Multiple Molecular Chaperones Interact with Apolipoprotein B during Its Maturation

THE NETWORK OF ENDOPLASMIC RETICULUM-RESIDENT CHAPERONES (ERp72, GRP94, CALRETICULIN, AND BiP) INTERACTS WITH APOLIPOPROTEIN B REGARDLESS OF ITS LIPIDATION STATE*

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The present study was undertaken to identify and characterize molecular chaperones that assist in the folding of apolipoprotein (apo) B, a secretory protein that requires assembly with lipids (lipidation) for its secretion. Both HepG2 cells, normally secreting full-length apoB (apoB-100), and C127 cells transfected to secrete truncated forms of apoB, apoB-41, apoB-29, and apoB-17, respectively, were employed. C127 cells were used to determine whether chaperone binding is dependent on apoB lipidation as they secrete both unlipidated and lipidated apoB forms despite their lack of microsomal triglyceride transfer protein (MTP), which mediates lipidation of apoB in HepG2 cells. The endoplasmic reticulum (ER)-resident molecular chaperones GRP94, calreticulin, and ERp72 were co-immunoprecipitated with apoB-100 from HepG2 cell lysates following cross-linking of proteins in living cells. The same chaperones including BiP/GRP78 were also associated with all truncated forms of apoB. Sequential immunoprecipitation with antibodies to MTP and apoB revealed the presence of ternary complexes containing apoB-100, MTP, and ERp72. However, MTP is not obligatory for the binding of ERp72 as it was associated with all truncated forms of apoB in C127 cells that lack MTP. The interactions between apoB-100 and ERp72 or GRP94 persisted for at least 2 h following a 30-min pulse. Thus, BiP/GRP78, calreticulin, ERp72, and GRP94 may participate in critical steps in the folding of apoB before any substantial lipidation occurs. ERp72 and GRP94 may also mediate the folding of more advanced folding intermediates and/or target the misfolded underlipidated pool of apoB for degradation.

Apolipoprotein B (apoB) is an atypical secretory protein. Its folding into a mature, secretion-competent form necessitates assembly with triacylglycerols (TAG) and phospholipids to produce TAG-rich lipoproteins such as very low density lipoproteins and chylomicrons. A major fraction of nascent apoB is degraded intracellularly when lipid availability is limited or when lipidation of apoB is inhibited. It was proposed that distinct amphiphilic elements in the secondary structure of apoB may initiate the binding of lipids to apoB co-translationally. Further, growing evidence suggests that the process of apoB maturation in the cell is mediated by molecular chaperones, which by binding nascent polypeptides prevent their aggregation and direct their folding into their mature, native form. Indeed, the recruitment of lipids to the nascent lipoprotein is significantly aided by microsomal triglyceride transfer protein (MTP) localized to the luminal surface of the endoplasmic reticulum (ER). To identify chaperones that interact with apoB, we used human hepatic-derived HepG2 cells, besides MTP (10), three other ER-resident molecular chaperones, calnexin, ER60/ER57, and BiP/GRP78, have been demonstrated to interact with apoB (11, 12). Calnexin, an integral membrane protein, initially identified as ubiquitin-dependent protein-binding protein, is the ER homologue of the cytosolic Hsp70 chaperone, and is thought to interact with unshielded hydrophobic domains of unfolded proteins (18). BiP has been suggested to facilitate the movement of nascent polypeptides through the translocon into the ER lumen (19) as well as the retrograde transport of misfolded proteins from the ER into the cytosol for proteasomal degradation (20). In a recent study in HepG2 cells, the binding of the cytosolic chaperone Hsp70 to apoB-100 was linked to ubiquitin-dependent proteasomal degradation (21).

In this report, we investigate whether other highly abundant ER-resident proteins known to function as molecular chaperones participate in the folding of apoB, and whether the chaperone-apoB interactions are dependent on the state of apoB lipidation. To identify chaperones that interact with apoB, we used human hepatic-derived HepG2 cells normally secreting the full-length apoB-100, as well as stably transfected C127 cells derived from murine mammary gland secreting truncated forms of apoB corresponding to the N-terminal 41, 29, and 17% of apoB-100, designated apoB-41, apoB-29, and apoB-17, respectively. The transfected C127 cells were chosen as a model
to study the dependence of the interactions on the lipidation state of apoB for two reasons: 1) these cells are devoid of endogenous apoB and MTP, and 2) apoB-41 is secreted by these cells exclusively on high density lipoprotein-like particles (22), whereas apoB-292 and apoB-17 (23) require little or no lipid to be secreted.

Here, we report that three other ER-resident molecular chaperones interact with apoB: calreticulin, ERP72, and GRP94. Calreticulin, a major calcium-binding protein, has been well documented to function as a molecular chaperone which can substitute for calnexin (reviewed in Ref. 24). However, the roles of ERP72, a member of the thio-redoxin family (25), and GRP94, a stress protein homologous to the cytosolic Hsp90 (26), in protein folding are poorly understood. Both proteins exhibit specific binding to various denatured proteins in vitro (27). They have also been found to associate intracellularly with nascent polypeptides such as thyroglobulin (28), thrombospondin (29), major histocompatibility complex class II molecules (30), and human chorionic gonadotropin α subunit (31). Further, their transcription can be induced under stress conditions (32, 33). Our results indicate that GRP94 and ERP72 interact with both early and more advanced folding intermediates of apoB and presumably mediate its folding into a secretion-competent form. All tested chaperones were found to interact with apoB whether it is lipidated or not.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum were obtained from Life Technologies, Inc. [35S]Methionine/cysteine ExprEase™S2S protein labeling mix with specific activity >1,000 Ci/mmol and reagents for enhanced chemiluminescent detection system (ECL) were from NEN Life Science Products. Goat polyclonal antibodies to human apoB were purchased from Biodesign. Rabbit polyclonal antibodies to murine ERP72 was kindly provided by Dr. Michael Green (St. Louis University, St. Louis, MO). Rat monoclonal antibodies to GRP94, rabbit polyclonal antibodies to BiP, rat monoclonal antibodies to calnexin, and rabbit polyclonal antibodies to calreticulin were obtained from StressGen Biotechnologies. Goat polyclonal antibodies to Hsc70, and rabbit polyclonal antibodies to GRP94, rabbit polyclonal antibodies to BiP, rat monoclonal antibodies to calnexin, and Hsp70) have been shown to co-immunoprecipitate with their substrates following non-denaturing solubilization of cells in buffers containing such detergents as CHAPS, sodium cholate, Triton X-100, or Nonidet P-40 (11, 28, 36, 37). However, some weak interactions between chaperones and their substrates were still disrupted by these detergents (28, 34). Therefore, additional manipulations were required to prevent the release of chaperones from their substrates. One such manipulation is based on the property of some chaperones to release their substrates upon binding of ATP (6, 7, 38) in the presence of divalent cations (29). Since little is known about chaperone-assisted folding of apoB (Fig. 1). The following buffers were used: lysis buffer, pH 7.4, 0.15 M NaCl, 1% CHAPS, same buffer supplemented with 0.5 M NaCl, and finally, PBS. When sequential immunoprecipitation was carried out, washed beads were heated to a 100 °C for 5 min in 1% SDS in PBS, diluted 5-fold with 1% Triton X-100 in PBS, incubated with a second antibody overnight, and recaptured on protein G-Sepharose beads. Beads were washed with 1% Triton X-100 and 0.5 M NaCl in PBS, and then heated at 100 °C for 5 min in sample buffer (35) containing 10% β-mercaptoethanol and 8 M urea and analyzed by 4–7% gradient SDS-polyacrylamide gel electrophoresis modified from Laemmli (35) followed by autoradiography using a PhosphorImager (Molecular Dynamics). For Western blot analysis, proteins were electrotransferred onto an Immobilon-P membrane, probed with respective antibodies, and visualized by the ECL technique (according to the manufacturer’s instructions).

RESULTS AND DISCUSSION

Identification of Chaperones That Interact with ApoB-100 in HepG2 Cells—Molecular chaperones bind to nascent polypeptides and mediate their folding into their mature native forms. Because these interactions are transient in nature, it is necessary to solubilize cells under conditions that preserve chaperone-substrate complexes. Indeed, some chaperones (e.g. BiP, calnexin, and Hsp70) have been shown to co-immunoprecipitate with their substrates following non-denaturing solubilization of cells in buffers containing such detergents as CHAPS, sodium cholate, Triton X-100, or Nonidet P-40 (11, 28, 36, 37). However, some weak interactions between chaperones and their substrates were still disrupted by these detergents (28, 34). Therefore, additional manipulations were required to prevent the release of chaperones from their substrates. One such manipulation is based on the property of some chaperones to release their substrates upon binding of ATP (6, 7, 38) in the presence of divalent cations (Mg2+, Ca2+, etc.). Thus, reducing the level of ATP and/or divalent cations during lysis of cells may prevent the release of these chaperones and thereby enhance their detection (28, 36). However, for chaperones that are ATP-independent, or when chaperone-substrate interactions are still disrupted, cross-linking of proteins could be required to preserve the integrity of chaperone-substrate complexes (28, 34, 39). Since little is known about chaperone-assisted folding of apoB, it was important to establish optimal lysis conditions that would allow us to detect as many apoB-associated chaperones as possible. To that end, HepG2 cells were pulse-labeled for 30 min and then subjected to various lysis conditions followed by immunoprecipitation with polyclonal antibodies to apoB (Fig. 1). The following buffers were used: lysis buffer (2% CHAPS in HEPESS-buffered saline) (lanes 1 and 7), lysis buffer containing: (a) EDTA and EGTA to chelate divalent cations (lanes 2 and 8), (b) an ATP-hydrolyzing enzyme, apyrase (lanes 9 and 3), or (c) ATP and MgCl2 (lanes 4 and 10). The membranes-permeable homobifunctional cleavable cross-linking reagent DSP was utilized in two methodologically distinct approaches: 1) during the first 30 min of lysis (lanes 5 and 11); and 2) prior to lysis, i.e. in situ cross-linking in living cells, to probe for in vivo associations of chaperones with apoB before lysis of cells

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that could potentially disrupt apoB-chaperone complexes (lanes 6 and 12).

Fig. 1 shows that the ER-resident chaperones: GRP94, the human homologue of murine ERp72 and calreticulin, and the cytosolic chaperone Hsc70, were co-immunoprecipitated with apoB (lanes 1–6). The control samples that were incubated in non-immune serum showed either no bands or bands with lower intensities of the corresponding chaperones (lanes 7–12). The levels of chaperones that co-immunoprecipitated with apoB varied substantially under different lysis conditions (Fig. 1), despite a relatively small variability (about 30%) in the efficiency of immunoprecipitation of radiolabeled apoB. The highest levels of GRP94 and ERp72 co-immunoprecipitated with apoB-100 were detected upon in situ cross-linking of proteins (lane 6). The lysis buffer seemed to promote the dissociation of a significant fraction of these apoB-bound chaperones even before the cross-linking reaction could take place (compare lane 5 to lane 6). Because these chaperones were cross-linked to apoB in living cells, we conclude that they interact with apoB in vivo. Kuznetzov et al. (28, 29) also observed that the interactions between GRP94 or ERp72 and thyroglobulin and thrombospondin in thyocytes were disrupted by lysis buffer, and cross-linking of proteins in situ enhanced their detection. Similarly, detection of GRP94 associated with immunoglobulin L chain in myeloma cells also required cross-linking of cellular proteins (39).

GRP94 was found to require ATP for its release from apoB-100, as higher amounts of GRP94 were co-immunoprecipitated with apoB upon a reduction of the ATP levels by the ATP-hydrolyzing enzyme apyrase (Fig. 1, lane 3). Lower levels of GRP94 were co-immunoprecipitated with apoB from the unsupplemented lysates or from the lysates supplemented with ATP (lanes 1 and 4, respectively). Thus, the unsupplemented lysates had sufficient levels of ATP should release of GRP94 occur. In contrast to our study, Melnick et al. (39) reported that a reduction of the intracellular levels of ATP in myeloma cells led to the release of GRP94 from immunoglobulin L chains. Despite this discrepancy, both our study and that of Melnick et al. suggest that GRP94 may be an ATP-binding protein. However, the binding of ATP to GRP94 also purported in other studies (40–42) was challenged recently by Wearch and Nichitta (43), who suggested that the binding and hydrolysis of ATP is not an inherent property of GRP94. However, ATP-binding sites were predicted by sequence analysis of GRP94 (40, 42) and inferred from the crystal structure of its cytosolic homologue Hsp90 (44). In light of this controversy, it remains undetermined whether the release of GRP94 from apoB occurs upon binding of ATP to GRP94 itself or to other proteins that may be present in the complexes. This idea is consistent with the proposal that chaperones in the ER form large heterocomplexes with their substrate polypeptides (45). Further, modulation of the “chaperone machinery” by accessory proteins has been documented for cytosolic chaperones (46, 47). Thus, the opposite effects observed following reduction of ATP levels on the interaction between GRP94 and apoB-100 or immunoglobulin L chains may be attributed to differences in the composition of chaperones present in GRP94-apoB-100- and GRP94-immunoglobulin L chain-containing complexes.

Unlike GRP94 and ERp72, the ER-lumenal chaperone calreticulin and the cytosolic chaperone Hsc70 (the constitutively expressed form of Hsp70) could not be detected after cross-linking (Fig. 1, lanes 5 and 6); however, they were observed under other conditions. Calreticulin was found to be elevated in the presence of the chelating agents EDTA and EGTA (lane 2), suggesting that the release of calreticulin from apoB was restricted when divalent cations in the lysates were sequestered. In contrast, the membrane-bound homologue of calreticulin, calnexin, appeared to require divalent cations (presumably Ca$^{2+}$) for its association with a secretion-incompetent variant of α1-antitrypsin (49). Zn$^{2+}$, on the other hand, was shown to cause dissociation of calreticulin-PDI complexes in vitro (50). Further proof for the interaction between calreticulin and apoB-100 in HepG2 cells was obtained in a separate experiment where nascent apoB was co-precipitated by antibodies to calreticulin following in situ cross-linking (data not shown). This implies that calreticulin interacts with apoB in vivo, and that the amount of calreticulin cross-linked to apoB on the presented blot was below a detectable level (lane 6).

The cytosolic Hsc70 was detected only when the concentration of ATP in the lysate was reduced by apyrase (lane 3). The ATP-dependent release of Hsp70 from its substrates has been demonstrated in other systems (38). The association of Hsc70 with apoB was further corroborated in a separate experiment, in which a 4-fold greater number of cells were pooled, subjected to in situ cross-linking, lysed, and then immunoprecipitated with antibodies to apoB. Hsc70 was detected by immunoblotting. Fisher et al. (21) have shown that transfection of HepG2 cells with cDNA encoding Hsp72 (the inducible form of Hsp70) led to increased ubiquitin-dependent degradation of apoB-100. However, in their study, antibodies recognizing both forms of Hsp70 were used, making it difficult to conclude whether only one or both forms are involved (21). Our findings provide evidence that Hsc70 interacts with apoB-100 and, therefore, like its inducible counterpart, may direct the misfolded pool of apoB for degradation. This proposal is consistent with a recent report implicating Hsc70 in the ubiquitin-dependent degradation of certain protein substrates in vitro (50).

The Interactions between ApoB-100 and GRP94 or ERp72 Persist for at Least 2 h—In the experiment shown in Fig. 1, the chaperones identified by Western blotting were presumably associated with both nascent apoB synthesized during the 30-min pulse and the pool that was present in the cells at the beginning of the pulse. A significant fraction of this pre-existing pool could be targeted for degradation. It was important to determine what fraction of the nascent apoB pool, which represents relatively early folding intermediates, was associated with these chaperones, and how long these chaperones associ-
samples, antibodies were omitted (panel A, panel B). Radiolabeled apoB-100 (indicated by arrowheads) was co-precipitated from the lysates with either antibodies to GRP94 (panel A, lanes 1 and 2), or antibodies to ERp72 (panel B, lanes 1 and 2). In the control samples, antibodies were omitted (panel A, lane 3) or rabbit non-immune serum was used (panel B, lanes 3 and 4).

To address these questions, we determined whether antibodies directed against GRP94 or ERp72 could co-precipitate nascent apoB. We first established that the antibodies to GRP94 and ERp72 do not cross-react with apoB using the following protocol. HepG2 cells were pulse-labeled for 30 min and then denatured by heating to 100 °C in 1% SDS with or without 20 mM dithiothreitol to release all associated proteins (31). Under these conditions neither antibody co-precipitated nascent apoB-100 (data not shown). Next, HepG2 cells were pulse-labeled for 30 min and then chased for 2 h. Following cross-linking of cellular proteins in situ, cells were lysed and subjected to immunoprecipitation with antibodies directed against GRP94, ERp72, or apoB. Fig. 2 shows that apoB-100 was co-precipitated by antibodies to either GRP94 or ERp72 both after the pulse and after a 2-h chase. Thus, these chaperones interact with apoB-100 relatively early during its biogenesis, and further, these interactions persisted for at least 2 h into the chase (Fig. 2, A and B). The percentage of the intracellular nascent apoB associated with either chaperone was similar at 0 and 2 h (at least 7 and 3% of the radiolabeled pool of apoB was co-precipitated by antibodies to ERp72 and GRP94, respectively). However, in the same time frame, the percentage of apoB bound to calnexin declined 4–5-fold (data not shown). These results were also confirmed by sequential immunoprecipitation. Therefore, GRP94 and ERp72 may play a role in both early and more advanced events in the folding of apoB and its assembly with lipids. Whereas no kinetic studies have been published on ERp72, Melnick et al. (39) demonstrated that GRP94 has a greater avidity toward more advanced folding intermediates of immunoglobulin L chains, whereas BiP and calnexin were shown to bind to very early folding intermediates and leave their substrates shortly thereafter, prior to the completion of folding (37, 39). However, if folding into a native form was not achieved, both BiP and calnexin remained bound to their substrates (11, 51) and the misfolded polypeptides were targeted for degradation (51). The prolonged association of GRP94 and ERp72 with apoB-100 suggests that these chaperones may target the misfolded apoB for degradation. Although a role for GRP94 in protein degradation has not been elucidated, its cytosolic homologue Hsp90 was suggested to facilitate protein degradation under certain conditions (52). ERp72, on the other hand, has been shown to possess a proteolytic activity that is sensitive to the inhibitor ALLN (53). Since ALLN blocks degradation of apoB-100 very efficiently, it is possible that ERp72 may be involved in the degradation of a subpopulation of misfolded apoB.

**MTP Interacts with Both GRP94 and ERp72 and Is Found in Ternary Complexes with Nascent apoB and ERp72**—The folding of apoB in the ER into a secretion-competent form is unique since it involves co-translational assembly with lipids. The ER-resident protein MTP plays an important role in the lipidation of apoB since it has been shown to catalyze the transfer of TAG and phospholipids in vitro, and mutations in the MTP gene or inhibition of MTP activity result in a substantial or complete block of apoB secretion (8). Furthermore, secretion of truncated forms of apoB corresponding to N-terminal 29% or longer in heterologous expression systems required co-transfection with MTPL (8). Thus, it was important to determine whether GRP94 and ERp72 interact with apoB in tandem with MTP. To that end, HepG2 cells were pulse-labeled for 30 min and cellular proteins were cross-linked in lysis buffer. This cross-linking protocol was preferred because it resulted in cleaner controls. Cross-linked lysates were subjected to immunoprecipitation with antibodies directed against MTPL. Fig. 3A shows that both GRP94 and ERp72 were co-immunoprecipitated with MTPL. The antibodies to MTPL did not co-precipitate either chaperone from C127 cell lysates that lack MTP; therefore we conclude that in HepG2 cells MTP interacts with both chaperones in vivo. Next, sequential immunoprecipitation of cross-linked cell lysates following a 30-min pulse was performed, first with antibodies to MTPL and then with antibodies to apoB followed by immunoblotting. Fig. 3B shows that ERp72 was detected in these samples demonstrating the presence of ternary complexes containing nascent apoB-100, ERp72, and MTP. These findings suggest that the putative function of ERp72 as disulfide isomerase may be coupled with the initiation of lipidation of early folding intermediates of apoB. Unlike ERp72, GRP94 was not detected in complexes containing both apoB and MTP upon sequential immunoprecipitation.

**Identification of Chaperones That Interact with Truncated Forms of ApoB in C127 Cells**—To address the question of whether the binding of chaperones to apoB is determined by the state of the apoB lipidation, we analyzed truncated forms of apoB expressed by transfected C127 cells. Despite the absence of MTP in these cells, they secrete apoB-41 exclusively on high density lipoprotein-sized TAG-rich particles. Moreover, apoB-41 in C127 and apoB-100 in HepG2 cells are similar in that their levels of secretion can be modulated by lipid availability (3, 22). Approximately 52% of the total mass of apoB-41-containing particles is lipid, whereas the shorter truncated form of apoB, apoB-29, is secreted with only 26% lipid, most of which is phospholipids and diacylglycerols. The shortest form, apoB-17, is efficiently secreted without any appreciable amount of lipid (23). Lysis conditions were selected based on the results obtained in HepG2 cells. Lysates were subjected to immunoprecipitation with antibodies to apoB. The membranes were probed to the chaperones identified in HepG2 cells as well as BiP, which has recently been reported to interact with apoB-100 in HepG2 cells (12).

The ER-lumenal chaperones GRP94, ERp72, and calreticulin, as well as BiP, were found to interact with all three truncated forms of apoB (Figs. 4A, 5, and 6). GRP94, ERp72, and...
Fig. 4. Panel A, molecular chaperones co-precipitated with apoB-41 in C127 cells. Cells were lysed under various conditions. Lysates were immunoprecipitated with antibodies to apoB (lanes 1–4) or non-immune goat serum in the control samples (lanes 5–7). Immunoprecipitated complexes were analyzed by Western blotting with antibodies against respective chaperones. The following lysis conditions were used: lanes 1 and 5, plain lysis in CHAPS; lanes 2 and 6, lysis in the presence of 5 mM EDTA and 5 mM EGTA; lane 3, lysis in the presence 50 units/ml apyrase; lanes 4 and 7, in situ cross-linking with 0.2 mM DSP prior to lysis in CHAPS. Panel B, ERp72 interacts with nascent apoB-41 in C127 cells. Cells were lysed in the presence of the cross-linking reagent DSP. Lysate was sequentially immunoprecipitated first with antibodies to apoB (lane 1), or with non-immune goat serum in control (lane 2) and then with anti-ERp72 (lanes 1 and 2).

BiP were cross-linked in situ to all three forms (Fig. 4A, lane 4; Fig. 5, lane 2; Fig. 6, lane 3), while calreticulin was cross-linked to apoB-41 and apoB-17. (Fig. 4, lane 4; Fig. 6, lane 3). Thus, we conclude that these chaperones interact with truncated forms of apoB in vivo, and their interaction with apoB is independent of the lipidation state of apoB. Therefore, these chaperones may be involved in early steps of apoB folding that take place before appreciable lipidation occurs.

Although all tested chaperones interact with all forms of apoB in two unrelated cell types, HepG2 and C127, the requirement for ATP for their release appeared to be dependent on the length of apoB, and perhaps the cell type. For example, ATP was required for the release of GRP94 from apoB-100 in HepG2 cells (Fig. 1, lane 3) but not from the truncated forms of apoB (Figs. 4A and 5, lane 3; Fig. 6, lane 2). Calreticulin on the other hand required ATP for its release from apoB-17 but not from apoB-41 or apoB-100 (Fig. 6, lane 2; Fig. 4A, lane 3; Fig. 1, lane 3, respectively). Since calreticulin is not an ATP-binding protein, it is conceivable that the ATP-dependent release from apoB-17 was due to other unidentified chaperone(s) present in apoB-17-calreticulin complexes. The observed differences lend support to the hypothesis that the chaperones identified in this study associate with apoB forms as a part of larger complexes containing other ( unidentified) chaperones or accessory proteins that may play an important regulatory role in the folding of apoB into a secretion-competent form.

As we observed in HepG2 cells, ERp72 is involved in both early (Fig. 2B, lane 1) and late (Fig. 2B, lane 2) events in the folding of apoB. We then demonstrated that the early folding intermediates of apoB can associate simultaneously with both ERp72 and MTP (Fig. 3B), suggesting that the two proteins may function co-operatively. Our results in C127 cells show that ERp72 interacts intracellularly with all truncated forms of apoB (Figs. 4A, 5, and 6); however, it was not established whether ERp72 binds to these truncated forms early in their biogenesis or it comes into play much later. To prove that ERp72 does interact with early folding intermediates of apoB even in the absence of MTP, C127 cells expressing apoB-41 were pulse-labeled for 30 min and lysed in the presence of the cross-linking reagent DSP. Radiolabeled apoB-41 was detected following sequential immunoprecipitation, first with anti-apoB and then with anti-ERp72 antibodies (Fig. 4B), indicating that MTP is not required for the interaction of ERp72 with nascent apoB. ERp72 is a member of the thioredoxin family of proteins, and therefore it may be involved in the disulfide bond formation in apoB. Of the eight disulfides in apoB-100, six are clustered within the first 500 amino acids corresponding to the N-terminal 11% of apoB (54). Previous studies by Shelness and Thornburg in HepG2 cells (55) and our own studies in C127 cells (56), suggested that the proper disulfide bond formation in the N-terminal region of apoB is critical for its folding into a mature secretion-competent form. These findings were further corroborated by later reports documenting that mutations of certain cysteine pairs within the N-terminal 5% of apoB (57, 58) resulted in the failure of apoB to associate with lipids and its accumulation in the cell as a misfolded protein. It is conceivable that members of the thioredoxin family such as PDI, ERp72, or ER60 would bind apoB very early during its biogenesis to catalyze the proper pairing of cysteines to allow the binding of MTP, which in turn may initiate the recruitment of lipids. It is unclear at present whether only one of these chaperones is involved in the process and whether there is a cooperation of two or more thioredoxin-like proteins. Both ERp72 (present report) and ER60 (12) were found to interact with apoB. It remains to be seen whether ERp72 and ER60 are in fact involved in disulfide bond formation, or whether their

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chaperone properties rather than their isomerase activity play a more significant role in apoB maturation.

In summary, the major players of the ER chaperone machinery, namely GRP94, ERp72, BiP, and calreticulin, were found to interact with apoB-100 in HepG2 cells and its truncated forms in C127 cells. Thus, the truncated forms were deemed to be an appropriate model to determine whether the chaperones can discriminate between different lipidation states of newly synthesized apoB. Our results suggest that these chaperones can bind apoB before any substantial lipidation occurs, indicating that they may be involved in the critical folding steps prior to assembly with lipids. We propose that one of these steps may be the binding of ERp72 to the N-terminal part of apoB to mediate disulfide bond formation to allow for MTP to bind and assist the assembly with lipids. The differences in the characteristics of the interactions between apoB forms and the chaperones indicate that the chaperones associate with apoB as a part of complexes containing a number of chaperones and accessory proteins. Since the interactions of ERp72 and GRP94 with apoB-100 persist for at least 2 h, it is possible that these chaperones are also involved in later events in the folding of apoB, and, perhaps, degradation of the misfolded apoB.

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