Retention of Mutant Low Density Lipoprotein Receptor in Endoplasmic Reticulum (ER) Leads to ER Stress*

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Familial hypercholesterolemia is an autosomal dominant disease caused by mutations in the gene encoding the low density lipoprotein receptor (LDLR). More than 50% of these mutations lead to receptor proteins that are completely or partly retained in the endoplasmic reticulum (ER). The mechanisms involved in the intracellular processing and retention of mutant LDLR are poorly understood. In the present study we show that the G544V mutant LDLR associates with the chaperones Grp78, Grp94, ERP72, and calnexin in the ER of transfected Chinese hamster ovary cells. Retention of the mutant LDLR was shown to cause ER stress and activation of the unfolded protein response. We observed a marked increase in the activity of two ER stress sensors, IRE1 and PERK. These results show that retention of mutant LDLR in ER induces cellular responses, which might be important for the clinical outcome of familial hypercholesterolemia.

Familial hypercholesterolemia (FH)2 is an autosomal dominant disease caused by mutations in the gene encoding the low density lipoprotein receptor (LDLR) that result in defective clearance of lipoproteins from the circulation (1). The disease is characterized by hypercholesterolemia, tendon xanthomas, and premature coronary heart disease. FH is one of the most common genetic disorders with a prevalence of heterozygotes of about one in 500 in most western countries. On a worldwide basis it has been estimated that more than 10 million people have FH, of which as many as 200,000 die of premature coronary heart disease each year (2). At present, more than 900 different mutations in the LDLR gene have been found to cause FH (www.umd.necker.fr/LDLR/mutation.html and www.ucl.ac.uk/fh/).

The LDLR is a cell surface glycoprotein composed of several structural domains that mediate the specific binding and uptake of apoB- and apoE-containing lipoproteins by receptor-mediated endocytosis (1). LDLR is synthesized in the endoplasmic reticulum (ER) as a partially glycosylated precursor with an apparent molecular mass of 120 kDa (3). Within 30–45 min the receptor is transported to the Golgi where the oligosaccharides are modified and the apparent molecular mass of the receptor is increased to 160 kDa. The 160-kDa form is transported to the cell surface, where it mediates the uptake of low density lipoprotein (LDL) particles. Different mutations in the LDLR gene have different effects on the receptor protein function. This has led to a classification of mutations into five functional classes: 1) null alleles that fail to produce receptor protein, 2) transport-defective alleles that encode receptors that are completely (class 2a) or partially (class 2b) blocked in the transport from ER to Golgi, 3) binding defective alleles that fail to bind LDL, 4) internalization-defective alleles that fail to internalize LDL by receptor-mediated endocytosis, and 5) recycling-deficient alleles that fail to release the ligand in the endosomes and thus do not recycle to the cell surface (4, 5). However, many mutant LDLR alleles produce receptors that fall into more than one of these classes.

Class 2 mutations are the most predominant class of mutations and account for more than 50% of the mutations causing FH (4). These mutations might not necessarily affect the ability of the receptor to bind LDL, but abnormal folding prevents it from reaching the cell surface. FH has therefore been classified as a protein conformation disease (6) or an ER storage disease (7, 8).

The mechanisms involved in the intracellular processing of proteins represent complex and not fully understood processes (9). On one hand, properly folded proteins must be guided from the ER to their final destination within the cell, and on the other hand, misfolded proteins that may be toxic to the cell must be disposed of without compromising normal cell function. This quality control system is based on common structural and biophysical features that distinguish native from non-native protein conformations. Folding is assisted by a variety of folding enzymes and chaperones with different properties and functions (9). The main role of these components is to prevent protein misfolding and aggregation, which could cause cellular dysfunction (10, 11).

Accumulation of misfolded proteins in the ER has been shown to cause ER stress and activation of a protective response known as the unfolded protein response (UPR) (12, 13). The UPR is mediated by three ER-resident transmembrane proteins that act as proximal sensors of ER stress: the type I transmembrane protein kinase and endoribonuclease (IRE1), the RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (14). UPR increases the folding capacity of the secretory pathway through transcriptional up-regulation of an array of genes required for protein folding, ER expansion, ER-Golgi trafficking, and ER-associated degradation (ERAD), which together act to relieve stress within the ER (15).

Although FH is a monogenic disease, the phenotypic expression, in terms of onset and severity of atherosclerotic disease, varies considerably. The phenotype differs substantially even among individuals who have the same genetic defect (16, 17). In addition to risk factors of environmental and metabolic origin, other genetic factors are suggested to contribute to the phenotypic variation of FH (16, 18). It is possible that differences in the quality control systems within the ER, as well as differences in the ERAD (19), could explain some of the phenotypic variation.

To test this hypothesis there is a need to understand how class 2 mutants interact with the ER quality control components.

To identify chaperones/folding enzymes interacting with the LDLR, we have used Chinese hamster ovary (CHO) cells transfected with either wild-type LDLR or a class 2a mutant LDLR where glycine in
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Confocal laser scanning microscopy was used to determine the cellular localization of the LDLR-EYFP and to analyze the LDLR activity by measuring internalization of LDL in transfected CHO cells. The cells were plated on fibronectin-coated glass slides (BD Biosciences) and allowed to adhere overnight. The expression of LDLR was induced by adding tetracycline (1 µg/ml).

LDLR Localization—The cells were fixed and permeabilized by incubation in 70% ethanol for 10 min and washed twice in phosphate-buffered saline (PBS). For staining of LDLR without EYFP tag, the cells were incubated with IgG-C7 mouse monoclonal antibody (Progen Biotechnik). Nonspecific binding sites were blocked by incubating the cells in blocking solution (PBS with 0.5% bovine serum albumin (BSA)) for 1 h at room temperature, before incubating with IgG-C7 (1:20 dilution) for 1 h at room temperature and washing three times in blocking solution. IgG-C7 was detected by incubating with Alexa Fluor® 488 goat anti-mouse IgG (1:400; Molecular Probes) for 30 min at room temperature. To visualize ER the cells were incubated with concanavalin A tetramethylrhodamine conjugate (1:100, Molecular Probes) for 1 h at room temperature. The cells were then washed three times in PBS with 0.5% BSA and 0.1% Tween 20 (Sigma-Aldrich).

LDLR Activity—Transfected cells were incubated in complete medium containing 20 µg/ml fluorescent LDL (LDL conjugated with Alexa Fluor 555; Molecular Probes) for 4 h at 37 °C. Following the incubation, the medium was removed, and the cells were washed twice in ice-cold PBS with 0.5% BSA before fixation in a 4% methanol-free formaldehyde solution (Sigma-Aldrich) for 15 min at room temperature. The slides were mounted with SlowFade Antifade solution including 4,6-diamino-2-phenylindole nuclear counterstain (Molecular Probes) to prevent photo bleaching. The slides were examined by a Leica TCS SP confocal microscope (Leica Microsystems) using the 100X oil objective. Image processing was carried out using Photoshop version 5.5 (Adobe Systems).

Metabolic Labeling and Immunoprecipitation

Transfected CHO cells stimulated with tetracycline (1 µg/ml) for 24 h were incubated in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5% diazylated fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 10 µg/ml bastidcin, 100 µg/ml zeocin, and 1 µg/ml tetracycline for 30 min to deplete intracellular pools of methionine and cysteine. 20 µl of 0.1 M Ci/ml Promix ([35S]methionine and [35S]cysteine; Amersham Biosciences) was added, and the cells were labeled for 10 min. The chase period was initiated with two washes with PBS followed by incubation in complete medium for 5–45 min. The cells were lysed at 4 °C for 30 min using lysis buffer A: 1% Triton X-100 (Sigma-Aldrich), 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and EDTA supplemented with a mixture of protease inhibitors (Roche Applied Science number 1,697,498) and harvested by scraping. The lysates were sonicated for 5–10 s and centrifuged to remove cell debris. The cell lysates were incubated with 25 µl of sheep anti-rabbit conjugated Dynabeads (Dynal Biotech) for 1 h at 4 °C with end-over-end mixing to remove nonspecific binding to the beads. LDLRs were then immunoprecipitated overnight at 4 °C using 1 µg of rabbit polyclonal anti-LDLR antibody (antibody 61099; Progen Biotechnik). To capture the immunocomplexes, the samples were incubated with sheep anti-rabbit IgG Dynabeads for 1 h at 4 °C. The beads with captured immunocomplexes were washed three times in lysis buffer A.
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and three times in PBS with 0.1% BSA and 0.1% Tween 20 and eluted by boiling for 5 min in Laemmli sample buffer (1% SDS, 30 mM Tris-HCl, pH 6.8, 12.5% glycerol, 0.005% bromphenol blue, and 2.5% mercaptoethanol). The immunoprecipitates were subjected to SDS-PAGE (7%). The gel was dried and exposed in a Phosphor screen, and the signal was analyzed using a PhosphorImager.

Cross-linking of Proteins, Immunoprecipitation, and Western Blot Analysis

The cells were lysed for 30 min at 4 °C using lysis buffer A and harvested by scraping. After thoroughly vortexing for 30 min, the lysates were centrifuged to remove the Triton-insoluble fraction. For cross-linking of proteins, the cells were incubated for 30 min at room temperature with 1 mM dithiobis succinimidyld propionate (DSP) (Sigma-Aldrich) and quenched with 100 mM Tris-HCl (pH 6.8) for 20 min before lysis in lysis buffer A. Immunoprecipitation was performed as previously described, except that increased amounts of sheep anti-rabbit conjugated Dynabeads (100 μl) and rabbit polyclonal anti-LDLR antibody (4 μg) or normal rabbit IgG (Santa Cruz) were used. The captured immunocomplexes were washed three times in lysis buffer.

The immunoprecipitates were run on 4–20% Tris-HCl gradient gel at 200 V for 1 h. The proteins were electrotransferred onto a polyvinylidene difluoride membrane (Bio-Rad) and blocked in 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline with 0.05% Tween 20. To omit detection of heavy and light chain of rabbit IgG, the blots were cut at approximately 60 kDa. The blots were probed with primary antibodies: polyclonal rabbit anti-LDLR (Progen Biotechnik) and rabbit polyclonal anti-Grp78, rat monoclonal anti-Grp94, rabbit polyclonal anti-ERp72, and rabbit polyclonal anti-calnexin (all four obtained from StressGen Biotechnologies) and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (Amersham Biosciences), before the proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). Chemidoc XRS (Bio-Rad) was used to detect the signals.

Protein concentrations in cell lysates were determined using BCA protein assay kit (Pierce). 20 μg of cell protein diluted to 20 μl in PBS was added 10 μl of 3× Laemmli sample buffer containing 5% β-mercaptoethanol. The samples were heated at 95 °C for 5 min and subjected to SDS-PAGE and Western blot analysis as described above. The Triton-insoluble fraction was solved by adding 50 μl of SDS-PAGE and Western blot analysis as described above. The TRIzol method was used to isolate total RNA from CHO cells expressing wild-type or G544V mutant LDLR. The mRNA was eluted in 0.05 M MOPS (pH 7.0) containing 1 mM EDTA, 5.6% formaldehyde, and 40% formamide. Loading buffer (2 mM sodium phosphate buffer, 1% Ficoll 400, and 0.025% bromphenol blue) was added, and the mRNA was separated on 1% agarose gels containing 6.7% formaldehyde. RNA was blotted onto nylon membranes (Hybond N+; Amersham Biosciences), and hybridization was carried out according to Church and Gilbert (23). For detection of Grp78, Grp94, and ERp72 mRNA, the cDNA clones obtained from ATCC were used (human Grp78, ATCC number 693965; mouse Grp94, ATCC number 5748980; and human ERp72, ATCC number 5297113). For the detection of β-actin mRNA, the mouse β-actin cDNA probe (A9715; Sigma-Aldrich) was used.

RT-PCR Analysis of XBP-1 Splicing

Total RNA was isolated from CHO cells expressing wild-type or G544V mutant LDLR using QIAamp RNA Blood Mini kit (Qiagen). RT-PCR was performed using a Qiagen one-step RT-PCR kit and the primers 5′-AGTGGCAGGTTCTGCTGAGT-3′ and 5′-AATCCAT-GGGGAGATGTTCTGG-3′. The conditions used for the RT-PCR were: reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. PCR products were analyzed by gel electrophoresis on a 3.5% MetaPhor gel (Cambrex Bio Science), which was stained with ethidium bromide. RNA isolated from CHO cells treated with 5 mM dithiothreitol for 24 h was used as a positive control for XBP-1 mRNA splicing.

RESULTS

T-Rex CHO cells were stably transfected with wild-type LDLR or G544V mutant LDLR. To enable induced expression of LDLR, we used a cytomegalovirus immediate-early promoter containing two tetracycline operator 2 elements. T-Rex CHO cells express a tetracycline repressor, which interacts with the tetracycline operator 2 elements, prevents expression in the absence of tetracycline, and enables induced expression by adding tetracycline.

To facilitate sorting of transfected cells and to visualize the LDL expression in living cells, an EYFP tag was added at the carboxyl terminus of the receptor. The EYFP tag introduced an ~30-kDa increase in the molecular size of the synthesized LDLR. To study whether the EYFP interfered with the biosynthesis of the LDLR, we performed [35S]methionine/cysteine pulse-chase experiments in CHO cells transfected with wild-type LDLR after incubation with tetracycline for 24 h. The maturation of wild-type LDLR from precursor to mature form occurred within 45 min, irrespective of whether the receptor had an EYFP tag (Fig. 1A). This indicates that the EYFP tag does not affect the normal transport of LDLR from the ER to the Golgi apparatus. The G544V mutant receptor has previously been shown to be completely retained in the ER (21). To confirm that the G544V mutant LDLR behaved like a class 2a receptor in our model system, Western blot analysis of cell
lysates from transfected CHO cells stimulated with tetracycline for 24 h was performed (Fig. 1B). Whereas all of the wild-type receptors were in the mature form, all of the mutant receptors with or without EYFP tags were in the precursor form. Thus, the G544V mutant LDLR was retained in ER and behaved like a class 2a mutant receptor. As shown, the EYFP tag did not alter the biosynthesis of the LDLR. To confirm that the cellular localization of the LDLR was unaffected, we performed confocal fluorescence microscopy. The wild-type LDLR, with or without EYFP tag showed a membrane-associated expression pattern (Fig. 1C). To analyze whether the biological activity of the LDLR was affected by the EYFP tag, the cells ability to internalize fluorescence labeled LDL was tested. As shown in Fig. 1C, CHO cells expressing wild-type LDLR with or without EYFP tag could efficiently internalize LDL. On the other hand, the G544V mutant LDLR exhibited an intracellular distribution with no active receptor on the cell surface. To study whether the intracellular localization was restricted to the ER, co-localization with the lectin concanavalin A was analyzed. Concanavalin A specifically binds to the mannose-rich glycans in the ER (24). The G544V mutant LDLR was found to co-localize with concanavalin A (Fig. 1D), indicating as shown previously that the G544V mutation give rise to a class 2a transport-deficient LDLR, which is retained in the ER (21). In all of the following experiments, we used CHO cells stably transfected with wild-type or G544V mutant LDLR containing the EYFP tag.
ones in ER have been demonstrated to operate in multiprotein complexes, and we therefore sought to determine whether Grp78 and other chaperones could be found associated with G544V-mutated LDLR. The interactions between chaperones and folding polypeptides are transient in nature. To prevent the release of chaperones from the substrates, cross-linking was therefore performed by adding DSP before the cells were solubilized in lysis buffer.

The Triton-soluble fractions were subjected to immunoprecipitation with antibodies to the LDLR, and the immunoprecipitated complexes were analyzed by Western blot analysis. The amount of immunoprecipitated LDLR from the Triton-soluble fraction was reduced in cells treated with DSP (Fig. 2A). Thorough analysis of the Triton-soluble and -insoluble fractions prior to immunoprecipitation confirmed that upon cross-linking with DSP a large fraction of the LDLR become Triton-insoluble. We were unable to solve the Triton-insoluble fraction of DSP-treated cells without boiling the fraction with mercaptoethanol. This treatment cleaves the disulfide bond in DSP and split the cross-linked complexes and thus makes the Triton-insoluble fraction unsuitable for further analysis by immunoprecipitation. The Triton-soluble fraction was subjected to immunoprecipitation and analyzed for co-immunoprecipitated chaperones. A small but detectable amount of Grp78 co-immunoprecipitated with the wild-type LDLR after cross-linking (Fig. 2B). On the other hand, Grp78 co-immunoprecipitated with the G544V mutant LDLR from cells treated with or without cross-linker. The chaperones Grp94, Erp72, and calnexin were only co-immunoprecipitated with G544V mutant LDLR in cells pretreated with cross-linker. These results indicate that the ER-resident chaperones Grp78, Grp94, and Erp72, and calnexin are involved in the retention of the G544V mutant LDLR in ER.

Expression of mutant, misfolded proteins has been shown to induce ER stress and activation of UPR. Accumulation of misfolded proteins in the ER might lead to exhaustion of the protein folding machinery. If so, the cell will adapt to the increased protein burden by up-regulation of the folding capacity of the ER through transcriptional induction of ER-resident chaperones (13). To study whether the expression of G544V mutant LDLR in CHO cells increased the expression of the ER chaperones, mRNA was isolated after CHO cells were stimulated to express G544V mutant LDLR or wild-type LDLR for 24 and 48 h. Northern blot analysis was performed, and as shown in Fig. 3 (A–C), expression of G544V mutant LDLR did increase the levels of the chaperone mRNAs. The increases in mRNA level after 24 h of tetracycline stimulation were 8.3, 4.3, and 2.5 for Grp78, Grp94, and Erp72, respectively. CHO cells expressing wild-type LDLR showed no increase in mRNA levels of Grp78, Grp94, or Erp72 (the effect on calnexin expression was not analyzed). The level of LDLR mRNA expression in CHO cells transfected with wild-type or G544V mutant LDLR was similar (data not shown). Tunicamycin causes ER stress by inhibition of N-glycosylation and is known to increase transcription of chaperones (12). In CHO cells stably transfected with G544V mutant LDLR, adding 2.5 μg of tunicamycin/ml for 24 h resulted in an increase in the mRNA levels of 9.5, 10.2, and 3.3 for Grp78, Grp94, or Erp72, respectively (data not shown). Thus, expression of G544V mutant LDLR for 24 h induces a transcriptional activation of Grp78 and Erp72 at a slightly lower level than tunicamycin.

In transfected CHO cells the expression of LDLR is induced by tetracycline. To examine how fast the cells react to the accumulation of misfolded G544V mutant LDLR in the ER, we stimulated the expression of G544V mutant LDLR for 4–12 h and measured the level of LDLR and Grp78 mRNA. As shown in Fig. 3D, the level of Grp78 mRNA starts to increase after 4 h of stimulation with tetracycline, which indicates a rather immediate UPR response to accumulation of misfolded G544V mutant LDLR.

To study whether the observed increase in Grp78 mRNA was due to increased transcriptional activity, we performed promoter studies using a chimeric construct containing 0.7 kb of the Grp78 promoter containing three ER stress response elements fused to the luciferase reporter (pGrp78-GL3). ER stress response element-mediated transcription has been described to be activated by ATF6 and X-box-binding protein (XBP-1) (26), and ER stress-induced transcription factors. In CHO cells transiently transfected with pGrp78-GL3 and induced to express the G544V mutant LDLR for 4 h, a 2.3-fold increase in transcriptional activity was observed (Fig. 3E). In cells expressing the wild-type LDLR, no significant increase was found. These results indicate that the observed increase in the chaperones mRNA levels is at least partly due to increased transcriptional activity.

To determine whether increased transcription of Grp78, Grp94, and Erp72 led to elevated levels of the corresponding proteins, we performed Western blot analysis of cell lysates prepared from the tetracycline-stimulated cells (Fig. 4). Protein levels of Grp78, Grp94, and Erp72 increased in the cells stimulated to express G544V mutant LDLR, whereas no such increase was observed in the cells expressing wild-type LDLR. In agreement with the mRNA analysis, the highest increase was
observed for Grp78. In addition, a modest increase in the level of calnexin was observed in cells expressing the G544V mutant LDLR.

Three ER-resident transmembrane proteins have been identified as proximal sensors of ER stress: the kinase and endoribonuclease IRE1, the PERK kinase, and the transcription factor ATF6. Grp78 associates with the luminal domains of these sensors, and the activation of all three sensors depends on the dissociation of Grp78. Grp78 is proposed to have higher affinity for unfolded or misfolded proteins than for the ER luminal domains of IRE1, PERK, and ATF6. Accordingly, accumulation of unfolded or misfolded proteins would drive the equilibrium of Grp78 binding away from the three sensor proteins (14). The dissociation of Grp78 would enable IRE1 and PERK to perform homodimerization and autophosphorylation and enable ATF6 to transit to Golgi and undergo proteolytic cleavage to become an active transcription factor. Upon phosphorylation IRE1 gains endoribonuclease activity and catalyzes the removal of a small intron of 26 base pairs from the mRNA encoding the XBP-1. This splicing event creates a translational frameshift in XBP-1 mRNA, which is translated into an active transcription factor (14). XBP-1 is a potent transcription factor involved in transcriptional induction of ER chaperones and proteins involved in ER-associated degradation (27).

To study whether expression of G544V mutant LDLR causes activation of the IRE1, splicing of XBP-1 mRNA was analyzed by RT-PCR of total RNA isolated from CHO cells induced to express wild-type or G544V mutant LDLR for 24 h. Primers flanking the XBP-1 mRNA splice site were used. Splicing of XBP-1 mRNA could be observed after expression of G544V mutant LDLR (Fig. 5A), indicating that the endoribonuclease IRE-1 has been activated. Dithiothreitol, a reducing agent known to cause ER stress, was used as a positive control. To confirm that the spliced XBP-1 is translated into an active transcription factor, we performed promoter studies using a chimeric construct containing five UPR elements (UPRE), identified to be a promoter element where XBP-1 binds (28), fused to the luciferase reporter (p5xUPRE-GL3). Expression of G544V mutant LDLR for 24 h increased the reporter

| FIGURE 3. Analysis of chaperone mRNA. | FIGURE 4. Western blot analysis of chaperones. |
|--------------------------------------|----------------------------------------------|
| The levels of Grp78 (A), Grp94 (B), and ERP72 (C) mRNA in CHO cells transfected with wild-type LDLR or G544V-mutated LDLR and stimulated with tetracycline. The mRNAs were isolated at indicated time points, divided in three equal aliquots, and subjected to Northern blot analysis. The membranes were sequentially hybridized with 32P-labeled cDNAs encoding Grp78, Grp94, or ERP72 and β-actin, and the level of chaperone mRNAs were correlated to the expression of β-actin mRNA. The fold increase was calculated relative to the basal level of chaperone mRNA in the cells transfected with wild type and not stimulated with tetracycline (0 h). One representative experiment of two performed in triplicate (± S.D.) is shown. D, the levels of LDLR and Grp78 mRNA in CHO cells transfected with wild-type LDLR or G544V-mutated LDLR and stimulated with tetracycline. The mRNAs were isolated at indicated time points, divided in two equal aliquots, and subjected to Northern blot analysis. The membranes were sequentially hybridized with 32P-labeled cDNAs encoding LDLR or Grp78 and β-actin, and the level of LDLR and Grp78 mRNAs were correlated to the expression of β-actin mRNA. The experiment was performed twice with similar results. E, the CHO cells stably transfected with wild-type or G544V-mutated LDLR were co-transfected with a luciferase reporter plasmid pGrp78-GL3 (see “Materials and Methods” for a detailed description), and the relative luciferase activity was measured following 24 h of incubation with tetracycline. pRL-SV40-expressing Renilla luciferase was used as an internal control vector, and the activity of the firefly luciferase was normalized against the Renilla luciferase activity. The means of three experiments (± S.D.) performed in triplicate are shown. | CHO cells transfected with wild-type LDLR or G544V mutant LDLR were incubated with tetracycline for the indicated time periods. The cell lysates (20 μg) were prepared and subjected to SDS-PAGE and Western blot analysis using anti-Grp78, anti-Grp94, anti-ERP72, and anti-calnexin antibodies. The experiment was performed twice with similar results. |
activity 2.8 times, whereas expression of the wild-type LDLR had no effect (Fig. 5B). These results indicate that expression of G544V mutant LDLR induces activation of the IRE-1 endoribonuclease, which catalyzes the removal of a small intron from XBP-1 mRNA creating a translational frameshift, which is translated into an active transcription factor.

Another ER-resident sensor for ER stress is PERK. PERK autophosphorylation leads to phosphorylation of the α subunit of the translational initiation factor eIF2, which inhibits the assembly of the 80 S ribosome and results in a general inhibition of protein synthesis. However, the transcription factor ATF4 requires phosphorylated eIF2 to be translated. ATF4 activates transcription of genes, which increases the susceptibility of the cells to various forms of stress, including amino acid deprivation, oxidative stress, and ER stress (29). To study whether expression of G544V mutant LDLR causes activation of PERK, we performed Western blot analysis of cell lysates using an antibody recognizing the phosphorylated (activated) form of PERK. Expression of G544V mutant LDLR for 15 h led to a 1.7-fold increase in the phosphorylation of PERK (Fig. 6), whereas expression of the wild-type LDLR did not have any effect.

**DISCUSSION**

In this study, we have shown that a fusion protein of LDLR and EYFP can be used in functional studies of a disease-causing mutation found in the LDLR gene. The EYFP tag did not have any effect on the maturation rate of the LDLR, nor did it affect the cellular localization or the activity of the receptor in transfected CHO cells. The G544V mutation has previously been described as a class 2a mutation (20, 21). In accordance with these previous observations, we showed that the G544V mutant LDLR is expressed in the precursor form co-localized with an ER marker.

Our aim was to identify chaperones associated with the LDLR in the ER and to analyze whether the retention of mutant LDLR in the ER had any cellular consequences. We examined four different chaperones (Grp78, Grp94, Erp72, and calnexin) to determine which ones associate with the wild-type LDLR and the G544V mutant receptor. Whereas only Grp78 was found to be weakly associated with the wild-type LDLR, Grp78, Grp94, Erp72, and calnexin were found to interact with the G544V mutant LDLR. The lack of observed interaction of Grp94, Erp72, and calnexin with the wild-type LDLR could be explained by the fact that the folding and maturation of wild-type LDLR in our model system was fast, and only diminutive amounts of the ER-localized precursor form were present at the time of immunoprecipitation.

Grp78 has previously been identified by Jorgensen et al. (25) to interact transiently with the wild-type LDLR and more firmly with two mutant forms (W556S and C646Y) of the LDLR in transfected Chang cells. The W556S and C646Y mutant LDLRs exhibit complete ER retention, which they have in common with the G544V-mutated LDLR. This indicates that Grp78 might be a general factor in ER retention of class 2a mutant LDLRs. The role of Grp78 in protein folding is well documented (30, 31). Grp78 is described to interact with hydrophobic protein regions exposed in unfolded or misfolded proteins, assisting their folding and assembly to prevent aggregation (31). Grp94 is the ER homologue to the cytosolic chaperone Hsp90, and it is abundant in ER (8). Although several proteins have been described to associate with Grp94 in ER, its role in protein folding is poorly understood. Erp72 is a member of the thioredoxin family, which catalyzes the formation of disulfide bonds in substrate proteins or performs isomerization and reduction reactions (32). Newly synthesized LDLR has been shown to fold in a nonvectorial way, forming transient non-native disulfide bonds between distant cysteines before isomerization and formation of native short range cysteine pairs (33). If not the only thioredoxin involved, Erp72 could be essential in this folding process. Calnexin is a lectin that recognizes monoglycosylated oligosaccharides (34). LDLR is a glycoprotein with N-linked oligosaccharides located at asparagine 135 and 251 (35) and is therefore a likely candidate for interaction with calnexin.

Meunier et al. (36) demonstrated that Grp78, Grp94, and Erp72 were organized in large multiprotein complexes in the ER together with several other chaperones. Calnexin was not found to be associated with these complexes. ER contains several different chaperone complexes or...
systems, and networks of chaperones are postulated to perform transient sequential interactions with folding proteins (9, 36, 34). Our results indicate that the G544V mutant LDLR associates both with a similar Grp78-containing chaperone complex, as described by Meunier et al. (36) and with calnexin. Whether the interaction is contemporary or sequential remains to be investigated.

Accumulation of misfolded proteins in ER have been described to cause ER stress and induction of a coordinated adaptive process called the UPR (12, 13). The overall aim for UPR is to augment the folding capacity of the ER and to increase the ability of the cell to eliminate misfolded proteins by ERAD. One hallmark of UPR is the transcriptional induction of genes encoding ER-resident chaperones and folding enzymes (37). Synthesis of G544V mutant LDLR in CHO cells caused a considerable increase in the expression of Grp78 and a smaller but significant increase in Grp94, ERP72, and calnexin, indicating that the accumulation of misfolded LDLR in the ER induced ER stress. Expression of all of these chaperones have previously been reported to be induced upon ER stress (38). The UPR is known to be mediated by three ER stress sensors located in the ER membrane, ATF6, IRE1, and PERK (14). Accumulation of misfolded LDLR in ER caused enhanced transcription from promoters containing ER stress response elements and UPREs, which are known to be mediated by activation of the ER stress sensors ATF6 and IRE1 (28). IRE1 induces gene expression by catalyzing a frameshift splicing of XBP-1 mRNA (39). The spliced XBP-1 mRNA encodes a potent basic leucine zipper transcription factor. An increase in frameshift splicing of XBP-1 mRNA was seen in the cells expressing mutant LDLR but not wild-type LDLR. In addition, ER retention of mutated LDLR caused a significant increase in PERK phosphorylation.

Taken together, induced synthesis of G544V mutant LDLR for 24 h in CHO cells caused ER stress and activation of UPR. However, the increased expression of chaperones did not improve the ability of the G544V mutant LDLR to fold and to be transported to the Golgi apparatus and further to the cell surface. Further analysis of fibroblasts and lymphocytes from FH patients with class 2a mutant LDLRs will reveal whether the observed effects are found in cells expressing endogenous mutant LDLR.

Travers et al. (15) demonstrated that the capacity of the ERAD system is readily saturated under conditions of ER stress and that the UPR was necessary for efficient ERAD function. Li et al. (40) showed that proteasomal degradation is the principal pathway for class 2 mutant LDLR degradation. The 26 S proteasome is described to be the main factor in the ERAD pathway where it degrades abnormal proteins translocated from the ER (41). The induction of the UPR caused by the expression of mutant LDLR in the CHO cell might be beneficial for the cell by enhancing the capacity of the ERAD system of the cell. Kim and Arvan (8) suggested that the cellular responses to the synthesis of proteins unable to fold correctly might differ between mutations, between tissues, between individual patients, and between different physiological states in the same patient. Thus, these variations might explain some of the phenotypic variations observed between patients with FH.

The pathological outcomes of a variety of diseases, including diabetes (42), cystic fibrosis (43), Alzheimer disease (44), and α1-antitrypsin deficiency (45), have been linked to ER stress. In α1-antitrypsin deficiency, accumulation of α1-antitrypsin in the ER has been shown to cause ER stress in the hepatocytes, and the ER stress is proposed to be the cause of the liver failure observed in severe cases of α1-antitrypsin deficiency (45). However, there exists no medical description of FH patients supporting the possibility that ER stress contributes to the pathological outcome of FH. The primary aim of UPR is to limit damage to the cell by adapting the cell to the situation causing the ER stress. However, increased UPR caused by prolonged stress or the additive effect of several inducers might induce apoptosis of the cell (12). In FH patients with class 2a mutations, the UPR response to ER retention of misfolded LDLR might protect the cells by increasing the ability of the cells to handle misfolded LDLRs. However, the cells might have increased sensitivity to other inducers of ER stress such as hypoxia, glucose deprivation, oxidative stress, and homocysteine (38, 46–48).

Treatment of heterozygous FH patients with agents that increase the LDLR expression might increase ER stress in the cells because of increased expression of the misfolded LDLR as well. A number of studies published in the last few years described a group of low molecular weight compounds known as chemical chaperones that are able to assist folding of mutant proteins and increase the fraction of correctly folded proteins located at the appropriate cellular destination (49, 50). As shown for cystic fibrosis conductance transmembrane regulator, the biological activity of an ER-retained protein might be preserved, although the protein is transport-deficient (51). If a LDLR mutation causes a situation of transport deficiency without damaging the biological activity of the receptors, chemical chaperones could represent a new concept of therapy to increase the level of LDLR at the cell surface, thus reducing the plasma LDL cholesterol.

In summary, we have in our model system of transfected CHO cells identified three chaperones associated with a class 2a mutated LDLR in ER and described for the first time that retention of misfolded LDLR in ER causes ER stress and activation of UPR.

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