CRT, these chaperones seem to facilitate the translocation of lipidated apoB into the microsomal lumen and to provide stability to the primordial intermediate in the lumen. c) ApoB is transported to the Golgi in complexes containing the same molecular chaperones as those interacting with the lumenal pool of apoB in the ER

except that chaperone/apoB ratio is lower. Thus, apoB leaves the ER as an incompletely-folded intermediate. d) Since GRP94 interacts with apoB that has gone through the TGN it is conceivable that this chaperone (and perhaps others) plays a role in late folding events in the maturation of apoB occurring in distal compartments of the secretory pathway.

On the basis of the data presented in this report we propose a model (Fig. 8) showing that the maturation of apoB is dependent not only on efficient MTP-mediated lipidation, but also on other ER-resident molecular chaperones that seem to play multiple roles in the biogenesis of apoB to form TAG-rich lipoproteins occurring throughout the secretory pathway, including the ER and distal Golgi compartments, contrary to current ideas assigning a role for molecular chaperones primarily in folding events occurring in the ER.

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Footnotes

- This work was supported by National Institute of Health grants HL-58833 and HL-26335.

- **List of abbreviations**

VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; MTP, microsomal triglyceride transfer protein; DMEM, Dulbecco's modified Eagle medium; DPBS, Dulbecco's phospate buffered saline; ALLN, N-Acetyl-L-leucinyl-L-leucinyl-L-norleucinal; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; DSP, Dithiobis(succinimidyl propionate); ECL. Enhanced chemiluminescence; CRT, calreticulin; cyclophilin B, CyPB; CNX, calnexin; TGN, trans-Golgi network.

- **Acknowledgements**

The authors are grateful to Dr. Donald M. Small for critically reading this manuscript and for helpful suggestions. The authors thank Dr. Milne and E. Marcel for the generous gift of the antibodies to apoB, 2D8, Dr. J. Fruchardt, Dr. C. Fievet, and Dr. Bazin for the generous gift of antibodies to apoB, B4, Dr. S. Cala for the generous gift of antiserum to GRP94, Dr. H. Hauri for the generous gift of antibodies to GPP130 and Dr. D. Gordon for the generous gift of BMS-197636. We are indebted to Wei Qiu for technical assistance.

Figure legends

Fig. 1 Molecular chaperones interact both with the membrane-bound and the lumenal pools of apoB.

(A) HepG2 cells were incubated for 2 h with 0.8 mM oleic acid and 25 μg/ml ALLN. Cell monolayers were then incubated with DSP to crosslink cellular proteins. Crude microsomes were prepared and then extracted with either carbonate (lanes 1,2) or carbonate/DOC/K (lanes 3,4). Lumenal contents were separated from membranes, then adjusted to 2% CHAPS and subjected to immunoprecipitation with goat antiserum to human apoB (lanes 1,3) or non-immune goat serum (lanes 2,4) followed by SDS-PAGE and Western blotting. (B) Cells were incubated as above and microsomes extracted with carbonate/DOC. Lumenal contents (lanes 5,6) and membranes (lanes 7,8) were adjusted to 1% SDS in PBS and heated to 65°C for 15 min. The samples were then adjusted to 0.1% SDS with 1% Triton and subjected to immunoprecipitation either with antiserum to apoB (lanes 5,7) or goat non-immune serum (lanes 6,8) followed by Western blotting. ApoB and chaperones were visualized by the ECL system as described under Methods. M, membranes; L, lumen; NI, non-immune

Fig. 2 Lipidated apoB present in the microsomal lumen is associated with molecular chaperones and has a peak density of HDL-like lipoproteins, while secreted apoB fractionates with VLDL/IDL and LDL-like lipoproteins.

HepG2 cells were incubated for 2 h with 0.8 mM OA, then pulse-labeled with

[^{35}S]methinoine/cysteine for 30 min and chased for 2h. Cells harvested after the pulse were subjected to crosslinking, and crude microsomes prepared and extracted with carbonate. Lumenal contents and conditioned media collected after a 2-h chase were adjusted to 1.25 g/ml with solid KBr and subjected to density gradient centrifugation. ApoB in density fractions was immunoprecipitated with antibodies to apoB, resolved by SDS-PAGE, visualized by autoradiography and quantified using Imagequant and expressed as percent of total in lumen (A) and media (B). Chaperones associated with apoB in lumen were identified by Western blotting (C).

Fig. 3 Molecular chaperones are associated with apoB localized to the ER and the Golgi. HepG2 cells were incubated for 2 h with oleic acid and ALLN, subjected to crosslinking, and homogenized as described in methods. Post-nuclear supernatants were adjusted to 1.3M sucrose, overlaid with 1.2, 1.15, 0.8 and 0.25M sucrose and centrifuged for 16 h at 25,000 x g. Fractions were collected, washed and extracted with carbonate/DOC/K. ApoB was immunoprecipitated with polyclonal antibodies to apoB. Marker proteins (A), apoB (B) and associated chaperones (C) were analyzed by immunoblotting. ApoB and molecular chaperones were quantified by densitometry. Chaperone/apoB ratio in the Golgi was expressed as percent of chaperone/apoB in the ER. Values (GRP94, ERp72 and CRT) represent the average of at least 3 experiments. Error bars indicate ± standard error. BiP and CyPB represent the average of 2 experiments

Fig. 4 The effects of MTP inhibitor on subcellular distribution of apoB and associated chaperones. HepG2 cells were incubated for 2 h with OA and ALLN without (lanes 1,2) or

with 100 nM MTP inhibitor BMS-197636 (lanes 3,4). Cells were then processed as in Fig. 3. ApoB and associated chaperones were identified by immunoblotting. G, Golgi

Fig. 5 Molecular chaperones co-immunoprecipitate with apoB in Golgi and ER without prior crosslinking of cellular proteins

Cells were incubated and processed as in Fig. 3 except that cellular proteins were not crosslinked prior to subcellular fractionation. Lumenal contents of Golgi (lanes 1,3,5,6,9,10) and ER (lanes 2,4,7,8,11,12) fractions obtained by carbonate extraction were washed and subjected to immunoprecipitation with monoclonal antibodies to apoB, 2D8 (lanes 1,2) or B4 (lanes 3,4,5,7), or rabbit antiserum to GRP94 (lanes 9,11). Control samples were incubated with non-immune mouse IgGs (lanes 6,8) or non-immune rabbit serum (lanes 10,12) under native conditions. Immunocomplexes were resolved on SDS-PAGE and apoB and molecular chaperones identified by immunoblotting. Three representative experiments are shown (Panels A, B and C). In Panel A, all lanes are from the same exposure time. In Panels B and C, Golgi and ER should not be compared directly as they are from different exposure times. Samples immunoprecipitated with antibodies and the corresponding controls within the same compartment are from the same exposure time and can be compared to each other. G, Golgi; E, ER; NI, non immune (IgG or serum)

Fig. 6 Density distribution of apoB in Golgi and ER. Cells were incubated, subjected to crosslinking, homogenized and subjected to subcellular fractionation as in Fig. 3. Golgi and ER were extracted with carbonate/DOC/K and lumenal contents adjusted to 1.25 g/ml with solid KBr and subjected to density gradient centrifugation. Twelve fractions were collected

from the top and density measured as described in Methods. Fractions were combined as follows: fraction # 1, d ≤ 1.02 g/ml; fraction #2, 1.02 < d < 1.08 g/ml; fraction #3, 1.08 < d < 1.21 g/ml and fraction # 4, d > 1.21 g/ml. ApoB in combined fractions was immunoprecipitated and quantified by densitometry as described in Fig. 3. (A) Distribution of apoB density fractions expressed as percent of total apoB in Golgi (squares) and ER (circles). (B) An immunoblot showing apoB and associated molecular chaperones in Golgi (left) and ER (right).

Fig. 7 GRP94 is associated with apoB that has gone through TGN. Cells were incubated with OA and ALLN, and then homogenized without prior crosslinking of proteins, and microsomes prepared and extracted with carbonate. Lumenal contents were then subjected to immunoprecipitation with rabbit antibodies to GRP94 (lanes 1,3) or non-immune serum (lanes 2,4) and immunocomplexes in duplicates resolved by SDS-PAGE and electro-transferred onto a membrane. Membrane was blocked with albumin and then one half was incubated with buffer alone (lanes 1,2) and the other half with buffer containing neuramindase (lanes 3,4) for 20 h at 37ºC. Both halves were then probed sequentially with the lectin SNA-I followed by antibodies to apoB. Bound lectin was visualized by extravidin-HRP. NIS, non-immune serum.

Fig. 8 A proposed model for chaperone-assisted folding of apoB to form lipoproteins

Molecular chaperones interact with apoB as it emerges through the translocon (shown in red) into the ER lumen. Following elongation, domains which are presumably enriched in amphipathic β–sheets interact with the membrane (43,44) cotranslationally, where initiation

of MTP-mediated lipidation leads to the formation of a small core (shown in yellow) (step I). Ongoing translation-coupled lipidation leads to elongation, enlargement of the core and recruitment of additional molecular chaperones (step II). Recruitment of additional neutral lipid molecules leads to completion of translation (41) followed by dissociation of the ribosome (III). At this stage enough lipids had been recruited and the lipidated intermediate begins to detach from the ER membrane. It appears however, that in order for it to completely detach, additional chaperone molecules, GRP94, ERp72 and CyPB, but not CRT are recruited to facilitate the release presumably by replacing the hydrophobic interactions with the membrane and to stabilize the primordial intermediate following its release into the lumen (step IV). This intermediate recruits more core lipids to form a larger particle (step V). It is then transported to the Golgi concomitantly with the release of about 20-50% of GRP94, ERp72 and calreticulin, and over 90% of BiP and CyPB (Step VI). The ER-resident chaperone proteins remain associated with apoB-containing lipoproteins in Golgi while additional modifications take place (step VII). Ultimately, all chaperones dissociate presumably before packaging into secretory vesicles (VIII) and nascent VLDL is secreted.

Figure 1

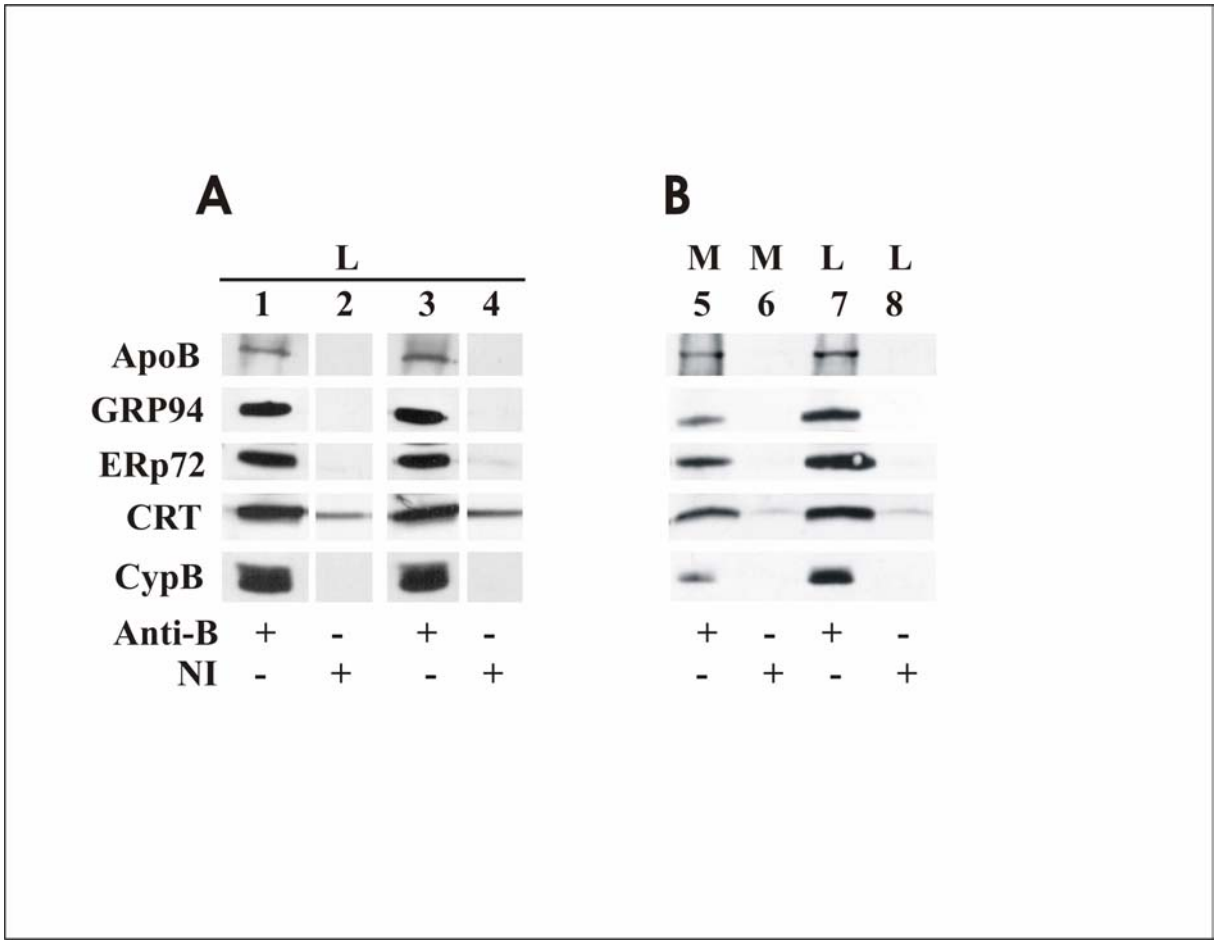

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Figure 2

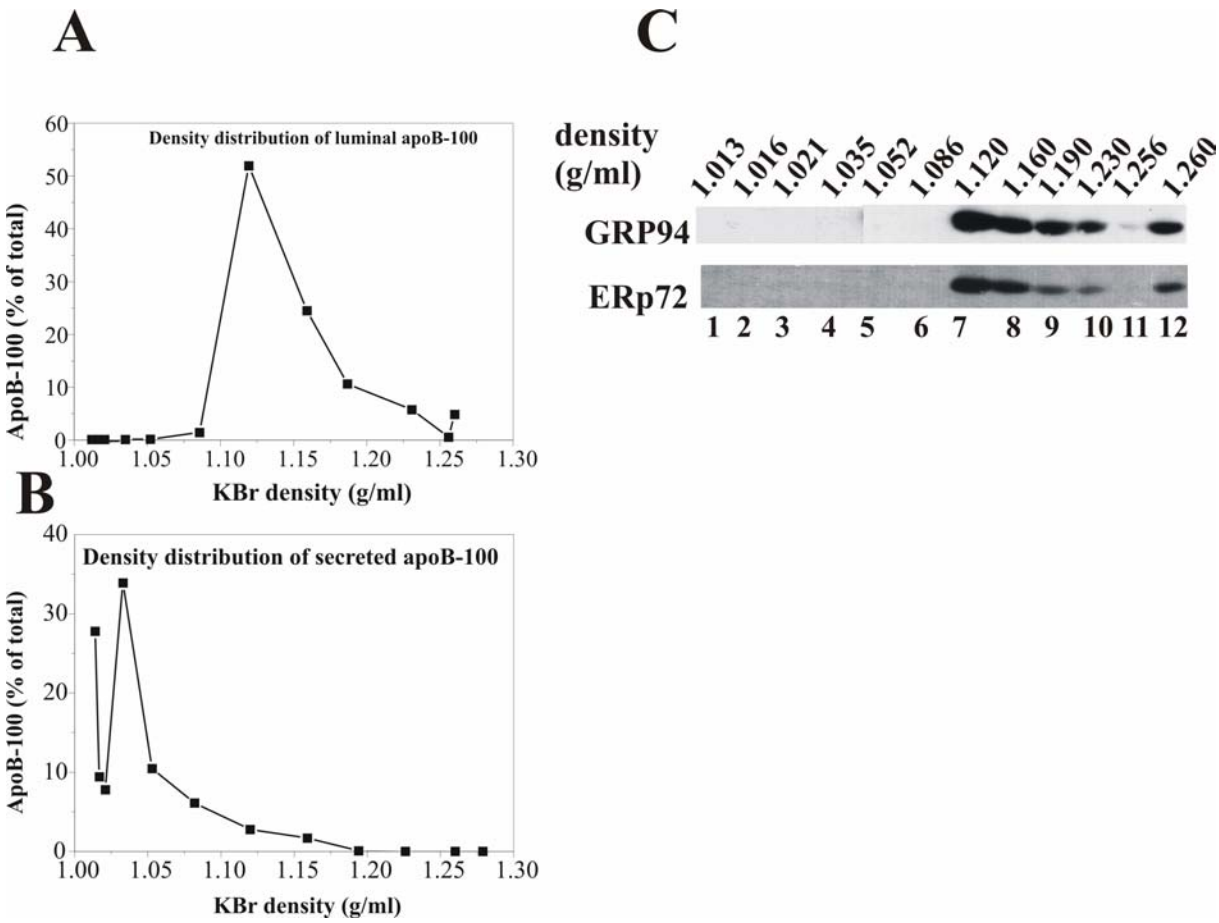

A
Density distribution of luminal apoB-100
ApoB-100 (% of total)
KBr density (g/ml)
B
Density distribution of secreted apoB-100
ApoB-100 (% of total)
KBr density (g/ml)
C
density (g/ml)
1.013
1.016
1.021
1.035
1.052
1.086
1.120
1.160
1.190
1.230
1.256
1.260
GRP94
ERp72
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3

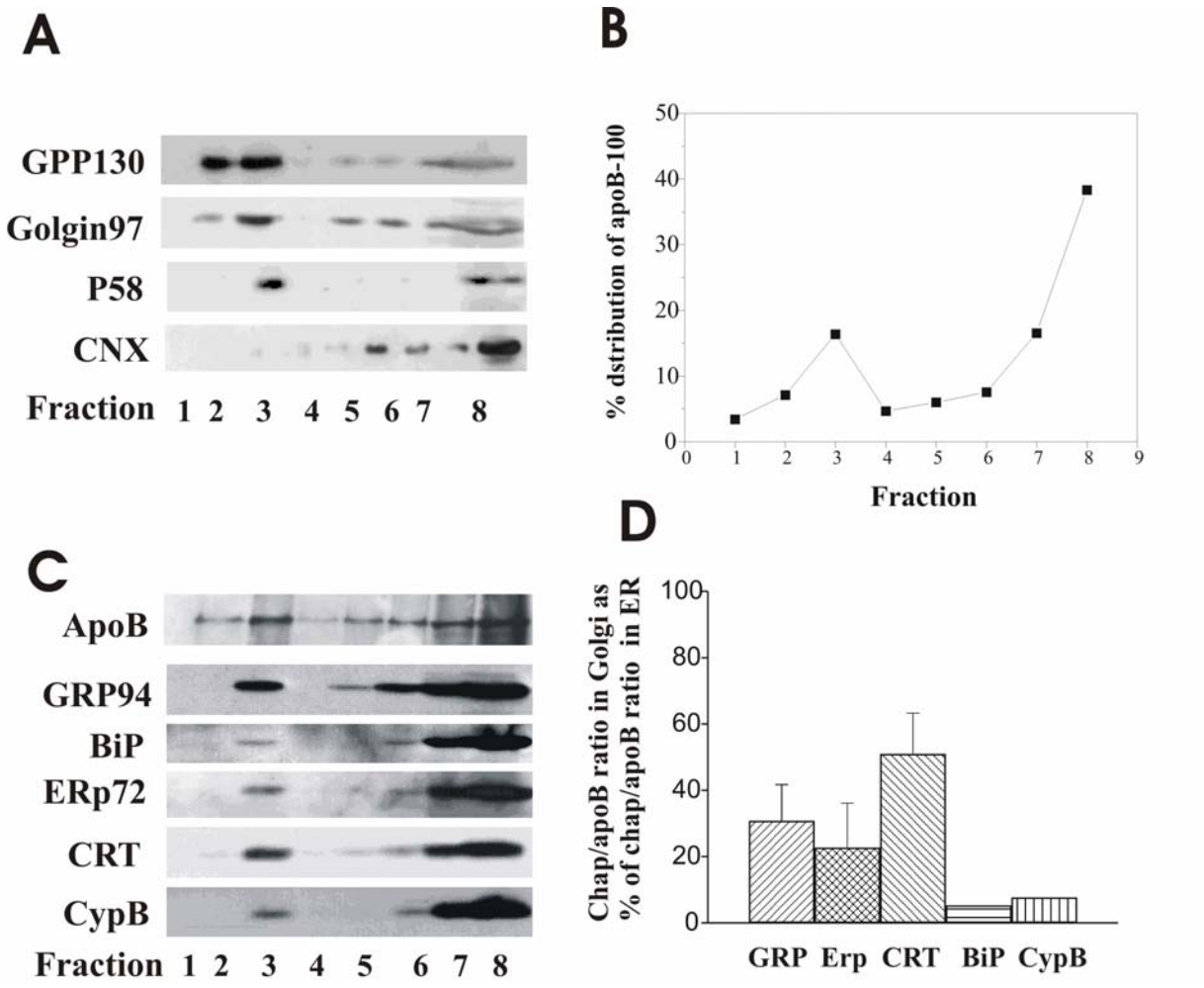

A
GPP130
Golgin97
P58
CNX
Fraction 1 2 3 4 5 6 7 8
B
% dstribution of apoB-100
Fraction
C
ApoB
GRP94
BiP
ERp72
CRT
CypB
Fraction 1 2 3 4 5 6 7 8
D
Chap/apoB ratio in Golgi as % of chap/apoB ratio in ER
GRP Erp CRT BiP CypB

Figure 4

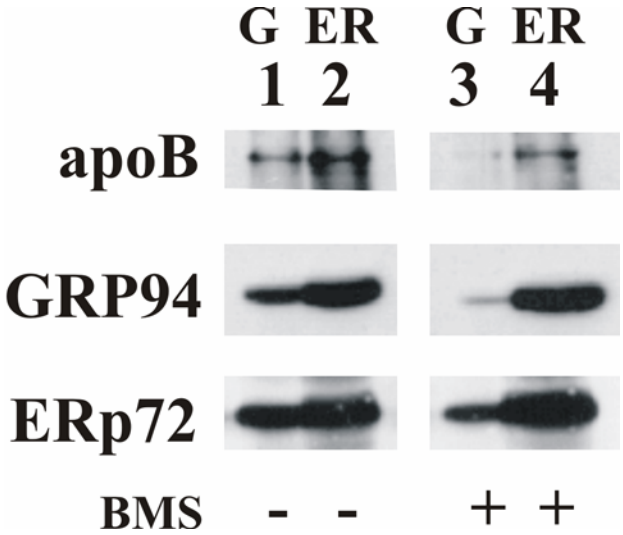


Figure 5

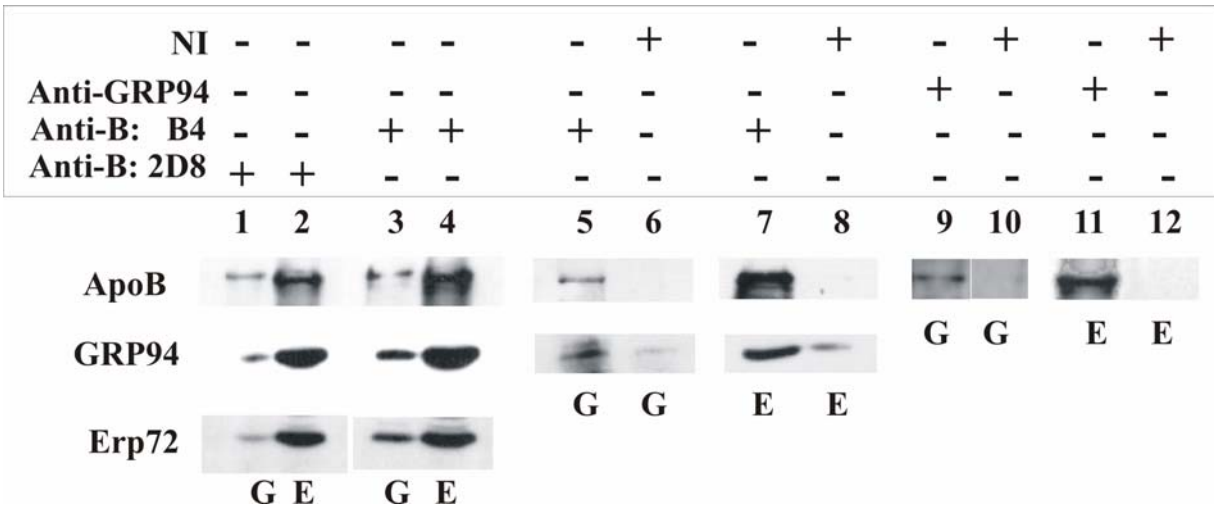

NI - - - - - + - + - + - +
Anti-GRP94 - - - - - - - - + - + -
Anti-B: B4 - - + + + - + - - - - -
Anti-B: 2D8 + + - - - - - - - - - -
1 2 3 4 5 6 7 8 9 10 11 12
ApoB
GRP94
Erp72
G E G E
G G E E
G G E E

Figure 6

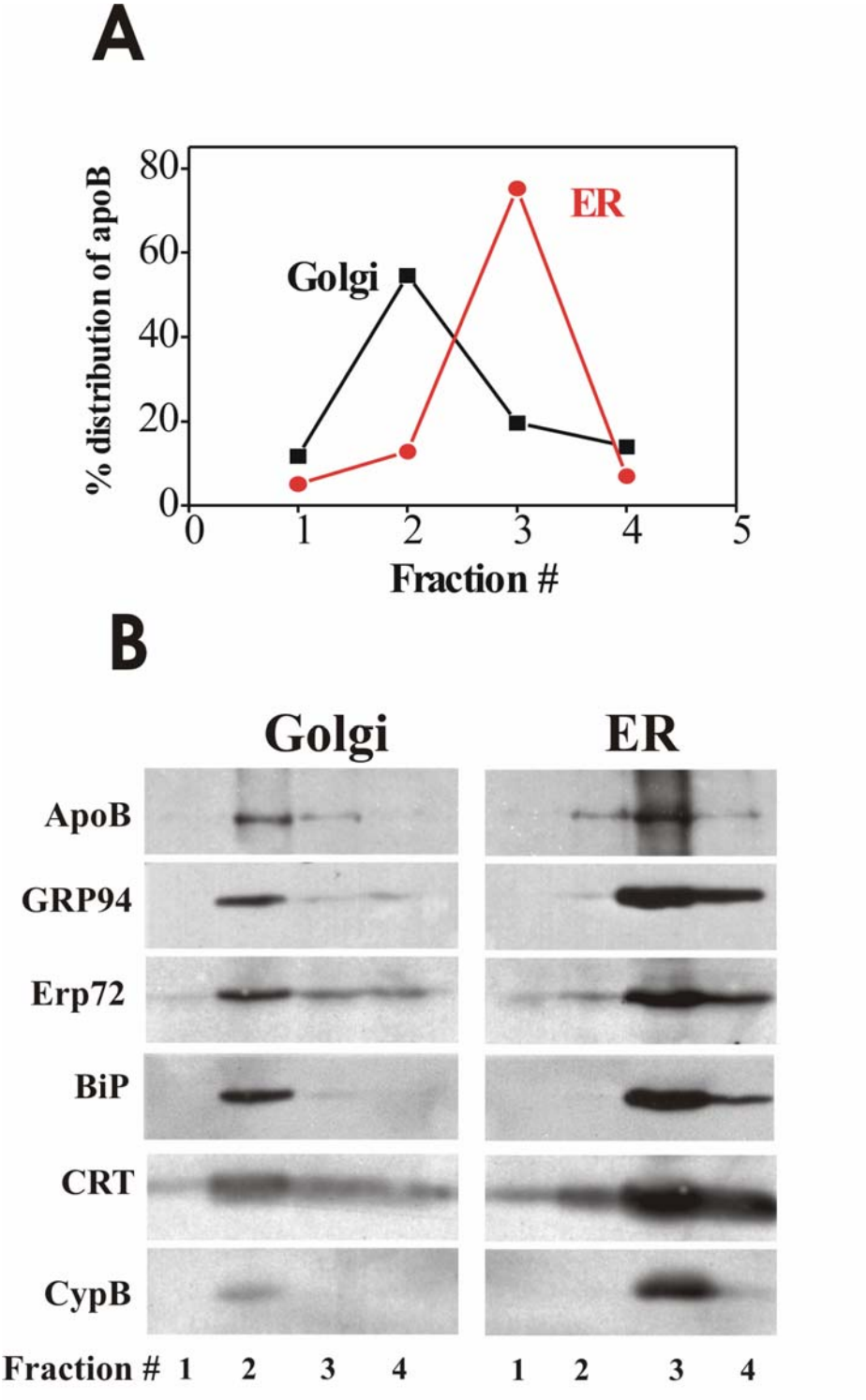

A
% distribution of apoB
80
60
40
20
0
0
1
2
3
4
5
Fraction #
Golgi
ER
B
Golgi
ER
ApoB
GRP94
Erp72
BiP
CRT
CypB
Fraction # 1 2 3 4 1 2 3 4

Figure 7

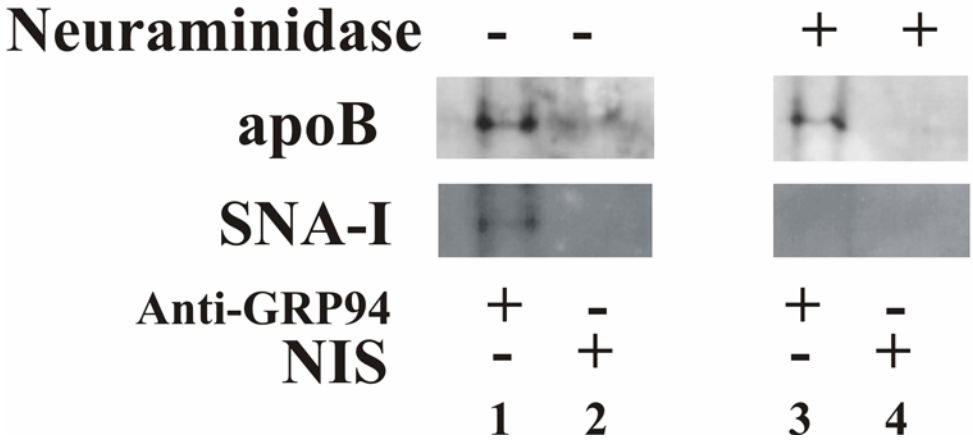
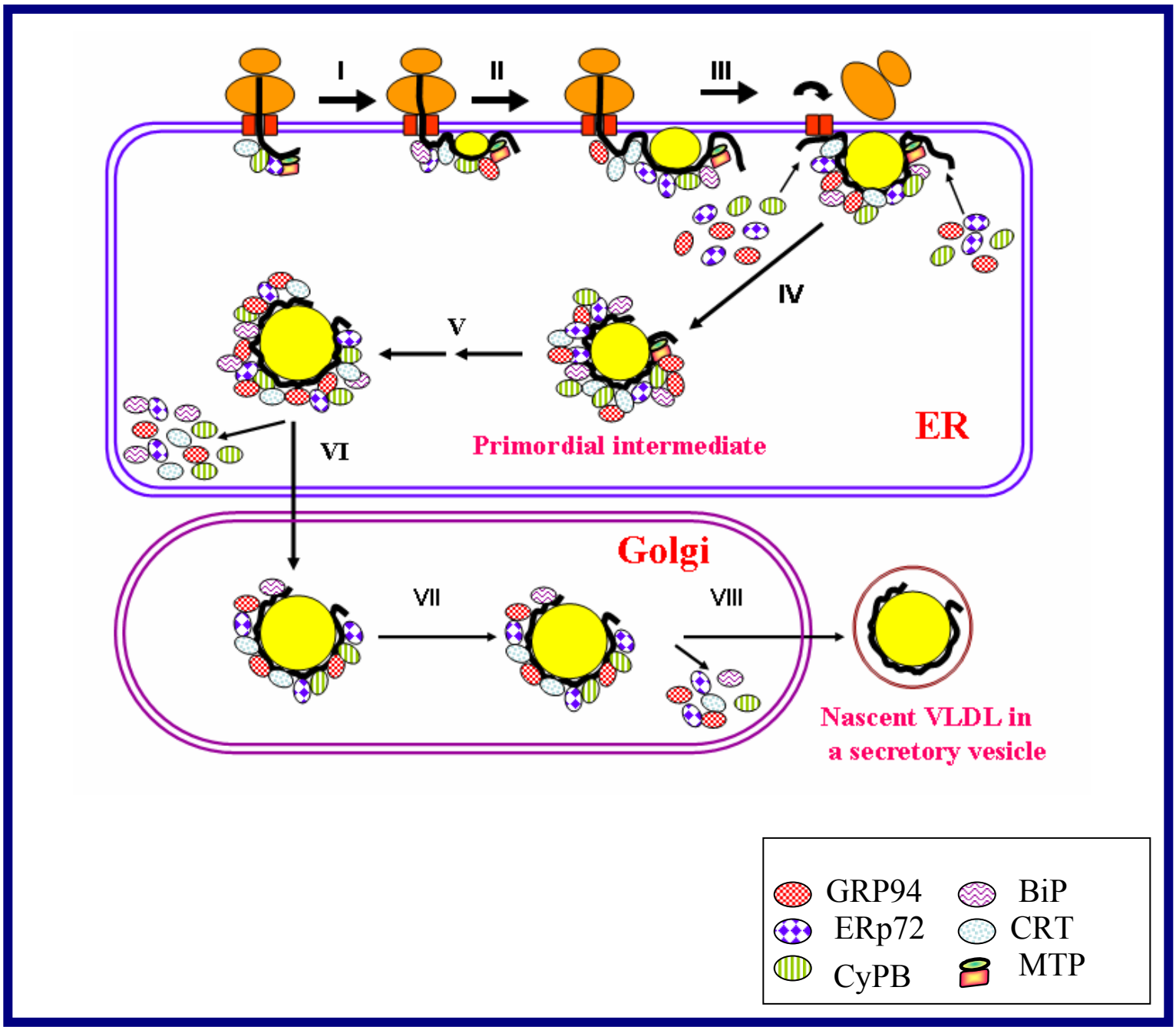


Figure 8


I
II
III
IV
V
VI
VII
VIII
ER
Golgi
Primordial intermediate
Nascent VLDL in
a secretory vesicle
GRP94
ERp72
CyPB
BiP
CRT
MTP