Grp78 Is Involved in Retention of Mutant Low Density Lipoprotein Receptor Protein in the Endoplasmic Reticulum*

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The low density lipoprotein (LDL) receptor is responsible for removing the majority of the LDL cholesterol from the plasma. Mutations in the LDL receptor gene cause the disease familial hypercholesterolemia (FH). Approximately 50% of the mutations in the LDL receptor gene in patients with FH lead to receptor proteins that are retained in the endoplasmic reticulum (ER). Misfolding of mutant LDL receptors is a probable cause of this ER retention, resulting in no functional LDL receptors at the cell surface. However, the specific factors and mechanisms responsible for retention of mutant LDL receptors are unknown. In the present study we show that the molecular chaperone Grp78/BiP co-immunoprecipitates with both the wild type and two different mutant (W556S and C646Y) LDL receptors in lysates obtained from human liver cells overexpressing wild type or mutant LDL receptors. A pulse-chase study shows that the interaction between the wild type LDL receptor and Grp78 is no longer detectable after 2 1/2 h, whereas it persists for more than 4 h with the mutant receptors. Furthermore, about five times more Grp78 is co-immunoprecipitated with the mutant receptors than with the wild type receptor suggesting that Grp78 is involved in retention of mutant LDL receptors in the ER. Overexpression of Grp78 causes no major alterations on the steady state level of active LDL receptors at the cell surface. However, overexpression of Grp78 decreases the processing rate of newly synthesized wild type LDL receptors. This indicates that the Grp78 interaction is a rate-limiting step in the maturation of the wild type LDL receptor and that Grp78 may be an important factor in the quality control of newly synthesized LDL receptors.

The low density lipoprotein (LDL) receptor is a transmembrane glycoprotein that binds and internalizes circulating particles of LDL by receptor-mediated endocytosis (1). Mutations in the LDL receptor gene cause familial hypercholesterolemia (FH), which is an autosomal dominant inherited disorder of lipoprotein metabolism. Heterozygous FH is a common disorder with an estimated frequency of about 1 in 500. Today, more than 500 different mutations have been identified in the LDL receptor gene. About 50% of the characterized mutations result in LDL receptor proteins that are retained in the endoplasmic reticulum (ER) (2). Since the existence of an ER quality control system ensures that only correctly folded, newly synthesized proteins are transported to the plasma membrane or secreted, it is likely that protein misfolding contributes to the pathogenesis of FH.

Protein misfolding is implicated in the pathogenesis of many genetic diseases, including familial hypercholesterolemia, Alzheimer’s disease, Creutzfeld-Jakob disease, a1-antitrypsin deficiency, and cystic fibrosis (reviewed in Refs. 3, 7, and 8). The ER retention of a variety of LDL receptor mutants suggests that FH may belong to the group of conformational diseases.

The folding and maturation pathway of the newly synthesized LDL receptor in the ER has not yet been characterized. In order to reach a better understanding of the molecular pathogenesis of FH, we embarked on identification of ER quality control components involved in prolonged interaction with mutant, ER-retained, and LDL receptors.

The ER quality control system includes a number of chaperones and folding enzymes localized in the lumen or in the membrane of the ER (reviewed in Ref. 9). It is likely that most, if not all, proteins synthesized in the ER interact with chaperones at some stage of the folding and maturation pathway. The chaperones bind non-native proteins and are thought to assist folding by preventing irreversible aggregation and misfolding. Exactly how chaperones act in concert to keep non-native proteins on the productive folding pathway, and selectively retain certain proteins, is not fully understood. Although some chaperones in the ER are well studied, including 78-kDa glucose-regulated protein (Grp78 or BiP), calnexin, calreticulin, and 94-kDa glucose-regulated protein (Grp94 or endoplasmic), it is at present impossible to predict which chaperones a specific...
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EXPERIMENTAL PROCEDURES

Generation of Constructs—Constructs expressing mutant LDL receptor were generated by site-directed mutagenesis and cloned in the vector (Invitrogen). Correct orientation and sequence of the Grp78 cDNA were confirmed by Northern analysis of transfected Chang cells.

Cell Culture and Transfection—Chang cells (ATCC, CCL-13) were cultivated in RPMI 1640 (In Vitro, Denmark) containing 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Inc.), 100 units/ml penicillin (Leo, Denmark), 0.1 mg/ml streptomycin (Leo, Denmark), and 0.01 mg/ml phenol red, in 5% CO2, 95% air atmosphere at 37 °C. Cells were passaged with TrypLE Express (Invitrogen) to check that they had reached 50% confluency. Transfection was performed according to suppliers’ recommendations. 16 h after transfection the immunocomplexes were dissociated and solubilized by incubating in 50 μl of two-dimensional lysis buffer (8 μm urea, 2% CHAPS, 0.5% IPG-buffer pH 3–10, 0.3% dithiothreitol, 1.25 μm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 μm leupeptin). Chang cell pellets were solubilized in the two-dimensional lysis buffer described above to a final protein concentration of 1.5 mg/ml. 25 μl of the solubilized immunocomplex and 100 μl of the Chang cell extract were diluted with 95 μl of rehydration solution (8 μm urea, 2% CHAPS, 0.5% IPG-buffer pH 3–10, 0.3% dithiothreitol, few grains of bromphenol blue) to ensure a sufficient amount of protein for the mass spectrometry analysis. Two-dimensional gel electrophoresis was carried out using a Multiphor apparatus (Amersham Pharmacia Biotech). The first dimension isoelectric focusing was performed on an immobilized pH gradient (immobiline dry strips, Amersham Pharmacia Biotech) with a total length of 11 cm. An Immobiline DryStrip Reswelling Tray (Amersham Pharmacia Biotech) was used for rehydration and loading of the sample. After the first dimension, the strips were equilibrated twice for 10 min in equilibration solution (50 mM Tris-Cl, pH 8.8, 6% urea, 30% glycerol, 1% SDS) supplemented with 16 mM dithiothreitol and the first incubation solution for the second incubation. The second dimension was carried out in horizontal SDS-PAGE using 8–18% Excel Gel. The gels were run according to suppliers’ recommendation, dried, and analyzed using the PhosphoImager and the ImageQuant software (Molecular Dynamics, CA) as described above.

Protein Digestion—Tryptic digestion of protein in excised two-dimensional gel slices was performed as described previously (17, 18). In brief, the excised gel slices were washed in 100 mM NH4HCO3/acetonitrile (1:1). The protein was reduced and S-alkylated with iodoacetamide, and the gel slices were dried by vacuum centrifugation. Modified porcine trypsin (12 ng/μl) (Promega, sequencing grade) in digestion buffer (50 mM NH4HCO3, 5 mM CaCl2) was added to the dry gel pieces, and they were incubated on ice for 1 h in order to reswell them. After removing the supernatant, 10–20 μl of digestion buffer was added, and the digestion was continued overnight at 37 °C. The peptides were extracted with 5% formic acid, 50% acetonitrile, dried by vacuum centrifugation, and redissolved in 20 μl of 5% formic acid prior to mass analysis.

Peptide Mass Mapping by Matrix-assisted Laser-Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry—A Bruker REFLEX delayed extraction MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with the SCOUT source and variable detector bias gating was employed for mass analysis of peptide mixtures in positive ion reflector mode. Ion acceleration voltage was 22 kV. Thin film matrices of α-cyano-4-hydroxycinnamic acid and nitrocellulose were prepared by the fast evaporation method (17, 18). An aliquot of peptide solution (0.3–0.5 μg) was mixed with a 0.6-μl droplet of 5% formic acid previously deposited onto the thin matrix layer, and the solvent was then allowed to dry. The analyte/matrix surface was washed with 10 μl of 5% formic acid and then with 10 μl of pure water prior to analysis by MALDI-TOF mass spectrometry. Mass spectra were calibrated by using matrix ion signals and trypsin autolysis peptide signals as internal mass calibrants (19).

protein will interact with and to predict the consequences of the interaction.

Grp78 is the ER-located Hsp70 analogue. It binds transiently to a variety of newly synthesized proteins and more persistently to some misfolded proteins. Furthermore, Grp78 is known to assist folding and assembly of newly synthesized proteins by recognition and binding of hydrophobic stretches of unfolded proteins. Binding of Grp78 prevents protein aggregation and maintains the proteins in a folding and oligomerization-competent state (reviewed in Ref. 10). Calnexin and calreticulin are lectin-like ER chaperones, which specifically recognize monoglycosylated N-linked core glycans. A major function of the two chaperones is to monitor glycoprotein folding and to prevent misfolded protein from leaving the ER (11). Grp94 is the ER homologue to cytosolic Hsp90. It is abundant in the ER and possesses Mg2+-dependent ATPase activity (12). Grp94 interacts with several nascent polypeptides and presumably mediates folding of apolipoprotein B (13).

The purpose of this study was first to identify ER chaperones interacting with the wild type and mutant LDL receptors. Second, it was to characterize possible differences in the chaperone-LDL receptor interaction for the mutant receptors compared with the wild type receptor. Third, it was to manipulate the amount of identified chaperones, by overexpression, in an attempt to influence the folding and maturation of the LDL receptor.

To identify chaperones/folding enzymes interacting with wild type and mutant LDL receptors, we performed expression studies in human liver epithelial cells (Chang cells). Two mutants, ER-retained, LDL receptors, and the wild type LDL receptor were analyzed. Both mutant receptors contain amino acid substitutions caused by missense mutations. The first causes a tryptophan to serine substitution at amino acid position 556 (W556S) in the receptor protein. About 12% of FH in Denmark is caused by this W556S mutation (14). The second was a missense mutation causing a cysteine to tyrosine substitution at amino acid position 646 (C646Y) (15).

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Protein Identification by Peptide Mass Mapping and Data Base Searching—High mass accuracy MALDI-TOF peptide mass maps were used to query a comprehensive protein sequence data base (NRDB, European Bioinformatics Institute, Hinxton, UK) utilizing the Pep- tideSearch software (20). Unambiguous protein identification was achieved by requiring a tryptic peptide mass error below 50 ppm.

Immunoblot Analysis—Equal proportions of immunoprecipitated proteins and aliquots of total cell protein extracts corresponding to 1/50 of the extracts subjected to immunoprecipitation were subjected to 4–15% gradient SDS gel electrophoresis, transferred to polyvinylidene difluoride membrane, blocked, and immunoblotted with anti-Grp78 antibody (Ab-108 from Gen-Probe, CA). The antibody signal was diluted 1:10,000 in PBS-Tween (0.15 M NaCl, 0.05% Tween 20, pH 7.2, 0.05% Tween 20) containing 5% skim milk powder. The membranes were incubated 15 h at room temperature. Subsequently, the membranes were incubated in horseradish peroxidase-conjugated anti-mouse antibody (DAKO, Denmark) diluted 1:25,000 in PBS/Tween containing 5% skim milk powder for 2 h at room temperature. Chemiluminescence detection was performed with ECL Plus (Amer-sham Pharmacia Biotech) and direct image analysis using the Molecular Dynamics Storm blot imaging system.

Flow Cytometry—Transfected cells for LDL receptor activity measurements were cultivated for 5 h in RPMI containing 5% lipid-deficient human serum and 2 μg/ml 1,1-diiodotyrosyl-3,3,3'-tetramethylindolo-carbocyanine perchlorate (DiI)-conjugated LDL (21) (Molecular Probes). Cells were harvested in PBS containing 0.6% EDTA followed by a short incubation in PBS containing 0.6% EDTA and 0.01% trypsin, washed 3 times in PBS, and analyzed by flow cytometry as described below. For surface staining cells were harvested 48 h after transfection by incubation in PBS containing 0.6% EDTA followed by a short incubation in PBS containing 0.6% EDTA and 0.01% trypsin. The cells were labeled with 2.5 μg/ml monoclonal anti-LDL receptor antibody (C7) for 30 min at 4 °C, washed in complete RPMI, and stained with Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes), and diluted 1:400 in RPMI for 20 min at 4 °C. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with an argon laser operating at 488 nm. Forward angle light scatter gates were established to exclude dead cells and cell debris from the analysis. 5 x 10^5 cells were analyzed in each sample.

RESULTS

A 78-kDa Protein Interacts Transiently with LDL Receptor Proteins and Displays Prolonged Interaction with Mutant LDL Receptor Proteins—Chang cells expressing wild type LDL receptor, W556S-LDL receptor, or C646Y-LDL receptor were pulse-labeled with [35S]methionine and [35S]cysteine for 30 min and chased in the presence of cycloheximide to inhibit further protein synthesis. Cell extracts were subjected to immunoprecipitation with anti-LDL receptor antibodies under non-denaturing conditions. Fig. 1 shows that within 1 h most of the immunoprecipitated wild type LDL receptors were processed from the faster migrating 120-kDa precursor form to the more slowly migrating 160-kDa mature form. In contrast, none of the mutant W556S-LDL receptors and C646Y-LDL receptors appeared to be processed after a 4-h chase (Fig. 1A). The band appearing approximately at 160 kDa at the early time points for the C646Y mutant is not present when the immunoprecipitation is performed under denaturing conditions, indicating that it does not represent mature LDL receptors (results not shown). These results confirm previously obtained results showing that the two mutant LDL receptors are retained in the endoplasmic reticulum (ER) (14, 15). The pulse-chase experiment reveals one protein with an apparent mass of 78 kDa that specifically co-immunoprecipitates with the newly synthesized wild type LDL receptor (Fig. 1A). The interaction between the wild type LDL receptor and the 78-kDa protein is detectable only in the initial phase of the chase. After 2½ h of chase, where most of the LDL receptor has been transported out of the ER, interaction is no longer detectable. In contrast, the association between the two mutant LDL receptors and the 78-kDa protein persists during 4 h of chase (Fig. 1A). Furthermore, the ratio between the intensity of the band representing the 78-
immunoprecipitation, and immunoblotted using an antibody recognizing both Grp78 and Grp94. The Western blot confirms that Grp78 co-immunoprecipitates with the LDL receptor. A faint band representing Grp78 could be detected when expressing wild type LDL receptor (Fig. 4, lane 1). A significantly larger amount of Grp78 co-immunoprecipitated with either of the two mutant receptors compared with the wild type (Fig. 4, lanes 2 and 3). Quantification of the bands representing Grp78 showed that in the steady-state situation five times more Grp78 co-immunoprecipitated with the mutant receptors compared with the wild type. Furthermore, aliquots corresponding to 1/50 of the total Chang cell extract were isolated and immunoblotted together with the immunoprecipitated proteins. The antibody detects both Grp78 and Grp94 in the Chang cell lysates (Fig. 4, lanes 5–8). However, using these specific co-immunoprecipitation conditions Grp94 was not co-isolated with the LDL receptor (Fig. 4, lanes 1–3), indicating no detectable association between the LDL receptor and Grp94.

Up-regulation of Grp78 by 6-Aminonicotinamide Treatment—6-Aminonicotinamide (6-AN) is an analogue of niacin that can be metabolized to 6-amino-NAD(P), a competitive inhibitor of NAD(P)-requiring processes including gluconeogenesis (22) and poly(ADP-ribose) synthesis (23). 6-AN has previously been used for specific up-regulation of Grp78 and for studying the effect from overexpression of Grp78 in colon cancer cell lines (24). Furthermore, previously obtained results show that Grp78 co-immunoprecipitated with the LDL receptor (Fig. 4, lanes 1–3), indicating no detectable association between the LDL receptor and Grp94.

Effects of Overexpression of Grp78 with Grp78 Co-transfection and 6-AN Treatment—Previous studies demonstrated that overexpression of wild type Grp78 can inhibit secretion of a variety of proteins in the ER (25–27). This indicates that one effect of overexpression of Grp78 is selective retention of proteins in the ER. In contrast, other studies have shown that overexpression of Grp78 can stimulate folding and improve secretion of other proteins (28, 29). Therefore, we studied the
The effect of Grp78 overexpression on the processing of wild type LDL receptor, both by Grp78 co-transfection and 6-AN treatment. In immunoprecipitates from cells expressing wild type LDL receptor, the intensity of the band representing the mature form has reached the same intensity as the band representing the precursor LDL receptor after about 30–45 min. Thus, at this point the amount of mature LDL receptor is equal to the amount of precursor receptor protein. However, when overexpressing Grp78 by co-transfection or by 6-AN treatment, more than 1 h is needed before half of the newly synthesized precursor protein is present in the mature form (Fig. 6). These results indicate that overexpression of Grp78 decreases the processing rate of the wild type LDL receptor precursor. Overexpression of Grp78 did not cause any detectable differences in the processing of the two mutant LDL receptors (results not shown). The mutant receptors were not detectable in the mature form, either during co-expression of Grp78 or during 6-AN treatment. The pulse-chase results were identical to the results in Fig. 1.

Since Grp78 overexpression decreases the processing rate of the wild type LDL receptor, overexpression of Grp78 may also influence the steady state level of the LDL receptors at the cell surface. To address this question Chang cells were transfected with a plasmid expressing the wild type LDL receptor alone, co-transfected with plasmids expressing the wild type LDL receptor and Grp78, or transfected with a plasmid expressing the wild type LDL receptor and treated with 6-AN to increase the endogenous level of Grp78. The cell surface expression and activity of LDL receptors were analyzed by flow cytometry. In order to measure the relative amount of LDL receptors at the cell surface, intact cells were stained with C7 antibody and Alexa-conjugated rabbit anti-mouse antibody at 4 °C (Fig. 7A). In parallel, the activity of the LDL receptors was determined as binding and uptake of Dil-conjugated LDL at 37 °C (Fig. 7, B and C), shows the median fluorescence above background for cells transfected with plasmids expressing the wild type LDL receptor with or without 6-AN treatment or co-transfection with plasmids expressing Grp78. Background was defined as the fluorescence value below which 99.75% of cells transfected with the pMP6 vector without insert was found. Accordingly, the population above the background represents cells overexpressing the wild type LDL receptor. Overexpression of Grp78 causes no major alterations of the steady state level of the LDL receptor quantity or function (Fig. 7, B and C). Nevertheless, one-way analysis of variance shows a significant difference between the three groups for the C7 labeling (p = 0.00097) as well as for the Dil-LDL incubation (p = 0.00055). The Dil-LDL binding and uptake measurements show no significant difference between the untreated and the 6-AN-treated cells (p = 0.09676) indicating that it is the decrease in the Grp78 co-transfected cells that causes the significant difference between the three groups.

**DISCUSSION**

Approximately 50% of the characterized mutations in the LDL receptor gene lead to mutant proteins that are partially or totally retained in the ER (2). However, the specific factors and mechanisms responsible for the retention of mutant LDL receptors are unknown. Our results are the first to identify a specific chaperone involved in retention of LDL receptors in the ER. The mass spectrometry (Fig. 3) and Western blot analysis (Fig. 4) unambiguously identify the 78-kDa protein co-immunoprecipitating with the LDL receptor as Grp78. In our system no other proteins showed specific co-immunoprecipitation with either the wild type or the mutant LDL receptors. This does not rule out the possibility that other chaperones are involved in the quality control of the LDL receptor. In fact previous studies demonstrate that Grp78 can cooperate with other chaperones. For example in the maturation of apolipoprotein B (apoB) Grp78 associates with apoB as a part of a complex including the chaperones Grp94, calreticulin, and Erp72 (13). It cannot be excluded that some of these chaperones also interact with the LDL receptor, but our results indicate that such interactions are weak if they are present. Calnexin and calreticulin react specifically with monoglycosylated N-linked core glycans, and
since the LDL receptor is a glycoprotein it is a potential substrate for calnexin and calreticulin. However, we detected no association between the LDL receptor and calnexin or the LDL receptor and calreticulin, indicating that these two chaperones are not major contributors in the ER retention and quality control of the LDL receptor. The LDL receptor contains one or two asparagine-linked glycans (30), which are not localized within the first 50 residues of NH2 terminus of the protein. This may explain why the LDL receptor is mainly associated with Grp78 and not with calnexin and calreticulin. This is in accordance with recent results where Molinari and Helenius (31) showed that direct interaction of asparagine-linked glycans with calnexin and calreticulin, without prior interaction with Grp78, occurs only if glycans were present within about 50 residues of the NH2 terminus of the protein.

During this study we have established a sensitive method for identification of proteins interacting with the LDL receptor. It may be expected that combining the already established protocol with expression of other processing-deficient mutant LDL receptors, or stabilization of the chaperone complexes by chemical cross-linking, may identify other chaperones. However, our results showing that Grp78 displays transient interaction with the wild type LDL receptor and prolonged interaction with two ER-retained, mutant, LDL receptors, indicate that Grp78 is a major factor in ER retention of mutant LDL receptors. This is supported by the observation of a significant increase in the

![Fig. 7. Characterization of expressed cell surface-located LDL receptor protein in transfected Chang cells using flow cytometric measurements.](image-url)
amount of Grp78 co-immunoprecipitated when expressing either of the two mutant receptors and compared with the wild type LDL receptor. This shows that Grp78 has an increased affinity for the mutant receptors and is consistent with previous results demonstrating that one function of Grp78 is selective retention of proteins in the ER (32). Grp78 is believed to function as a chaperone via cyclic on and off associations with hydrophobic protein stretches, coupled to ATP hydrolysis (33, 34). The increased affinity of Grp78 for the mutant LDL receptors implicates that the two mutations cause misfolding resulting in exposed hydrophobic areas in the LDL receptor protein. The W556S and the C646Y mutations are both localized in the second domain of the LDL receptor. This domain is characterized by being 33% identical to a portion of the human epidermal growth factor precursor (35). The C646Y mutation disrupts the correct formation of a disulfide bond in one of the three growth factor repeats. The W556S mutation results in an amino acid substitution in one of the conserved YWTD repeats (35). Mutations resulting in disruption of disulfide bonds or mutations in conserved regions are likely to cause folding problems. However, since the two mutations are not localized in the ligand binding domain of the LDL receptor (14, 15), it is possible that the mutant receptors are in position to bind LDL if they could escape the ER quality control system. Taken together, our results support the hypothesis that protein misfolding contributes to the pathogenesis of FH, and thereby FH can also be regarded as a conformational disease.

The Grp78 overexpression experiments show that increased levels of Grp78 decrease the processing rate of the wild type LDL receptor (Fig. 6). Since Grp78 binds through cyclic on and off associations, the decreased processing rate may be due to a more frequent binding of the LDL receptor by Grp78, owing to the increased concentration of Grp78 in the overexpressing cells. Therefore, the average association free time for the LDL receptor during overexpression of Grp78 is shortened. It is well known that when unfolded proteins accumulate in the ER, Grp78 transcription is induced. This response is a part of the cellular unfolded protein response. The signal for induction is believed to be a decrease in the concentration of the free Grp78 owing to binding of Grp78 in complexes with unfolded proteins (36, 37). This indicates that an effective regulation of Grp78 transcription, resulting in increased amounts of Grp78, is important for the ability of the cells to cope with stress situations. The observed decrease in the processing rate of the wild type LDL receptor (Fig. 6) is consistent with the notion that an increased amount of Grp78 can influence the folding and maturation of the LDL receptor.

Overexpression of Grp78 did not have any detectable influence on the folding and maturation of the mutant LDL receptors. They stayed totally retained in the ER, and no significant alterations in degradation could be observed. We suggest that the available amount of Grp78 is already sufficient to prevent transport of the mutant receptors to the plasma membrane. Therefore, a further increase of Grp78 by overexpression will not significantly alter the situation. However, many LDL receptor mutations resulting only in delayed transport of the newly synthesized receptor through the ER have been identified. It might be expected that overexpression of Grp78 could influence the partial retention of these “less severe” mutant receptors.

Overexpression of Grp78 is known to cause selective retention of a variety of proteins (25–27). According to these results and our observation that overexpression of Grp78 caused delayed processing of the wild type receptor, we expected overexpression of Grp78 to decrease the steady state level of active LDL receptors. Surprisingly, we observed no major decrease in the steady state amount of cell surface LDL receptors or in the binding and internalization of DiI-LDL (Fig. 7). This shows that decreasing the folding and maturation rate of the LDL receptor had no major influence on the steady state level of the wild type LDL receptor. However, it cannot be excluded that overexpression of Grp78 influences the steady state level of the LDL receptor but that the effects may be too small for detection in our system. Actually, one-way analysis of variance showed a significant difference in the number and the activity of cell surface-located LDL receptors between Chang cells overexpressing the LDL receptor alone, overexpressing the LDL receptor together with Grp78, or overexpressing the LDL receptor after 6-AN treatment. We especially observed a decrease in the activity of cell surface-located LDL receptors when co-expressing Grp78. Overexpression of Grp78 decreases the processing rate of the LDL receptor, indicating an increase in the average time each LDL receptor molecule spends in the ER. This may lead to an increased risk of degradation of the receptor and thereby decreased steady state level at the cell surface. We did not observe the same decrease in the 6-AN-treated cell.

However, overexpression of recombinant Grp78 by co-transfection and up-regulation of endogenous Grp78 by 6-AN are two distinct mechanisms. A direct mechanistic link between 6-AN induction of Grp78 expression has not been established. However, an association has been identified between deficiency of the NAD-poly(ADP-ribose) synthesis system and induction of Grp78, suggesting that 6-AN induces Grp78 indirectly by inhibition of poly(ADP-ribose) polymerase (38). 6-AN also affects the cellular ATP levels by secondary inhibition of glycolysis (22). Since protein dissociation from Grp78 can be blocked by depletion of cellular ATP levels (39), the 6-AN treatment may cause induction of Grp78 by accumulation of a variety of Grp78-bound unfolded proteins in the ER. Therefore, induction of Grp78 by 6-AN treatment might not increase the level of available Grp78 in the ER. This might explain why we do not observe a significant effect on the steady state level and activity of cell surface-located LDL receptors in the 6-AN-treated cells. Taking into account that only about 10% of the cells express the recombinant Grp78, the individual co-transfected cells express approximately 10 times more Grp78 when compared with the 6-AN-treated cells (Fig. 5, lanes 1–4). It cannot totally be excluded that the decrease in the number and activity of cell surface-located LDL receptors is linked to the simultaneous expression of two recombinant proteins from strong promoters, leading to a possible overload of cellular protein synthesis capacity.

In summary, Grp78 interacts transiently with the wild type LDL receptor and displays prolonged interaction with two mutant LDL receptors. This indicates that misfolding of the two mutant LDL receptors causes retention in the ER and that Grp78 is involved in the specific retention of the mutant receptors in the ER. Accordingly, protein misfolding contributes to the pathogenesis of familial hypercholesterolemia. Our results suggest that Grp78 is a potential key factor in the ER quality control of the newly synthesized LDL receptor.

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