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Grp78: An Important Factor in the Protein Quality Control of the Low Density Lipoprotein Receptor

Jørgensen, Malene Munk

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Malene Munk Jørgensen, October 2002
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>6-AN</td>
<td>6-aminonicotinamide</td>
</tr>
<tr>
<td>6-FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>aa</td>
<td>amino acids.</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ATTC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BiP</td>
<td>Immunoglobulin Heavy Chain Binding Protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CEPT</td>
<td>Cholesterylester transfer protein</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Cnx</td>
<td>Calnexin</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1´-di-octadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotides</td>
</tr>
<tr>
<td>DSP</td>
<td>[Dithiobis(succinimidylpropionate)]</td>
</tr>
<tr>
<td>DTE</td>
<td>Dithioerythritol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>eIF1α</td>
<td>Translation initiation factor 1α</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation</td>
</tr>
<tr>
<td>ERSD</td>
<td>Endoplasmic reticulum storage disease</td>
</tr>
<tr>
<td>ERSE</td>
<td>Endoplasmic reticulum stress element</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FH</td>
<td>Familial Hypercholesterolemia</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>Grp</td>
<td>Glucose regulated protein</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HMG CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IRE</td>
<td>Inositol requiring</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome elongation sequence</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KDEL</td>
<td>The amino acid sequence: Lys-Asp-Glu-Leu.</td>
</tr>
<tr>
<td>KKXX</td>
<td>The amino acid sequence: Lys-Lys-X-X (X: any amino acid)</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lichithin-cholesterol-acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLr</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor related protein</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser-desorption ionization-time-of-flight</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polycrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like Endoplasmic Reticulum kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>Interferon-inducible protein kinase</td>
</tr>
<tr>
<td>PPI</td>
<td>Peptidylprolyl isomerase</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor-associated Protein</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol response element</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TESS</td>
<td>Transcription element search software</td>
</tr>
<tr>
<td>TM</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>UGGT</td>
<td>UDP-glucose:glycoprotein-glucosyltransferase</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Xbp1</td>
<td>X-box binding protein 1</td>
</tr>
</tbody>
</table>
Introduction

It is widely believed that elevated plasma levels of cholesterol play a central role in the development of atherosclerosis. The risk of coronary heart disease is directly related to the plasma level of low-density lipoprotein (LDL) cholesterol and inversely related to plasma high-density lipoprotein (HDL) cholesterol levels [1, 2, 3]. Lowering the level of LDL cholesterol diminishes the risk of coronary events and improves survival [4], underscoring the importance of identifying individuals with elevated plasma cholesterol levels, especially those with concomitant risk factors for developing premature coronary heart disease. Genetic and dietary factors as well influence plasma LDL cholesterol levels, but detailed knowledge of their complex interplay is not yet well defined. Twin and family studies suggest that about 50% of the observed inter-individual variation of the LDL and HDL levels is caused by genetic factors [5, 6].

Mutations in the LDL receptor gene are responsible for the relatively common Mendelian inherited disorder Familial Hypercholesterolemia (FH), occurring with a frequency of 1 in 500 in most populations, but accounting only for about 5% of coronary artery disease in middle aged patients [7]. Similar to the general population a significant inter-individual variation is observed in the degree of hypercholesterolemia, and in the onset of atherosclerotic disease symptoms among heterozygous FH patients [8, 9, 10]. In FH and in the general population as well the variation is caused by a combination of environmental factors and genetic factors. The genetic variation cannot be ascribed to sequence variations in the LDL receptor gene alone [11, 12]. Actually, in most families segregation of plasma LDL concentrations is not consistent with simple Mendelian inheritance, suggesting that inherited variation in LDL concentrations presumably reflects a combination of common sequence polymorphisms in genes that modulate the regulation, biosynthesis, and clearance of cholesterol.

Most of LDL cholesterol is cleared from plasma by LDL receptor mediated up-take. After synthesis the LDL receptor is folded and modified in the lumen of the endoplasmic reticulum (ER). The folding process is assisted by molecular chaperones; a diverse group of proteins, which in addition to assist protein folding, also takes part in quality control reactions. The term “quality control” has been coined to describe the phenomenon whereby only properly folded or assembled proteins are exported from the ER. About half of the characterized mutations in the LDL receptor gene lead to LDL receptor protein which is retained in the ER, indicating that misfolding is a frequent cause of defective LDL receptors [13], and that protein folding and processing events are directly implicated in the pathophysiology of FH. We hypothesized that individual variations in the regulation or function of the quality control system could contribute to explain the observed variations in the plasma LDL concentration both among FH patients and in the general population. Nevertheless, when this project was initiated only limited information about which factors assisted the folding and maturation of the LDL receptor was available. Therefore, a main purpose of this study was the identification of chaperones interacting with the newly synthesized LDL receptor.
Background

Lipoprotein Metabolism
The lipids cholesterol and triglycerides have very important cellular functions owing to the fact that triglycerides are an energy source, while cholesterol is the raw material for the manufacturing of steroid hormones and bile acids. A drawback for cholesterol is that the very same property making it useful in cell membranes, namely its absolute insolubility in water, also makes it lethal. Simplified, the insolubility of cholesterol in water can cause an accumulation of cholesterol in the wrong place, for example within the wall of an artery. In order to maintain a low concentration of cholesterol in the blood and to lower its tendency to escape from the bloodstream as well, multicellular organisms esterify the sterol with long-chain fatty acids. These esters are packed inside the hydrophobic cores of plasma lipoproteins. The function of the lipoproteins is primarily to transport lipids, mainly the triglycerides and cholesteryl esters from their sites of synthesis and absorption, to sites of utilization and storage.

The lipoproteins are classified into five main types according to their densities (reviewed in [14]). The largest and least dense lipoprotein consists in the chylomicrons, after this the very low density lipoproteins (VLDL), the intermediate density lipoproteins (IDL), the low density lipoproteins (LDL), the most abundant cholesterol-carrying lipoprotein in human plasma, and finally the high density lipoprotein (HDL).

The lipid transport system can be divided into three pathways (Fig. 1). The exogenous pathway transports dietary lipids and fat-soluble vitamins from the gut to the tissues, while the endogenous pathway transports the lipids synthesized by the liver to the tissue. Finally, the reverse cholesterol transport transports excess cholesterol and cholesteryl esters from peripheral tissues back to the liver for excretion (reviewed in [14]). The three pathways are briefly described below.

The Exogenous Pathway: After absorption from the intestine, dietary triglyceride and cholesterol are secreted into lymph as chylomicrons, which carry apo B-48 as their structural protein. In plasma, an exchange of apolipoproteins between chylomicrons and HDL takes place, and an increase in the amount of apo C and apo E on chylomicrons will be noted. Apo C-II activates the enzyme lipoprotein lipase (LPL), which is present on endothelial cells in adipose tissue and muscle. This enzyme hydrolyses triglycerides from the chylomicron core, and liberated fatty acids are taken up in adipose tissue and muscle in which they are re-esterified to triglycerides and stored for energy reserve. The remaining particles, chylomicron remnants, return some apolipoproteins and surface structures such as phospholipids to HDL, and they are then taken up by the liver through receptor-mediated endocytosis dependent on apo E.

The Endogenous Pathway: In the liver, endogenous lipoprotein is synthesized, especially VLDL is a characteristic particle of this pathway. It consists mainly of apo B-100 and triglycerides.
These triglyceride-rich particles go through a corresponding successive catabolism through the influence of LPL, and a remnant particle, IDL, is formed. IDL may either be taken up by hepatic LDL receptors, or progressively further catabolysed to LDL, which solely contains apo B-100 as apolipoprotein. LDL may then be taken up by specific LDL receptors [15] localized in the liver and in the peripheral tissue. A problem with LDL is that it is cleared very slowly and shows a tendency of accumulating in the plasma.

The LDL receptor is responsible for taking up most of the LDL, and it in position to recognize, bind and remove chylomicron remnants, VLDL remnants, and IDL too. The apolipoproteins enable the LDL receptor to distinguish and bind the different lipoproteins. If the LDL receptor is defect, the rate of clearance of LDL is decreased, and consequently the concentration in plasma is raised. When the LDL receptor activity is defective, less VLDL remnants are cleared, and therefore a higher amount is available to be converted to LDL. The removal of LDL is impaired, resulting in an increased concentration of LDL in plasma and an increase in its half-life in plasma, which shows in the peripheral tissues in xanthomata and an accelerated rate of atherosclerosis.

Reverse Transport: Reverse cholesterol transport is the process in which peripheral cells release cholesterol to an extra cellular acceptor such as high-density lipoprotein (HDL) which then mediates cholesterol delivery to the liver for excretion (reviewed in [16]). Reverse cholesterol transport represents a physiological mechanism by which peripheral tissues are protected against excessive accumulation of cholesterol. The enzyme lecithin-cholesterol acyltransferase (LCAT) is important in this transport through its action in the removal of free cholesterol on the HDL surface into the core of the HDL particle. The esterified cholesterol can then be transferred to other lipoproteins, such as IDL and chylomicron remnants, under the influence of the cholesterylester transfer protein (CETP).

The LDL receptor is not the only way to remove LDL from plasma; in fact one-third of plasma LDL is degraded by LDL receptor independent pathways [7]. However, only limited information about the responsible cellular and biochemical mechanisms is available. It has been suggested that the receptor independent removal of LDL is divided about equally between the liver and the extra hepatic tissues [17, 18]. Some of the plasma LDL is taken up by macrophages which express receptors for LDL that has been altered by for example oxidation or by complexing with other molecules. These receptors are called “scavenger receptors” [19]. This uptake of modified LDL results in conversion of macrophage to foam cells as seen in vivo in xanthomata and atherosclerotic plaques [19, 20].
Figure 1. Simplified scheme of lipoprotein metabolism in man showing the exogenous pathway for chylomicron mediated transport of dietary lipids and fat-soluble vitamins, the endogenous pathway for VLDL mediated transport of liver synthesized lipids, and finally, the reverse cholesterol transport of excess cholesterol from peripheral tissue and back to the liver. LPL: Lipoprotein lipase. LCAT: Lecithin-cholesterol acyltransferase.

Familial Hypercholesterolemia
Familial hypercholesterolemia (FH) is an autosomal dominant inherited disorder, caused by mutations in the LDL receptor gene. The disorder is clinically characterized by elevated concentration of LDL cholesterol, the presence of tendon xanthomata, and a family history of identical symptoms in a first degree relative. FH is one of the most common inherited metabolic disorders, occurring in about 1 in 500 individuals in most populations. Heterozygous FH patients tend to have a 2- to 3-fold increase in the concentration of LDL in plasma (6.0-14.0 mmol/l)
resulting in a 25-fold increased risk of premature CHD compared to the general population [21]. Consequently, heterozygous FH is associated with a substantial excess mortality from CHD especially among young adults [22]. Homozygous FH is a rare disorder with an estimated frequency of 1 in 10^6. However, it is a more severe disorder compared to heterozygous FH, characterized by a 6- to 8-fold (≥15 mmol/l) increase in plasma LDL levels and often resulting in death from myocardial infarction before age 20.

Treatment of FH is aimed at reducing levels of LDL cholesterol in order to retard progression of atherosclerotic lesion and decrease risk of CHD. The transcription of the LDL receptor gene is maintained under tight feedback regulation by cellular levels of sterols, and it is possible to up-regulate the LDL receptor gene by keeping the cholesterol level inside the hepatocytes low (reviewed in [7]). Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which catalyzes the committed step of the cholesterol synthesis in the cell. Suppressing the intracellular cholesterol synthesis by statin treatment significantly lower plasma LDL cholesterol levels in heterozygous FH patients. A recent study showed that high dose treatment of FH patients with Simvastatin reduced LDL cholesterol levels by 48% to mean levels of 4.29 mmol/l [23]. Combined with diet and other lipid lowering drugs, such as bile-acid binding resins, the treatment of heterozygous FH is quite effective. In contrast, patients with homozygous FH respond little to any drug therapy owing to the fact that they posses no or only few functionally LDL receptors to be stimulated. The preferred treatment in homozygous FH today is selective removal of apo B-containing lipoproteins by LDL aphaeresis, combined with statin treatment [24].

The Low-Density Lipoprotein Receptor Gene Structure
The LDL receptor gene is located at the distal end of the short arm of chromosome 19 (19p13.1), it is approximately 45 kb in length, and it is comprised of 18 exons and 17 introns [25]. The cDNA is 5.3 kb long of which 3.5 kb is coding. More of the exons share evolutionary history with exons of other genes and a correlation from the exons to the functional domains of the protein can be drawn. Exon 1 encodes the signal sequence responsible for targeting of the receptor to the ER (Fig. 2). Exon 2-6 encode the ligand binding domain, and exon 7-14 encode a region that shares sequence identity to the human epidermal growth factor (EGF) precursor gene. This domain contains three 40 amino acid cysteine-rich growth factor repeats, which are designated A, B, and C. The A and B repeats are separated from the C repeat by five repeats containing the conserved amino acid motif Tyr-Trp-Thr-Asp usually referred to as YWTD-repeats. Exon 15 encodes 58 amino acids that are enriched in serine and threonine residues, many of which serve as attachment sites for O-linked carbohydrate chains. Exon 16 and the 5’ end of exon 17 encode the membrane-spanning domain that anchors the protein to the cell membrane. The remainder of exon 17 and the 5’ end of exon 18 encode the cytoplasmic part of the protein containing the signal that clusters the receptor in clathrin-coated pits.
The Promoter Region of the LDL Receptor Gene

Expression of the LDL receptor is regulated by the abundance of sterols within the cell. When cholesterol is plentiful, transcription of mRNA for the receptor becomes minimal. Conversely, when the cell is deprived of cholesterol, transcription is strongly stimulated. The basic regulatory region of the LDL receptor is located within 177 bp of the proximal promoter and consists of two TATA-like sequences and three imperfect direct repeats of 16 bp (Fig. 3).

Figure 2. Domain structure of the LDL receptor. The domains of the 839 amino acid receptor protein are shown at left and the corresponding exons are shown in blue. (Redrawn from Hobbs et al. 1990 [13].

Figure 3. Simplified schematic representation of the promoter region of the LDL receptor gene. See text for details.

Repeat 1 and 3 contain binding sites for the transcription factor Sp1 and contribute to basal transcription of the LDL receptor gene. The target for sterol regulation is a 10 bp sterol response.
element (SRE-1), which is recognition site for the transcription factor sterol regulatory element-binding protein (SREBP), a conditionally positive transcription factor that is active only under conditions of sterol deprivation [26]. In response to sterol deprivation SRERP is activated by the Golgi localized site-1 protease (S1P) and site-2 protease (S2P) [27]. In order to be processed by S1P, SREBPs must be transported to the Golgi complex by the escort protein: SREBP cleavage-activating protein (SCAP) (Fig. 4). Sterols block the exit of the SCAP/SREBP complex from the ER, thereby blocking SREBP processing and repressing their own synthesis and the LDL receptor synthesis [28]. The S1P and S2P proteases are also known for being important factors in activation of the “unfolded protein response” (UPR) system by cleavage of the ATF6 transcription factor [29], which will be discussed later in this thesis. In addition, the LDL receptor transcription can be regulated by non-sterol mediators such as growth hormone and cytokines [30, 31].

**Figure 4.** Model for the sterol-mediated proteolytic release of SREBP from membrane. SCAP is sensor of sterols and an escorter of SREBP. When cells are depleted of sterols, SCAP transports SREBP from the ER to the Golgi. Release of SREBP from the membrane is initiated by S1P. S2P cleaves the N-terminal basic helix-loop-helix (bHLH) domain of SREBP, which is transported to the nucleus, where it activates transcription of the LDL receptor and multiple other genes involved in the biosynthesis of cholesterol and fatty acids as well. W: protein/protein interacting domain. R: Regulatory domain of SREBP. (Redrawn from Goldstein *et al.*, 2002 [32]).

**The LDL Receptor Protein**

The LDL receptor is a cell surface glycoprotein which is in a position to recognize and bind apo B-100, the sole protein of LDL and apo E, which exists in multiple copies in IDL and a subclass of HDL. The LDL receptor consists of 860 amino acids including a N-terminal signal sequence of 21 amino acid residues which is cleaved from the receptor immediately after it is translated,
leaving a receptor protein of 839 amino acids (Appendix 1). Based on its primary sequence the LDL receptor is proposed to consist of 5 different domains (Fig. 2) [13]. Although the structure of different parts of the LDL receptor is elucidated, the overall 3D structure is still unknown.

- **Ligand binding domain.** The most N-terminal located domain is the ligand binding domain. It consists of seven complement-like ligand binding repeats, each with a sequence length of about 40 amino acids. The repeats are stabilized by three disulfide bonds and a Calcium ion. High affinity binding of apo B-100 and apo E requires different combinations of repeats, but repeat 5 is crucial for binding of both lipoproteins [33].

- **Epidermal growth factor (EGF) precursor homology domain:** The second domain consists of about 400 amino acids, and contains three EGF repeats (Fig. 2 A, B and C). The A and B repeats are separated from the C repeat by five YWTD repeats. This domain is necessary for dissociation of lipoprotein from the LDL receptor at low pH in the lysosomes and recycling of the receptor to the cell surface.

- **O-linked glycan domain:** About two third of the eighteen O-linked glycans that are present in the mature LDL receptor is clustered in the O-linked glycan domain. The role of the O-glycosylation in the LDL receptor is not totally elucidated. Deletion does apparently not affect expression, binding or recycling of the receptor, at least not in fibroblasts [34]. Furthermore, deletion of exon 15 of the LDL receptor gene is associated with a mild form of FH [35]. It has been suggested that the O-glycosylation protects the receptor against proteolytic cleavage or serve to keep the receptor protein in an upright position to ease the ligand binding [34].

- **Transmembrane domain:** The fourth domain is the membrane spanning domain, consisting of 22 amino acids. Receptors lacking this domain are directly secreted into the extra cellular medium.

- **Cytosolic domain:** The cytosolic domain comprises the 50 C-terminal amino acids, and contains two discrete signals, one for localizing the receptor in clathrin coated pits and another for efficient basolateral targeting of the receptor [36].

**The Life Cycle of the LDL Receptor**

The synthesis of the LDL receptor occurs on membrane bound ribosomes attached to the endoplasmic reticulum (ER) with the N-terminus of the nascent peptide inserted into the ER (Fig. 5). The 21 amino acid long precursor protein is cleaved off in the ER, afterwards N-linked carbohydrate chains and the core sugar (N-acetylgalactosamine) of the O-linked sugar chains are added resulting in a protein which runs with an apparent molecular mass of 120 kDa on reducing SDS-PAGE gels. After approximately 30 minutes the precursor is transported to the Golgi complex, where the N-linked oligosaccharide chains are converted to the complex endoglycosidase H-resistant form. At the same time, each O-linked chain is elongated by addition of a galactose residue and one or two sialic acid residues. This change in mass and conformation results in a change in electrophoretic mobility causing the receptor to run with an apparent mass
of 160 kDa. Approximately 45 minutes after synthesis, the LDL receptor appears on the cell surface. Attached to the plasma membrane the LDL receptor is in a position to bind LDL, gather in coated pits, and be taken up by the cell by endocytosis. The gathering in coated pits and the endocytosis occurs whether LDL is bound to the receptor or not. After endocytosis the clathrin coat dissociates and multiple endocytic vesicles fuse and create larger sacs of irregular contour called “endosomes” or “receptosomes”. An ATP-driven proton pump placed in the membrane of the endosome lowers the internal pH to about 6.5 resulting in release of the ligand from the receptor. The empty receptor molecules tend to cluster in one end of the endosome. The receptors are returned to the membrane by recycling vesicles. These vesicles are formed when a segment of the endosome membrane containing the LDL-receptors is pinched off. Once the LDL-receptors reach the cell surface they can bind another molecule of LDL and initiate another cycle of endocytosis. The receptors recycle in this fashion more than one hundred times, making a complete trip in about 10 minutes. The LDL released from the receptor is transported to a lysosome when the membranes of the endosome and lysosome fuse. In the lysosome the cholesteryl esters are hydrolysed by a lysosomal acid lipase and cholesterol is liberated. The free cholesterol released in this way is used for membrane synthesis or stored in the cell as droplets of oil for later use. Furthermore, the free cholesterol mediates a sophisticated system of feedback control stabilizing the intracellular cholesterol concentration (Fig. 5). Firstly, the cholesterol suppresses the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) and

Figure 5. The Life Cycle of the LDL Receptor. See text for details. (Redrawn from Brown and Goldstein, 1985, [37].
thereby down-regulates the cellular cholesterol synthesis. Secondly, free cholesterol activates acyl CoA:cholesterol acyl transferase (ACAT), the enzyme catalyzing reesterification of the cholesterol for storage inside the cell. Thirdly, the free cholesterol suppresses the synthesis of new LDL receptors. The overall effect of this regulatory system is to coordinate the intracellular and extra cellular sources of cholesterol so as to maintain a constant level of cholesterol within the cell.

**Mutations in the LDL Receptor Gene**

Over the past 30 years more than 700 different mutations have been identified in the LDL receptor gene comprising mostly point mutations introducing missense, nonsense, promoter or intronic splice site mutations. In addition, more deletion and insertion mutations have also been reported. (http://www.umd.necker.fr, http://www.ucl.ac.uk/fh). The mutations are distributed over the entire gene and some of the mutations have been classified into different classes based on LDL receptor function in cultured cells. Mutations in one particular domain are often associated with each class. However, there are exceptions and many mutations do not fall clearly into one single category. Due to the huge number of newly identified, different mutations in the LDL receptor, most of the mutations have not been tested in cells grown in culture, and therefore they are not classified with respect to the five different classes described below (Fig. 6).

- **Class 1 mutations**: In this class no mRNA is detectable or no receptor protein or only trace amounts is synthesized. The mutations causing this phenotype are mutations leading to a premature termination codon, a deletion of the LDL receptor gene or mutations in the promoter region of the LDL receptor gene. The premature termination codon or a deletion of the LDL receptor gene may lead to a destabilization of the mRNA. These kinds of mutations are located in all domains of the LDL receptor.

- **Class 2 mutations**: Encode LDL receptor proteins which are retained either partially or totally in the ER. This class is subdivided into two classes; 2A (no mature receptor protein reaches the cell surface) and 2B (maturation of precursor to mature protein is delayed). The mutations leading to this phenotype are primarily located in the EGF homology domain and in the ligand binding domain.

- **Class 3 mutations**: The mutations in this class are the mutations leading to a receptor which is not in a position to bind LDL. The mutations that lead to this phenotype tend to be clustered in the ligand binding domain.

- **Class 4 mutations**: Encode a mutant receptor protein that can bind LDL but fails to localize in clathrin-coated pits, leading to an internalization-defective LDL receptor. These mutations are found in the cytoplasmic tail of the receptor.

- **Class 5 mutations**: The mutations in this class lead to a recycling-defective receptor in which the LDL receptor fails to dissociate from the ligand in the endosomes, and thereby fails to return to the cell surface. The mutations that lead to this phenotype tend to be clustered in the EGF homology domain.
**Figure 6:** Classification of LDL receptor mutations based on abnormal function of the mutant protein in cell grown in culture. The numbers given in red denote the class of mutation, and the X denotes the step in the LDL receptor “life-cycle” that is disrupted by each class of mutations. (Redrawn from Hobbs et al., 1990 [13]).

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Protein Folding and Quality Control in the Endoplasmic Reticulum

The endoplasmic reticulum (ER) is the largest organelle of the endomembrane system with respect to its surface. It encloses a lumen which forms a continuum with the lumen of the nuclear envelope and it possesses two essential functions. First, secretory and membrane associated proteins are synthesized on the ER surface. During translation the nascent polypeptides are translocated into the ER lumen via the translocon (reviewed in [38]). In the lumen they are folded

Figure 7: Overview over the co-translational protein folding and degradation in the ER. Chaperones bind immediately to the newly synthesized polypeptide (1), which initiates folding co-translationally (2). If the protein folds into a transport competent conformation the chaperones will dissociate from the protein (6) and it can be transported to the Golgi complex (7). Alternatively, if the protein is unable to fold correctly, for example due to mutations in the gene encoding the protein, or due to cellular stress, the chaperones will stay associated with the protein (8). The chaperone/protein complex will accumulate in the ER until it has been transported out of the ER and degraded (9-11). Accumulation of mutant proteins in the ER induces the synthesis of more chaperones by the unfolded protein response pathway (12) (From Kuznetsov et al., 1998, [39]).
and modified. Second, synthesis of lipids and cholesterol takes place on the cytoplasmic site of the ER membrane, making the ER the production site for essential component of cellular membranes, proteins, lipids, and steroids. The lumen of the ER comprises a unique milieu for the folding and secondary modifications of newly synthesized proteins. The lumen contains a specialised subset of molecular chaperones and protein-modifying enzymes assisting folding and maturation. In addition, the chaperones are part of a quality control system ensuring that only properly folded proteins exit the ER. Other unique features are the more oxidizing environment as compared to the cytosol, allowing the formation of disulfide bridges, a unique glycosylation apparatus, and the presence of high Ca$^{2+}$ concentration. Conclusively, the ER can be regarded as specialized environment supporting the biosynthesis of proteins which cannot be produced in the cytosol. (Fig. 7).

**Molecular Chaperones and Folding Enzymes of the ER**

![Figure 8](image)

**Figure 8.** Schematic representation of ER resident chaperones and folding enzymes. The proteins have been grouped according to their function. Some overlaps between the groups can be found; e.g. PDI, P5 and ERp72 have been described both as folding enzymes and classical chaperones. Further to this, the lectins calnexin, calreticulin, and calmegin act as classical chaperones but bind preferentially glycosylated proteins.

The folding process of the nascent polypeptide chain starts immediately after translocation of the N-terminus into the lumen of the ER. The overall speed and efficiency of exportable protein folding is enhanced through a combination of interactions with ER resident chaperones and folding enzymes. Molecular chaperones are defined as proteins assisting the self-assembly of other polypeptides through transient interactions, and preventing unwanted inter- and intramolecular interactions that may induce aggregation [40]. Folding enzymes are true enzymes,
which during interaction with substrate proteins lower the activation energy required for a
discrete conformational change. A well known example is peptidylprolyl isomerase (PPI), which
catalyses the cis-trans isomerisation of proline side chains.

To ensure ER residency of chaperones and folding enzymes in the ER lumen, most of the
chaperones harbour specific retrieval signals. The best characterized signals are the amino acid
sequences KDEL and KKXX (X is any amino acid) motifs, which ensure that the chaperones are
retrieved to the ER from the Golgi through receptor mediated transport. Retention signals exist
as well but are less well defined [41, 42].

**Glucose Regulated Protein 78 (Grp78)**

Grp78, also known as Immunoglobulin Heavy Chain Binding Protein (BiP), is one of the best
characterized proteins in the lumen of the ER. Grp78 is a member of the heat shock protein 70
(Hsp70) family which is a family of proteins localized in all major compartments of the
eukaryotic cell [43]. The Grp78 protein is distinguished from the other family members by the
KDEL ER retrieval sequence, but shares remarkably homology with the other Hsp70 family
members especially in the substrate-binding domain. Similar to the other members of the Hsp70
family, the substrate binding domain of Grp78 most likely possesses a peptide-binding channel
[44]. Grp78 is known to interact optimally with hepta-peptides harbouring the amino acid motif
Hy(W/X)HyXHyXHy, where Hy is a bulky aromatic or hydrophobic residue (Trp, Phe, Leu, Met
or Ile), W is tryptophan, and X is any amino acid [45]. In properly folded proteins such stretches
are normally buried in the core of the protein. However, some misfolded proteins were found to
show prolonged interaction with Grp78 [46, 47, 48]. Such prolonged interaction may occur via
persistent re-association of Grp78 with the substrate protein, in which the hydrophobic binding
sites are not buried due to the misfolding. Cyclic binding and release of substrate polypeptides is
ATP dependent. Like the other member of the Hsp70 family, Grp78 consists of two major
domains; an ATPase-domain and a substrate-binding domain (Fig. 9). The two domains
communicate to regulate the affinity and duration of the polypeptide binding, resulting in cyclic
binding and release of unfolded proteins chaperoned by Grp78. The duration of each cycle
depends on the rate of which Grp78 undergoes exchange of ATP for ADP and ATP hydrolysis.
Thus an unfolded polypeptide chaperoned by Grp78 may undergo cycles of binding and release
until it is properly folded and that the Grp78 binding motif is no longer exposed.

In addition to being a classical chaperone Grp78 function is considered being important at
multiple stages during the synthesis and maturation of transmembrane and secretory proteins.
Grp78 is involved in post-translational translocation of nascent polypeptides into the lumen of the
ER [49]. Grp78 seals the luminal side of the translocon when no ribosomes are attached [50], and
during integration of transmembrane segments during protein translocation [51]. Grp78 is also
involved in retrograde translocation of proteins, which are transported out of the ER for
degradation by the proteasome [52]. Further to this, Grp78 is a major calcium binding protein in
the ER lumen [53]. Calcium plays an important role in the regulation of ER function and hence protein folding.

Grp78 can be post-translationally modified by ADP ribosylation and by phosphorylation. However, only unmodified monomeric Grp78 molecules are identified to be associated with unfolded proteins [55, 56]. Thus post-translational modification of Grp78 is not important for the chaperone function of Grp78, rather it may play a role in the tight regulation of cellular Grp78 protein level. Taken together, Grp78 is an essential gene product that even the simplest eukaryotes cannot live without [54].

Grp78 is one of the key components of the unfolded protein response (UPR) pathway, which will be discussed later in this thesis. The level of Grp78 transcription is extensively increased by accumulation of unfolded proteins in the ER lumen. However, Grp78 is not regulated solely at the transcriptional level. A functional internal ribosome elongation sequence (IRES) was identified in the Grp78 mRNA, suggesting that Grp78 is further regulated at the translational level [57, 58]. Further to this, a recent study showed that Grp78 is tightly regulated at the translational level during cellular stress, and that this stress-dependent regulation is independent of 5′ and 3′ un-translated regions, including the IRES sequence [59].

**Figure 9.** Structure of Grp78. Like other members of the Hsp70 family, Grp78 is composed of an ATPase domain which is highly conserved and a ligand binding domain, held together by a small, flexible linker sequence. In addition the newly synthesized Grp78 possesses an N-terminal leader peptide that target Grp78 to the ER and is cleaved from the mature protein, and a C-terminal KDEL sequence ensuring that Grp78 it retrieved in the ER. The schematic structure of the 3-dimentional structure show the ATPase domain from bovine hsc70 (PDB: 3HSC) and the substrate binding domain of E.coli DnaK (PDB: 1DKX), in the ATP binding conformation (substrate shown in green), from which high resolution X-ray diffraction data was available.
Glucose Regulated Protein 170 (Grp170)
Grp170 is member of a sub-class of the Hsp70 super-family. It is retrieved in the ER due to a NDEL sequence and it is co-regulated with the other glucose regulated proteins by the UPR pathway [60]. The function of Grp170 is poorly characterized. However, Grp170 was shown to bind peptides efficiently and it may be involved in the folding/assembly of immunoglobulins [61] [62], suggesting a chaperone function of Grp170.

Glucose Regulated Protein 94 (Grp94)
Grp94, also termed endoplasmin, is the ER luminal member of the Hsp90 protein family. It has been demonstrated that Grp94 functions as a molecular chaperone. Grp94 is one of the most abundant proteins in the ER, and it was suggested that Grp94 plays an essential role in the maturation of a number of secretory pathway proteins including immunoglobulin [63] and epidermal growth factor receptor [64]. It has been reported that Grp94 displays an unusual broad range of intrinsic enzymatic activities. Included in this list are ATP hydrolysis [65], self-directed kinase activity [66], and amino peptidase activity [67]. It was suggested that substrate binding by Grp94 was coupled to ATP binding and hydrolysis, in a cyclic mechanism similar to the one observed for Grp78 [65]. Nevertheless, a more recent study showed that peptide binding to Grp94 occurs by a hydrophobic binding pocket whose accessibility is conformationally regulated in an adenine nucleotide-independent manner [68]. Further to this, very recent results showed that the kinase and amino peptidase activity is due to co-purification of contaminant enzymes with Grp94. In conclusion, so far it remains unclear how Grp94 interacts with substrate proteins [69].

Grp94 is transcriptional co-regulated with Grp78 as part of the UPR [70]. However, there are notable examples where disproportionate changes in one chaperone over the other is observed [71], indicating subtleties in transcriptional or translational regulation that are not well understood.

Calnexin, Calreticulin and Calmegin
The calnexin family of molecular chaperones is conserved among plants, fungi, and animals [72], and in addition to calnexin it includes calreticulin and calmegin. Calnexin is a type-1 integral membrane protein promoting folding and oligomeric assembly, preventing degradation, and supporting the quality control of N-linked glycoproteins. Calnexin is a monovalent lectin that recognizes and binds monoglycosylated core glycans (Glc1Man5-9GlcNAc2). The structure of the luminal domains has been solved [72]. The luminal part of calnexin consists of two domains; a globular lectin domain harbouring the glycan binding capabilities, and the P-domain that is a long, extended arm, which probably interacts with other proteins, one of which is ERp57 [72].

The molecular chaperone calreticulin is a soluble homologue to calnexin. The primary structure of calreticulin shares high sequence similarities with the luminal domain of calnexin (Fig. 10). Calreticulin is one of the most abundant proteins in the ER. It is a multifunctional protein, which
in addition to the ER has been found in other membrane-bound organelles, at the cell surface, and in extra-cellular environments (reviewed in [73]). In the ER it functions as chaperone and plays an important role in the Ca\(^{2+}\) homeostasis. Calreticulin is a lectin with similar oligosaccharide specificity as its membrane-bound homologue calnexin, and it binds transiently to many glycoproteins in the ER. Since calreticulin has the same oligosaccharide specificity as calnexin it is not surprising that the two chaperones bind to similar sets of proteins. Although there is no observed difference between the oligosaccharide-binding specificity of calnexin and calreticulin, both lectins may not interact \textit{in vivo} with the same glycoproteins. There exist several examples identifying association with one of the chaperones and not the other, during folding and maturation of specific glycoproteins (reviewed in [74]). Further to this, it was suggested that the substrate specificity of the two chaperones was dependent on the topological environment of calnexin and calreticulin, referring to membrane location of calnexin and luminal location of calreticulin [75].

Calmegin is a type-1 integral membrane protein, which is mainly expressed in spermatids of the testis [76]. It is a Ca\(^{2+}\) binding protein, which shares high sequence similarities with calnexin (Fig. 10). It is not well characterized. Nevertheless, it was shown that calmegin is required for

**Figure 10.** Schematic representation of calnexin, calreticulin, and calmegin. The regions A, B and C show high sequence identity. The P-domain contains two types of sequence repeats designated 1 and 2, each repeated four times in calmegin and calnexin and repeated three times in calreticulin. The transmembrane domain (TMD) is designated in black.

**Figure 11.** The core oligosaccharide is transferred to the asparagine residue of the growing polypeptide chain, possessing the structure shown in the figure. Terminal glucose residues are removed by glycosidase I and II. Monoglycosylated core oligosaccharides are substrates for calnexin and calreticulin. All three glucose residues and the indicated mannose residue are removed before transport of the protein to the Golgi complex. (Modified from Helenius and Aebi, 2001 [78]).

Calmegin is a type-1 integral membrane protein, which is mainly expressed in spermatids of the testis [76]. It is a Ca\(^{2+}\) binding protein, which shares high sequence similarities with calnexin (Fig. 10). It is not well characterized. Nevertheless, it was shown that calmegin is required for
fertilin α/β heterodimerization and sperm fertility, suggesting a chaperone function of calmegin [77].

The calnexin/calreticulin cycle: N-linked glycans are added to growing polypeptide chains as 14-residues oligosaccharides (Glc3Man9GlcNAC2) (Fig. 11). Immediately after coupling to the polypeptide chain, terminal glucose and mannose residues are removed by ER glycosidase I and II. Calnexin and calreticulin interact with the glycan moieties of substrate glycoproteins after they have been trimmed to the monoglucosylated form. Interaction with calnexin and calreticulin exposes the unfolded glycoprotein to the co-chaperone ERp57, which is a thiol oxidoreductase and may catalyze the formation and shuffling of disulfide bridges [79] (Fig. 12).

Removal of the last glucose residue by glycosidase II terminates the association between the glycoprotein and calnexin/calreticulin. If the glycoprotein has reached its native conformation it can be transported to the Golgi complex. On the other hand, if it is not properly folded it can be re-glycosylated by the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT). In this way the unfolded glycoprotein becomes a substrate for calnexin and calreticulin again. UGGT is considered being a folding sensor of the calreticulin/calnexin cycle. Although it is not known exactly how UGGT distinguishes folded glycoproteins from non-folded ones. Nevertheless, it

**Figure 12.** The calnexin/calreticulin folding cycle. See text for details. CNX: Calnexin, CRT: Calreticulin, UGGT: UDP-glucose:glycoprotein glucosyltransferase (Modified from Helenius and Aebi, 2001 [78]).
was shown that UGGT mainly recognizes partially folded glycoproteins [80], and in partially folded glycoproteins harbouring multiple domains, UGGT selectively recognizes glycans in the misfolded domains [81]. Therefore, it may be that UGGT recognizes exposed hydrophobic stretches and thereby the enzyme resembles the classical molecular chaperones in substrate binding and recognition.

Calnexin, calreticulin, ERp57, glycosidase II, and UGGT work together to increase the folding efficiency, to prevent premature oligomeric assembly and to prevent export of misfolded glycoproteins from the ER. Timing of calnexin and calreticulin binding is dependent on the position of the N-linked core glycans within the polypeptide chain. In general, when the N-linked core glycans are present in the first 50 amino acid residues association with calnexin and/or calreticulin may occur prior to interaction with Grp78. On the other hand, proteins that contain N-linked core glycans further down-stream the peptide chain may bind Grp78 first [82].

It remains unclear whether calnexin can act as a chaperone for non-glycoproteins. Calnexin has been co-immunoprecipitated with naturally occurring non-glycosylated proteins, and non-glycosylated proteins generated by tunicamycin inhibition of glycosylation [83]. Furthermore, \textit{in vitro} experiments showed that calnexin could prevent aggregation of non-glycosylated proteins [84, 85], suggesting a chaperone function of calnexin for non-glycosylated proteins as well.

\textbf{The Protein Disulfide Isomerase (PDI) Family}

The oxidative environment present in the lumen of the ER allows the formation of disulfide bonds. In this context protein folding is associated with formation of native disulfide bonds. This process is catalyzed by the members of the PDI family including PDI [86], ERp72 [87], P5 [88], ERp57 [89], PDIp [90], and PDIR [91]. All members of the PDI family identified to date are localized in the ER, they contain thioredoxin-like folds, and they show activity in thiol-disulfide exchange assays. PDI was the first catalyst of protein folding to be identified [86] and it is the most well characterized member of the PDI family. PDI comprises four domains a, b, b’, a’, plus a linker region between b’ and a’, and the acidic, KDEL ER retrieval signal (Fig. 13). The a domains contain the two active-site motifs, consisting of the amino acids WCGHC, which are redox active and cycle between the dithiol and disulfide form. PDI catalyzes the formation and isomerization of disulfide bonds in compact folding intermediates. A model of PDI function has been suggested in which the domains function synergistically. The two a domains catalyze the chemical isomerization while the b’ domain recognizes and binds unstructured regions of the polypeptide [92]. PDI is recognized as a multifunctional protein. In addition to catalyzing formation/isomerising of disulfide bonds it is a component of the prolyl-hydroxylase complex involved in collagen synthesis [93], and it heterodimerizes with the 97 kDa subunit of the microsomal triglyceride transfer protein (MTP) [94]. The role of PDI in the function of MTP is not entirely clear. Recently it was demonstrated that mammalian PDI may act as a redox-
regulated chaperone. In reduced state PDI bound and unfolded its substrate, while oxidized PDI released the substrate [95].

The folding enzymes PDI, PDIp and ERp57 all possess a linear sequence of four thioredoxin-like domains in an a-b-b’-a’ pattern (Fig. 13), and it was suggested that PDIp and ERp57 are isoforms of PDI with specialized substrate binding properties [96]. PDIp is exclusively expressed in pancreas, suggesting that PDIp may be a protein specific folding enzyme involved in the folding of only a subset of proteins secreted from the pancreas.

As previously mentioned ERp57, also known as ER60, interacts with calnexin and calreticulin and is involved in isomerization of disulfide bonds especially in N-glycosylated proteins. ERp57 does probably not possess a general binding site for non-native proteins, rather it has a specialized binding site for calnexin and calreticulin [97].

ERp72 is known as a calcium binding protein and a member of the PDI family. ERp72 contains three copies of the -CXXC- active site amino acid motif found in PDI [87]. Over-expression of mammalian ERp72 in yeast can rescue nonviable cells deficient for PDI, suggesting a PDI-like activity of ERp72 [98]. Nevertheless, more studies have suggested that ERp72 acts more as a classical chaperone. For instance it was shown that ERp72 interacted with apo B-100 and various truncated variants of apo B during protein maturation [99]. A more recent study identified ERp72 as a peptide binding protein, recognizing and binding various peptides in an ATP dependent manner [62].

P5 is another recently characterized member of the PDI family which has been suggested to possess both isomerase and chaperone activity [100]. New members of the PDI family are still identified, indicating that unidentified folding enzymes and chaperones in the ER exist. Recently,
ERp44 was identified as a new member of the PDI family and it was suggested to be involved in the control of oxidative folding in mammalian cells.

ERp29 is another recently identified ER localized protein, which is a structural homologue of PDI. It consists of two domains of which the N-terminal domain resembles the thioredoxin domain of PDI, although without the active site motif, indicating that it lacks isomerase folding enzyme activity. However, it was co-purified with the thyroglobulin folding complex, and a chaperone function of ERp29 was suggested [101].

At present the exact mechanism of substrate specificity of the different members of the PDI family is not elucidated. In addition, it is unclear, how or if they are in a position to act both as folding enzymes and classical chaperones, and if a redundancy in the oxidative folding pathway of newly synthesized proteins exists, such that the different isoforms could substitute each other. Such a redundant system has been described for the chaperone system. For instance it was shown that Grp78 can serve as back-up for calnexin of calreticulin in retention of misfolded proteins [102].

Receptor-Associated Protein (RAP)

The LDL receptor family is composed of several endocytic receptors that share structural homology and function in cellular uptake of various ligands including lipoproteins. Mammalian members of the LDL receptor family include: The LDL receptor, LDL receptor related protein (LRP), megalin, VLDL receptor, Apo E receptor 2, and LR11. RAP was originally discovered as a protein that co-purifies with LRP [103]. At present RAP is known to bind with high affinity to all members of the LDL receptor family, except for the LDL receptor, in which only low affinity binding was observed [104]. Two roles of RAP in maturation of LDL receptor family proteins have been suggested. First, RAP is an escort protein preventing premature binding of the receptor ligands, which are also synthesized in the ER. Second, RAP serves as a specialized chaperone assisting LDL receptor family members in folding correctly (reviewed in [105]). The mechanism by which RAP prevents premature binding of the ligand is not yet understood. It may involve steric hindrance, conformational changes in the receptor or a combination of the two. The interaction of RAP with the LRP receptor is the one which is best characterized. In the ER, RAP binds to LRP and escorts the LRP receptor to the Golgi complex, where it dissociates in the Medial-Golgi due to the low pH. RAP is retrieved back to the ER by the KDEL receptor. It is assumed that the presence of the low pH in the Medial-Golgi prevents premature association with LRP ligands within the terminal part of the secretory pathways. In addition, RAP acts as a classical chaperone protecting LRP, the VLDL receptor, and the LDL receptor against aggregation. Further to this, recent data show that over-expression of RAP could promote folding and processing of some class 2 mutant LDL receptors [106]. Finally, RAP has been shown to associate with receptors, which are not members of the LDL receptor family, e.g.
sortilin [107] and lipolysis-stimulated receptor [108], suggesting that RAP plays a role in the biogenesis of other proteins as well.

**Microsomal Triglyceride Transfer Protein (MTP)**
Apo B is synthesized in the ER of hepatocytes and enterocytes, where it is lipid-loaded as a consequence of interaction with the MTP complex. MTP consists of a 97-kDa large subunit and a 58-kDa PDI subunit. Both subunits are essential for efficient lipid transfer activity [109]. MTP acts as a protein specific chaperone for apo B. MTP was proven to associate with newly synthesized apo B, and recent results showed that the interaction between MTP and apo B creates a lipid transfer pocket required for lipoprotein assembly [110]. In the absence of lipid-loading of apo B by MTP, apo B is subjected to rapid degradation mediated by the by ER associated degradation (ERAD) [111, 112].

By assisting the lipid-loading and preventing premature degradation of apo B, MTP plays an important role in the assembly of VLDL and chylomicron particles (Fig. 1). Mutations in MTP cause abetalipoproteinemia, a rare disorder where VLDL and chylomicron production is impaired. It is noteworthy that MTP is the only ER chaperone in which natural occurring disease causing mutations have been reported. Other disorders owing to protein misfolding, termed conformational diseases [113, 114, 115] or ER storage diseases [116], are caused by mutations in a specific secretory protein, resulting in retention, accumulation or premature degradation of critically important proteins that are unable to reach their target sites. For example retention of class 2 mutant LDL receptors in the ER, which is due to misfolding of the LDL receptor.

**ER Associated Degradation (ERAD)**
During the synthesis and folding of proteins in the ER, both mutant and wild-type proteins are subjected to degradation. The quality control system of the ER ensures that mutant ER-retained proteins are degraded and thereby it prevents inexorable accumulation of proteins in the ER, which would probably become toxic to the cell. Further to this, wild-type proteins are also substrates for ER degradation. A well-known example is the degradation of the wild-type cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, in which 75% of the wild-type newly synthesized protein is degraded and never reaching the cell surface [117]. Furthermore, the ERAD system may be involved in the post-transcriptional regulation of specific proteins in the cell. For instance the regulation of apo B assembly into lipoproteins, and the secretion from liver occur partially at the post-transcriptional level. Apo B protein degradation was suggested to be dependent on the availability of lipids. In the absence of lipids, the newly synthesized apo B protein was subjected to rapid degradation mediated by the ERAD system [112, 118]. However, other results have shown that apo B secretion from liver cells was unaffected by changes in cellular cholesteryl ester levels, showing that the role of lipids in the assembly and secretion of apo B-containing lipoproteins is not fully understood.
For many years it was a common perception that proteases localized in the lumen of the ER were responsible for degrading misfolded proteins in the ER. At present no degradative pathway within the ER itself has been found. In contrast, a major breakthrough came when Ward et al. discovered that the cytoplasmic localized ubiquitin-proteasome system was involved in degradation of newly synthesized wild-type CFTR protein [117]. Today it has been proven that the ubiquitin-proteasome system is an essential part of the ERAD system responsible for degradation of many misfolded ER proteins (Fig. 14). This process involves recognition of misfolded or unassembled proteins in the ER, retrograde translocation of proteins from the ER back to the cytoplasm, and finally degradation by the ubiquitin-proteasome system (reviewed in [119]).

**Figure 14.** Schematic representation of the ERAD system. Newly synthesized polypeptides enter the lumen of the ER via the translocon. In the ER chaperones bind to the folding protein and if the protein reaches a transport competent conformation it will be transported to the Golgi complex. However, if the protein is misfolded and retained in the ER, inter-chain disulfide bonds are reduced and it is exported through the translocon, polyubiquitinated and degraded by the 26S proteasome. (Modified from Rutishauser and Spiess, 2002 [120]).

Exactly what targets proteins for degradation by the ERAD system is not well characterized. Nevertheless, prolonged retention of incompletely folded proteins in the ER results in degradation [119]. It is likely that unfolded regions of polypeptide chains serve as recognition
motifs that specify degradation, and that molecular chaperones play a role in targeting proteins for degradation by the ERAD system. Furthermore, trimming of the mannose residue on the N-linked core glycan by mannosidase I leads to Man₈GlcNac₂ structures, which were shown to serve as degradation signal of glycoproteins (Fig. 11) [121].

Misfolded proteins targeted for degradation by the ERAD system are retro-translocated through the same pore-forming complexes serving to translocate nascent polypeptide chain into the lumen of the ER (Fig. 14). It was demonstrated that Grp78 was necessary for degradation of several soluble proteins in yeast, and not required for degradation of others. Furthermore, recent results have shown that Grp78 and PDI may be necessary for efficient degradation of the purely folding β-secretase isoform BACE457. It was suggested that especially PDI was involved in unfolding the partially folded proteins, by reducing the disulfide bridges before retrograde translocation [122].

The ER associated degradation takes place in the cytosol using the proteasome, which is also responsible for degradation of cytosolic proteins. Activated ubiquitin molecules are coupled as a chain to the polypeptide substrate mediated by ubiquitin conjugating enzymes and ubiquitin ligases. The polyubiquitinated substrate is recognized and degraded by the 26S proteasome, a large multisubunit protease complex composed of a 20S proteolytic core and two 19S regulatory particles.

The Unfolded Protein Response (UPR)
Cellular perturbations disturbing ER homeostasis disrupt protein folding in the ER and eventually they lead to accumulation of misfolded proteins in the ER. These perturbations include expression of mutant and misfolded secretory proteins, disturbed calcium homeostasis, glucose/nutrient deprivation, and altered redox status. Such conditions may be detrimental to the cell, and to overcome such stressful situations the cell has evolved the “unfolded protein response” (UPR) signalling pathway (reviewed in [11]). On the cellular level, activation of the UPR pathway results in activation of transcription of a large set of genes and in attenuation of the translation of newly synthesized proteins. Genes harbouring an ER stress response element (ERSE) in the promoter region are known for being induced by the UPR pathway, including e.g. the genes encoding the chaperones: Grp78, Grp94, PDI and calreticulin. Nevertheless, genes without well defined ERSE elements in the promoter region are also induced by the UPR pathway. In yeast more than 350 different genes have been shown to be UPR-regulated, many of which encode proteins that function to increase protein translocation, folding and ERAD mediated degradation [123]. Additionally, the UPR induced genes also included genes encoding factors involved in lipid metabolism, vesicle trafficking, and glycosylation [123].

The complex network of physiological responses to ER stress in mammalian cells seems to be regulated by few transmembrane proteins: Activating transcription factor 6 (ATF6), Inositol requiring 1 α/β (IRE1α / IRE1β), and PKR-like endoplasmic reticulum kinase (PERK). In
addition, Grp78 and Grp94 were the first proteins that were recognized to be regulated by glucose deprivation or what today is known as the UPR pathway [124]. Actually, Grp78 is a key element in sensing the folding capacity in the ER. Considering the ER-stress sensing cells there exist two main physiologically outcomes, either they overcome the stress-full situation or they go into apoptosis. Recently the transcription factor CHOP was shown to be an important part of the UPR-dependent programmed cell death [125].

**Figure 15.** The mammalian UPR Pathway. Accumulation of misfolded proteins in the ER lumen catalyzes dissociation of Grp78 from ATF6, PERK and IRE1. ATF6 is activated by the S1P and S2P proteases, transported to the nucleus and it activates transcription by binding to the ERSE element in the promoter region of the response genes. IRE1 forms oligomers, it is trans-activated, and it cleaves Xbp1 mRNA, resulting in formation of a potent Xbp1 transcription factor capable of binding ERSE elements and activating transcription. PERK is activated in an IRE1 like manner and stall protein synthesis by phosphorylation of eIF2α (Modified from Spear, E. et al, 2001 [126]).

The ATF6 transcription factor is synthesized as a membrane-bound precursor and it is activated by the S1P and S2P Golgi localized proteases, which are also responsible for activating SREBP
as a response to intracellular sterol deprivation (Fig. 4). Recent results showed that Grp78 is bound to ATF6 under normal growth conditions, and during cellular stress, Grp78 dissociates from ATF6 resulting in transport of ATF6 to the Golgi complex [127]. Cleavage by the S1P and S2P proteases liberate the active ATF6 transcription factor from the membrane. The processed ATF6 migrates to the nucleus and activates transcription by binding to the ERSE of the UPR responsive genes (Fig. 15). Alternatively, information about ER stress is communicated by two transmembrane kinases (IRE1α and IRE1β) which are activated by oligomerization and trans-autophosphorylation. It is assumed that association between Grp78 and IRE1 prevents oligomerization and activation of IRE1. During cellular stress Grp78 dissociates from IRE1 activating the endonuclease of IRE1. One substrate for mammalian IRE1 is the mRNA encoding the bZIP transcription factor X-box binding protein 1 (Xbp1). Cleavage of Xbp1 mRNA removes a 26 nucleotide intron, generating a transcription frame shift and creating a new open reading frame. Transcription of the cleaved mRNA results in the production of a potent Xbp1 transcription factor, which is translocated to the nucleus and it binds to the ERSE elements in the promoter region of the target genes (Fig. 15).

Activation of the UPR pathway includes attenuation of protein synthesis. The translation of new proteins is stalled by PERK mediated phosphorylation of the general translational initiation factor eIF2α (Fig. 15). This translational slow-down appears to protect cells against overload of unfolded proteins in the ER.

The Unfolded Protein Pathway and Disease

ER stress followed by activation of the UPR pathway has been linked to human diseases. One example is early-onset cases of familial Alzheimer’s disease (FAD). Mutations in the gene presenilin-1 (PS1) were linked to early-onset FAD. Furthermore, in transfected cell lines, mutant PS1 decreased UPR [128] and increased vulnerability to spontaneous and induced apoptosis in neurons over-expressing mutant PS1 [129]. This suggests that neurons harbouring the PS1 mutations are more sensitive for perturbations causing ER stress. However, these results were brought into question by another study, according to which cell lines lacking PS1 exhibited UPR similar to wild-type [130].

Hyperhomocysteinemia is a common, independent risk factor for cardiovascular diseases [131]. It was shown that activation of the UPR by homocysteine induced SREBP-dependent genes that are essential for biosynthesis and uptake of cholesterol and triglycerides and leads to the accumulation of cholesterol in cultured cells [132]. Furthermore, the transcription factors SREBP and ATF6 are both activated by the S1P and S2P proteases (Fig. 4 and 15). However, only UPR elicits ATF6 processing, and sterol deprivation alone induces SREBP binding. Interestingly, homocysteine induced ER stress is linked to induction of both and it was suggested that the stress-induced disturbance of the lipid metabolism caused hepatic steatosis observed in some patients suffering from severe hyperhomocysteinemia [132].
Endoplasmic Reticulum Storage Disease (ERSD)
ERSD is a group of genetically based disorders in which mutant proteins fail to pass the ER quality control system, mainly due to protein misfolding or defects in the transport apparatus. Protein misfolding is implicated in many different diseases causing the clinical phenotype of ERSDs to be very heterogeneous (reviewed in [120]). However, the pathogenesis is caused by equivalent mechanism, such as retention of functional protein due to ER retention and toxic effects caused by aggregation of misfolded proteins.

Table 1. Examples of lipoprotein disorders classified as ERSDs

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Defect protein</th>
<th>Pathogenic effect</th>
</tr>
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<tbody>
<tr>
<td>Familial Hypercholesterolemia</td>
<td>LDL receptor (Class 2)</td>
<td>Lack of functional receptor.</td>
</tr>
<tr>
<td>Familial hyperchylomicronemia</td>
<td>Lipoprotein lipase</td>
<td>Lack of functional protein</td>
</tr>
<tr>
<td>Lipoprotein(a) Deficiency</td>
<td>Lipoprotein(a)</td>
<td>Decreased Lp(a) level</td>
</tr>
<tr>
<td>Abetalipoproteinemia</td>
<td>MTP</td>
<td>Defective transport machinery</td>
</tr>
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</table>

Several disorders affecting the lipoprotein metabolism have been defined as ERSDs (Table 1). FH, Familial hyperchylomicronemia [133], Lipoprotein(a) deficiency [134], can all be caused by ER retention of mutant proteins, while abetalipoproteinemia caused by mutations in MTP represents a defect in the transport apparatus.

It is assumed that the UPR system together with the ERAD system constitute the cellular defence mechanism, protecting the cell against the harmful effects caused by accumulation of mutant proteins in the ER. It is likely that genetic variations in the ER quality control system, the UPR and ERAD system may influence the ability of the cell to recognize and retain mutant proteins, and eventually degrade them.
Aims of the Study

The risk of CHD is directly related to the level of LDL-cholesterol and inversely related to the level of HDL. A significant inter-individual variation in the plasma cholesterol levels exists in the general population and among heterozygous FH patients as well. Most of the cholesterol is transported as a constituent of LDL or HDL particles, and genetic factors strongly influence the levels of plasma lipoproteins. However, all the genes and sequence variations contributing to the most common forms of dyslipidemias are not detected. We hypothesized that some of the unexplained variations may be due to genetic variations in ER localized chaperones and folding enzymes involved in the quality control of the LDL receptor. The ER quality control may influence the total number of LDL receptor at the cell surface thereby influencing the capacity for removing LDL from the blood. Further to this, variations in the ER quality control system may influence the synthesis and amount of apo B containing lipoprotein particles released to the blood.

When this study was initiated only little information was available about the folding and quality control of the LDL receptor, so the main aims of this study were:

- To identify chaperones and folding enzymes interacting with the newly synthesized LDL receptor.
- To investigate if the identified chaperones were involved in the quality control of the newly synthesized LDL receptor.
- To study if inter-individual variation in the relevant component of the quality control system may influence the plasma cholesterol levels among heterozygous FH patients.
Results and Discussion

Identification of Chaperones Involved in the Folding and Maturation of the LDL Receptor

A main purpose of this Ph.D. project was to identify chaperones involved in the protein quality control of the LDL receptor. This Ph.D. study showed that the general ER localized chaperone Grp78 co-immunoprecipitated with both the wild-type and two mutant, ER retained (W556S & C646Y) LDL receptors and that Grp78 showed prolonged interaction with the ER retained, mutant LDL receptors (see article in appendix 3). In brief, a pulse-chase study showed that the interaction between the wild-type LDL receptor and Grp78 was no longer detectable after 2½ hours, while it persisted for more than 4 hours with the mutant receptors. Furthermore, about five times more Grp78 co-immunoprecipitated with the mutant receptors than with the wild-type receptor suggesting that Grp78 was involved in retention of mutant LDL receptors in the ER. Over-expression of Grp78 caused no detectable alterations on the steady state level of active LDL receptors at the cell surface. Over-expression of Grp78, nevertheless, decreased the processing rate of newly synthesized wild-type LDL receptors. This indicated that Grp78 interaction may be a rate-limiting step in maturation of the wild-type LDL receptor, and that Grp78 may be an important factor in the quality control of newly synthesized LDL receptors.

Using the co-immunoprecipitation system described in Appendix 3, no other chaperones associating with the LDL receptor were identified. However, in order to increase the sensitivity of the system the metabolic labelled cells over-expressing wild-type, C646Y-LDLr or W556S-LDLr were subjected to chemical cross-linking with [Dithiobis(succinimidylpropionate)] (DSP) before lysis. Immunoprecipitation was performed using anti-LDL receptor antibodies, anti-calnexin antibodies, or anti-KDEL antibodies, which recognizes mammalian Grp78, Grp94, and an unidentified protein at about 55 kDa on SDS-PAGE immunoblots. Using anti-LDL receptor antibodies three major proteins were precipitated representing the 120 kDa precursor LDL receptor, the 160 kDa mature LDL receptor and a 78 kDa band representing Grp78 (Fig. 16, lane 1-3).

The KDEL-antibody immunoprecipitated a protein with an apparent mass of 78 kDa representing Grp78, and a faint band which may represent the 120 kDa band of the LDL receptor (Fig. 16, lane 4-7). The KDEL antibody was inefficient for immunoprecipitation of antigens subjected to chemical cross-linking by DSP. This observation may be due to the fact that the KDEL-antibody is a monoclonal antibody raised against an only 6 amino acid residue (Ser-Glu-Lys-Asp-Glu-Leu). It is likely that DSP, which reacts with the e-amine of lysine, reacts with the lysine in the SEKDEL sequence and thereby altering the epitope of the antigen. Alternatively, cross-linking may bury the epitope inside large protein complexes and thereby preventing the antibodies from binding.
The LDL receptor is co-immunoprecipitated with calnexin following chemical cross-linking. Chang cells were transfected with plasmids expressing wild-type, W556S, or C646Y-LDL receptor or mock transfected (pMP6). The cells were metabolically labelled for 2 hours, subjected to chemical cross-linking with 2 mM DSP, and lysed. The extracts were subjected to immunoprecipitation with polyclonal anti-LDL receptor antibodies (lane 1-3), monoclonal anti-KDEL antibodies (lane 4-7), or monoclonal anti-calnexin antibodies (lane 8-11).

The monoclonal calnexin antibody precipitated a major protein with an apparent mass of 90 kDa (Fig. 16, lane 8-11) corresponding to calnexin. In addition, a band at 120 kDa could be detected in the lanes representing cell lysates from cells over-expressing wild-type or W556S-LDL receptor. This indicated that the 120 kDa LDL receptor precursor may co-precipitate with calnexin. Nevertheless, no band representing calnexin was detected when performing the immunoprecipitation with anti-LDL receptor antibodies (Fig. 16, lane 1-3). In order to investigate...
if calnexin was co-isolated with the LDL receptor a Western blot was performed. Chang cells transiently expressing wild-type or W556S-LDL receptor were cross-linked, lysed, subjected to immunoprecipitation using LDL receptor specific antibodies, and immunoblotted using an antibody against calnexin. A significant band representing calnexin could be detected when expressing both the wild-type and the mutant LDL receptor (Fig.17, lane 4-6). These results indicated that calnexin associates with both the wild-type and the W556S-LDL receptor to comparable degree.

Figure 18. Overexpression of calnexin did not alter the processing rate of the wild-type LDL receptor. Chang cells expressing the Wild-type LDL receptor (WT-LDLr), co-expressing wild-type LDL receptor and Grp78, or co-expressing wild-type LDL receptor and calnexin were pulse-labelled for 30 minutes and chased for the indicated periods. The cells were lysed and subjected to immunoprecipitation using a polyclonal antibody against the LDL receptor. The immunoprecipitated proteins were analysed by 4-15% SDS PAGE and the intensity of the bands representing the precursor LDL receptor protein (120 kDa) and the band representing mature LDL receptor protein (160 kDa) were quantified using a phosphorImager. Intensity of the band representing the mature LDL receptor relative to each band representing the precursor protein is shown with time.

I have previously observed that over-expression of Grp78 altered the processing-rate of the wild-type LDL receptor indicating that Grp78 is involved in the quality control of Grp78 (Appendix 3). In order to study if over-expression of calnexin also influenced the processing of the LDL
receptor, Chang cells were co-transfected with plasmids expressing wild-type LDL receptor and calnexin. The cells were subjected to a pulse-chase analysis and the LDL receptor was purified by immunoprecipitation (Fig. 18). The pulse-chase study showed that over-expression of calnexin did not noteworthy alter the processing rate of the wild-type LDL receptor.

The results showed that calnexin can associate with wild-type and class 2 mutant LDL-receptors. Nevertheless, in contrast to the results concerning Grp78 (Appendix 3), the association was not very stable since chemical cross-linking was needed to isolate the complexes. No significant difference was observed in the amount of protein co-isolated with the wild-type LDL receptor compared to the mutant W556S-LDL receptor. Finally, over-expression of calnexin did not influence the processing-rate of the wild-type receptor. Considering these results it remains an open question if calnexin acts as a chaperone for the LDL receptor. Calnexin recognizes and binds monoglycosylated core glycans. Indeed the newly synthesized LDL receptor contains two N-linked glycans (Personal communication I. Braakman), localized in the YWTD repeats, making it a potential substrate for calnexin. However, it was demonstrated that when the N-linked glycans are not present within the first 50 amino acids, the newly synthesized peptide may be bound by Grp78 first [82]. Since the N-linked glycans in the LDL receptor are not localized within the first 50 amino acids (Appendix 1), it is likely that Grp78 may be the first choice of chaperone considering the newly synthesized LDL receptor protein, and that calnexin is in a position to bind the receptor, although the interaction seems less stable. In accordance with this, it was previously demonstrated that Grp78 and calnexin can act sequentially in the folding of the newly synthesized proteins [135], and it was shown that there exists a degree of redundancy in the quality control system of the ER ensuring that if one chaperone fails to interact with an incompletely folded protein, another chaperone likely will [102]. It may be that Grp78 and calnexin act together in the folding of the LDL receptor either sequentially or that calnexin possesses the ability to compensate for Grp78 during LDL receptor folding.

At the present time the exact mechanism for LDL receptor folding and maturations remains unclear. This study identified Grp78 as being an important factor for the quality control of the LDL receptor and calnexin to be a potential factor. Li et al. demonstrated that the specialized chaperone RAP promotes LDL receptor folding and maturation [106]. Finally, Garcia et al. demonstrated that mutations in a putative LDL receptor adaptor protein may be responsible for defective LDL receptor activity, causing an autosomal recessive form of hypercholesterolemia [136]. Nevertheless, there is no indication that this putative LDL receptor adaptor protein influences the folding and maturation of the LDL receptor. At present Grp78, calnexin and RAP are the only known chaperones to associate with the newly synthesized wild-type and mutant LDL-receptor.
Accumulation of Unfolded Mutant LDL Receptors in the ER Induces the Transcription of the GRP78 Gene.

It is well known that accumulation of unfolded proteins in the ER induces the Unfolded Protein Response System (UPR) and Grp78 is a classical marker for UPR activation. We have previously shown that the mutant W556S-LDL receptor is totally retained in the ER [137], and this study has shown that Grp78 is likely to be an important factor in the quality control of the LDL receptor. According to this, it is likely that accumulation of mutant ER retained LDL receptors in the ER results in activation of the UPR system. In order to investigate if mutant LDL receptors induced transcription of the GRP78 gene, a 587 bp fragment of the GRP78 promoter region was inserted into the pGL3 Luciferase Reporter Vector (Appendix 2). The 587 bp fragment included the three identified ERSE1 elements, two Sp1 sites and the TATA box. The constructs were co-expressed with constructs expressing wild-type or different mutant LDL receptors in human epithelial cells (Chang) as described in the section “Materials and Methods”. Two of the expressed mutant LDL receptors were known to be totally retained in the ER (W556S and C646Y), and two were binding deficient (E119K), or recycling deficient (W66G) (Table 2). The E119K-LDL receptor and W66G-LDL receptor were both processed similar to the wild-type LDL receptor in the Chang cell expression system (Fig. 19).

Table 2. Mutant LDL receptors used for investigations of the induction of GRP78 transcription.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Class</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>W556S-LDL receptor</td>
<td>2</td>
<td>[137, 138]</td>
</tr>
<tr>
<td>C646Y-LDL receptor</td>
<td>2</td>
<td>[139]</td>
</tr>
<tr>
<td>W66G-LDL receptor</td>
<td>3 or 5</td>
<td>[139, 140]</td>
</tr>
<tr>
<td>E119K-LDL receptor</td>
<td>3</td>
<td>[141]</td>
</tr>
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</table>

Figure 19. Processing of the W66G-LDL receptor and the E119K-LDL receptor in Chang cells. Chang cells were transfected with pMP6 expression vector containing the wild-type LDL receptor cDNA (WT-LDLr) or the mutant cDNA encoding the W66G-LDL receptor or E119K-LDL receptor. The cells were pulse-labelled for 30 minutes and chased for the indicated periods, according to the method given in Appendix 3. The cells were lysed and subjected to immunoprecipitation using a polyclonal LDL receptor antibody. The immunoprecipitated proteins were analysed by 4-15% SDS PAGE followed by phosphor imaging.
Forty-eight hours after transfection the cells were washed, lysed, and the luciferase and the β-galactosidase activities were measured as described in the section “Materials and Methods”. Chang cells co-transfected with the constructs expressing the two mutant, ER retained, LDL receptors (W556S and C646Y) showed a two-fold induction of the GRP78 promoter compared to cells transfected with the wild-type LDL receptor construct (Fig. 19). However, cells co-transfected with the non-ER retained mutant LDL receptor constructs (E119K and W66G) did not induce the transcription more than cells expressing the wild-type LDL receptor. This result indicated that accumulation of mutant ER retained LDL receptors in the ER induced the UPR system. Co-expression of wild-type, E119K- and W66G-LDL receptor did cause significant induction compared to the cells co-transfected with the empty pMP6 vector and the pGL3 construct (Fig. 19, pMP6). Expression of the LDL receptor proteins is controlled by a strong CMV promoter, resulting in high expression of recombinant LDL receptor proteins in the cell. Hence over-expression of wild-type LDL receptor protein may be enough to cause a milder stress condition in the cell, resulting in some induction of the GRP78 promoter.

![Graph showing effects of co-expression of wild-type and mutant LDL receptors on the GRP78 promoter activity.](image)

**Figure 19. Effects of co-expression of wild-type and mutant LDL receptors on the GRP78 promoter activity.** 587 bp of the GRP78 promoter region (-587 to -1; numbers indicate the nucleotide position relative to the translation start site) was inserted in the pGL3-basic vector. Chang cells were co-transfected with the pGL3-construct and plasmids expressing wild-type LDL receptor (WT-LDLr) or various mutant LDL receptors (W556S-LDLr, C646Y-LDLr, W66G-LDLr or E119K-LDLr). As control the cells were transfected with the pGL3-construct and the empty pMP6 vector (pMP6) or the empty pGL3-basic vector and the WT-LDLr construct (Control). Relative activity was determined as described in the section “Materials & Methods”. Average of four independent transfections is presented with S.D. values (bars).

Treatment of the cells with tunicamycin is a well described method for induction of the cellular UPR system. Tunicamycin inhibits specifically asparagine-linked (N-linked) glycosylation of newly synthesized proteins in the ER. Inhibition of N-linked glycosylation disrupts folding of newly synthesized proteins resulting in accumulation of unfolded proteins in the ER and
induction of the UPR system. To investigate if co-expression of ER retained mutant LDL receptors induced the GRP78 transcription to the same level as tunicamycin, Chang cells were co-transfected with the reporter construct and wild-type LDL receptor or W556S-LDL receptor. Half of the cells were treated with tunicamycin for 16 hours before harvesting and measurement of luciferase activity.

Cells treated with tunicamycin showed a 2-3 fold induction of GRP78 transcription when compared to the untreated cells (Fig. 20). Interestingly, the level of induction in the tunicamycin treated cells, expressing wild-type LDL receptor, did not reach the same level of induction as the tunicamycin treated cells expressing W556S-LDL receptor. Tunicamycin treatment of the cells is considered a harsh treatment of the cells resulting in a significant stress response. Previous studies have shown a five to eight fold induction of GRP78 transcription [142]. However, in this publication a shorter segment of the promoter and alternative cell lines were used, resulting in the fact that the results are not completely comparable. The results showed that extent of induction caused by accumulation of W556S-LDL receptor in the ER caused an induction comparable to the induction caused by tunicamycin.

**Transcript Levels and Protein Levels of Grp78 during Over-expression of LDL Receptor**

Transcription of the GRP78 gene was induced during accumulation of mutant LDL receptors in the ER indicating that the cells sense a cellular stress and that the UPR system is turned on. To cope with the stress situation an increased synthesis of for example chaperone proteins must occur. However, induction of the GRP78 transcription is not tantamount to an equivalent translation and synthesis of Grp78 protein. The expression level of Grp78 protein is not solely
regulated at the transcriptional level. The 220 nucleotide 5’ non-coding region of the human Grp78 mRNA contains an internal ribosome entry site (IRES) that mediates the translation of the second cistron in a dicistronic mRNA in cultured mammalian cells [143]. The presence of the IRES sequence suggests that Grp78 expression is further regulated at the level of translation.

In order to investigate if accumulation of mutant LDL receptors leads to increased levels of Grp78 RNA and protein, Chang cells were transfected with constructs expressing wild-type, W556S, C646Y, W66G or E119K LDL receptors. As control, cells were mock-transfected or treated with tunicamycin. LDL receptor over-expression and transfection efficiency was estimated by immunostaining and fluorescence-microscopy. The transfection efficiency was 10-12% (data not shown). Forty-eight hours after transfection the cells were harvested, and the pellets were split. Total RNA was isolated from 9/10 of the cells and 1/10 of the cells were lysed and subjected to immunoblotting using a KDEL antibody as described in the section “Materials and Methods”. The Northern-blot analyses showed no significant difference between the steady state RNA levels of cells over-expressing ER retained, mutant LDL receptors (W556S and C646Y) (Fig. 21 A, lane 2 and 3) when compared to cells over-expressing wild-type LDL receptor or non-ER retained mutant LDL receptors (W66G and E119K) (Fig. 21A, lane 4 and 5). Over-expression of LDL receptors did not cause a detectable increase of Grp78 RNA levels compared to non-transfected Chang cells. (Fig. 21A, compare lane 1-5 to lane 8). Tunicamycin treatment of the cells induced the mRNA levels about 15 fold, which is consistent with previous reports [142].

In order to investigate if over-expression of ER retained, mutant LDL receptors changed the level of Grp78 protein, a Western-blot was performed. Considering the Grp78 protein level, no significant difference was observed between cells over-expressing wild-type or mutant LDL receptors (Fig. 21B, lane 1-5) when compared to mock-transfected (Fig. 21B, lane 6) or non-transfected cells (Fig. 21B, lane 7). Tunicamycin treatment induced the endogenous Grp78 protein level about 2 fold (Fig. 21B, lane 8). It is noteworthy that a 15 fold induction at the RNA level results in no more than 2 folds induction at the protein level. This observation underscores the fact that regulation of Grp78 not solely occurs at the transcriptional level.

The results described in figure 21 showed no detectable induction of Grp78 protein due to over-expression of mutant, ER retained LDL receptors. However, the transfection-efficiency of the cells was only estimated to be 10-12%, thus almost 90% of the cells did not express recombinant LDL receptors. For the blotting analyses, total RNA and proteins were isolated creating a large background originating from the non-transfected cells and resulting in low sensitivity of the Northern and Western blotting analyses. In contrast, in the luciferase-assay only transfection-positive cells possessed the ability to catalyze the conversion of luciferin, and thereby only the transfected cells contributed to the measurements. Furthermore, differences in transfection
efficiencies were accounted for by a β-galactosidase assay, resulting in an accurate and sensitive assay.

Figure 21. Grp78 RNA and protein levels in cells transfected with wild-type and various mutant LDL receptors. Chang cells were transfected with pMP6 constructs expressing wild-type or mutant LDL receptors. 48 hours after transfection the cells were harvested, the pellets were split. 9/10 of the cells were used for purification of total RNA and analyzed by Northern blotting using a GRP78 specific probe or a GAPDH specific probe, which served as internal standard (A). 1/10 of the cells were lysed, and the protein extracts were analyzed by Western blotting using an anti-KDEL antibody (B). A: 1: WT-LDLr, 2: W556S-LDLr, 3: C646Y-LDLr, 4: W66G-LDLr, 5: E119K-LDLr, 6: pMP6, 7: Chang + 5 µg/ml tunicamycin, 16 hours prior to harvesting, 8: Chang. B: 1: WT-LDLr, 2: W556S-LDLr, 3: C646Y-LDLr, 4: W66G-LDLr, 5: E119K-LDLr, 6: pMP6, 7: Chang, 8: Chang + 5 µg/ml tunicamycin, 16 hours prior to harvesting.

To increase the sensitivity of the Northern and Western blotting assays HepG2 cells stably over-expressing wild-type and two mutant, ER retained LDL receptors (W556S and C660X [144]) were obtained. (The cells were generously provided by Marie S. Andersen and Thomas G. Jensen, University of Aarhus). The cells were grown in DMEM until 90% confluent. HepG2 without over-expression of LDL receptors were used as controls (HepG2÷). Some of the control cells were induced with 5µg/ml tunicamycin 16 hours before harvesting. The pellets were split, and total RNA was isolated from 9/10 of the cells. 1/10 of cells were lysed according to the protocol given in the section “Materials and Methods”, and the supernatants were subjected to immunoblotting using the anti-KDEL antibody. Northern blotting followed by hybridization with a Grp78 specific probe showed no detectable differences of cells over-expressing mutant LDL receptors when compared to cells over-expressing wild-type LDL receptors and HepG2÷ cells (Fig. 22A, compare lane 2 and 3, with lane 1 or 5). The tunicamycin treatment resulted in a 15 fold induction of the Grp78 RNA level (Fig. 22A, lane 4) and a 7 fold induction of the protein level (Fig. 22B, lane 4). These results are in accordance with results reported previously [142]. Apparently, a large cell to cell variation exists among HepG2 cell and Chang cells, considering the induction of Grp78 protein. Both cell lines were original derived from liver cells and are epithelial cell. However, it turned out that Chang cells (ATTC, CCL-13) was originally established via HeLa contamination. Hence they and are not true liver epithelial cells. The Chang cell line was originally chosen for these investigations because it handled the over-expression of
the LDL receptor efficiently, it showed better transfection efficiency when compared to the HepG2 cells. Nevertheless, considering the stress-response experiments, the HepG2 cells may have been a better choice. The large variation (2-7 folds) in the stress response, considering Grp78 protein level, is in accordance with results published previously [59, 142]. Most interestingly, recently it was suggested that the stress induced activation of Grp78 and the stress induced translation are independent events, and that a part of the increased levels of Grp78 protein is due to increased translation efficiency, independent of increased amounts of Grp78 mRNA and independent of the IRES sequence included in the 5’untranslated region of Grp78 [59].

![Figure 22. GRP78 RNA and protein levels in HepG2 cells stably over-expressing wild-type or mutant LDL receptors.](image)

The cells stably over-expressing wild-type and mutant LDL receptor showed no detectable differences in the steady state expression of Grp78 protein. This indicated that the degree of over-expression of wild-type or mutant LDL receptor protein does not induce a detectable unfolded protein response. Nevertheless, the luciferase assay is a more sensitive assay compared to the Northern and Western blot techniques. It may be that accumulation of mutant LDL receptors in the ER induces a small stress response, and that a slight increase in the Grp78 protein synthesis is enough to allow an efficient removal and degradation of the mutant proteins from the ER. However, in order to conclude if accumulation of ER retained mutant LDL receptors induce the UPR system more investigations must be performed. It should be attempted to obtain a higher percentage of liver cells over-expressing mutant and wild-type LDL receptors, for instance by using a retroviral system for transduction of HepG2 cells. More sensitive measurements of total RNA than the Northern blotting procedure is required. This could be obtained using the
Lightcycler (Roche A/S) for quantitative real time PCR. Finally, it is necessary to investigate how expression of mutant LDL receptors influences the expression of other proteins known to be induced by the UPR system, for instance CHOP.

Hyperhomocysteinemia is a common, independent risk factor for cardiovascular disease. Homocysteine causes protein misfolding in the ER, and activates the unfolded protein response leading to increased expression of the ER stress-response genes including GRP78 [145]. The homocysteine-induced ER stress causes dysregulation of the endogenous sterol response pathway, and this mechanism was suggested in order to explain the development of hepatic steatosis and atherosclerotic lesions observed in hyperhomocysteinemia [132]. Considering my results showing that retention of over-expressed mutant LDL receptors in the ER did not lead to a huge stress response in human cell lines, it is unlikely that accumulation of mutant LDL receptors in the ER will cause a similar stress response in patients suffering from FH. Further to this, liver damages caused by accumulation of mutant LDL receptors in the ER, have according to my knowledge never been reported. However, the general chaperone Grp78 is a key component of the UPR system, it is involved in the quality control of the LDL receptor, and it interacts with apo B during its maturation [99], indicating that indirectly it could influence receptor mediated uptake of plasma LDL cholesterol. A large inter-individual variation exists in plasma cholesterol levels in heterozygous FH patients. A part of this variation is ascribed to genetic factors. We hypothesized that potential polymorphisms in the promoter region or in the coding region of GRP78 gene may influence the plasma cholesterol level and contribute to explain the observed inter-individual variation among heterozygous FH patients.

**Characterization of a Length Polymorphism in the GRP78 Promoter Region.**

The promoter region of the GRP78 gene (-370 to +223; numbers indicate the nucleotide position relative to the transcription start site) in 30 FH patients was already sequenced in our laboratory. Only one polymorphism was identified by Research Assistant Naseer Shukri). It was a length polymorphism consisting of a stretch of Gs -390(G)7-9, localized in a putative Sp1 site (Transcription Element Search Software (TESS): http://www.cbil.upenn.edu/tess) (Appendix 2). To investigate if the -390(G)7-9 polymorphism influenced the induction of GRP78 transcription, promoter regions containing the G7 row, the G8 row or the G9 row were inserted into the pGL3 vector as described in the section “Materials and Methods”. The constructs were termed pGL3-G7, pGL3-G8, and pGL3-G9. The various constructs were expressed in Chang cells, and a luciferase assay was performed. The measurements showed that the relative activity of luciferase in the cells expressing the G7 genotype was about twice the activity of the cells expressing G9 genotype, and about 36% higher than cells expressing the G8 genotype (Fig. 23A). One-way analysis of variance showed a statistical significant difference between the three groups (p ≤ 0.004). Induction of the UPR by tunicamycin treatment resulted in an about 3 fold induction of transcription (Fig. 23B). Interestingly, in the tunicamycin treated cells the relative activity was again higher in the cells expressing the G7 variant compared to cells expressing the G9 variant.
However, the difference between the three groups was no longer statistically significant ($p \leq 0.055$). The “fold induction” caused by tunicamycin treatment was about equal when comparing cells expressing the G7, G8, or G9 variants (Fig. 23B). This indicated that the -390(G)7-9 polymorphism did not influence the stress response. More likely, the -390(G)7-9 polymorphism may influence the constitutive transcription of the GRP78 gene. This is in accordance with the fact that no known stress-response elements are overlapping with the stretch of Gs. In contrast, the G-stretch is a part of a consensus sequence for a Sp1 site. Sp1 is a general transcriptional activator found in all cells and may be involved in constitutive expression of Grp78. Nevertheless, using a mathematical, matrix based, prediction model (TESS: http://www.cbil.upenn.edu/tess) the -390(G)7-9 polymorphism localized in a putative Sp1 site, did not disrupt the consensus sequence of the putative Sp1 site. So it remains an open question exactly how the -390(G)7-9 polymorphism influences the transcription of GRP78, and if the polymorphism affects the expression level of Grp78 protein.

**Figure 23. Functional characterization of the -390(G)7-9 polymorphism localized in the GRP78 promoter region.** 587 bp of the GRP78 promoter region (-364 to +223; numbers indicate the nucleotide position relative to the transcription start site) containing the G7, G8 or G9 variants were amplified by PCR from FH patients and inserted into the pGL3 vector. The constructs were transiently expressed in Chang cells and when indicated the cells were induced with 5µg/ml tunicamycin, 16 hours prior to harvesting (+TM). “Relative activity” and “fold induction” were calculated as described in the section “Materials and Methods”. Average of four independent transfections is presented with S.D. values (bars). The results showing that mutant, ER retained LDL receptors induced the transcription of Grp78 were obtained using the luciferase construct with a GRP78 promoter fragment containing the G7 variant (pGL3-G7). To test whether the induction of GRP78 transcription, caused by ER retention of mutant LDL receptors, was independent of the -390(G)7-9 polymorphism, Chang cells were co-transfected with pGL3 constructs, and the LDL receptor expression constructs, as previously described. The luciferase assay showed that the cells expressing mutant, ER retained LDL
receptors induced the transcription 2-3 fold when compared to cells expressing wild-type or non-ER retained LDL receptors (Fig. 24). This is in accordance with previous observations (Fig. 19 and 20). There were no noteworthy differences in the level of induction comparing cells expressing the different variants of the -390(G)7-9 polymorphism (Fig. 24). The induction of the transcription caused by retention of mutant LDL receptors in the ER, seemed to be independent of the -390(G)7-9 polymorphism.

Figure 24. Effect of the -390(G)7-9 polymorphism localized in the GRP78 promoter region on the induction caused by accumulation of mutant LDL receptors in the ER. Chang cells were co-transfected with the different pGL3-constructs and wild-type or mutant LDL receptor plasmids as indicated. When indicated, the cells were treated with 5 µg/ml tunicamycin (+TM) 16 hours prior to harvesting. Relative activity was determined as described in the section “Materials & Methods”. Average of two independent transfections is presented with S.D. values (bars).

Investigations of a Possible Association of the -390(G)7-9 Polymorphism in the GRP78 Promoter Region and Plasma Lipid Concentrations

In order to test if the -390(G)7-9 polymorphism was associated with the total cholesterol level in heterozygous FH patients, DNA samples were obtained from 168 Danish FH patients, positive
for the mutations: W23X, W66G or W556S, which are prevalent in the Danish population (Table 3). A 180 bp fragment of the GRP78 promoter including the -390(G)7-9 polymorphism was amplified by PCR and the genotype was determined by Genescan analysis as described in the section “Materials and Methods”. A multivariate analysis was performed with the variables: Grp78 genotype, gender, age, index case, and type of LDL receptor mutation. (This statistical analysis was performed by Chief Physician, Ulrik Gerdes, M.D., D.M.Sc.). The analysis showed no statistical significant association between the GRP78 polymorphism and the dependent variable: Total plasma cholesterol (p=0.684). Hence, the -390(G)7-9 polymorphism does not contribute significantly to the variation in total plasma cholesterol level in this specific population of Danish FH patients (Table 4). It is a well known fact that cholesterol levels rise with age. Accordingly, this study showed a significant association between age and plasma cholesterol levels (p<0.0001). This suggests that age accounts for the majority of the observed variation in total plasma cholesterol levels among the FH patients included in this study. A minor effect caused by the type of LDL receptor mutation was observed (p=0.028). The W66G mutation seems to be associated with lower total plasma cholesterol levels, when compared to the effect of the W23X and W556S mutations (table 3). This is in accordance with more studies showing that receptor-negative causing mutations (Class 1), like the W23X mutation, are more severe than receptor-positive mutations, like the W66G mutation, which possess some residual activity [146, 147, 148]. Nevertheless, previous studies of Danish FH patients harbouring either the W23X mutation or the W66G mutation, showed unexpectedly no significant difference in the plasma LDL cholesterol levels among the two groups of patients [149, 150].

Finally, no statistically significant effects of gender (p=0.746) or index case (p=0.401) were observed. In conclusion, the results suggested that the observed genetic variation in this specific region of the GRP78 promoter did not modify the cholesterol level in this population of Danish heterozygous FH patients (Table 4).

Table 3. Distribution of LDL receptor mutation, index patients, and gender of FH patients included in the multivariate analysis.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Class</th>
<th>Index patients</th>
<th>Total</th>
<th>Gender (f/m)</th>
<th>Mean Chol.</th>
<th>Confidence Interval (mmol/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W23X</td>
<td>1</td>
<td>24</td>
<td>55</td>
<td>32/23</td>
<td>10.2</td>
<td>9.5-10.9</td>
<td>[151]</td>
</tr>
<tr>
<td>W556S</td>
<td>2</td>
<td>12</td>
<td>25</td>
<td>14/11</td>
<td>10.5</td>
<td>9.6-11.4</td>
<td>[137]</td>
</tr>
<tr>
<td>W66G</td>
<td>3 or 5</td>
<td>32</td>
<td>88</td>
<td>57/31</td>
<td>9.6</td>
<td>9.0-10.2</td>
<td>[139, 140, 151]</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>68</td>
<td>168</td>
<td>103/65</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean Chol.: Mean total plasma cholesterol levels (mmol/l).
The DNA samples were generously provided by Ph.D. Lillian G. Jensen, Department of Clinical Biochemistry, Aarhus Amtssygehus, Chief Physician Ole Færgeman, Professor, and Chief Physician Dr.med. Mogens L. Larsen, Department of Medicine and Cardiology, Aarhus Amtssygehus.
Table 4. Mean total plasma cholesterol levels in heterozygous FH patients, adjusted for effects of age, LDL receptor mutation, gender, and index case.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>7/7 (n=56)</th>
<th>7/8 (n=83)</th>
<th>8/8 (n=23)</th>
<th>7/9 (n=3)</th>
<th>8/9 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total cholesterol level (mmol/l)</td>
<td>10.1</td>
<td>10.0</td>
<td>10.3</td>
<td>11.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Confidence Interval (mmol/l)</td>
<td>9.6-10.5</td>
<td>9.6-10.4</td>
<td>9.6-11.0</td>
<td>9.1-13.0</td>
<td>7.2-11.1</td>
</tr>
</tbody>
</table>

Number of individuals is given in parentheses. Evaluated at covariates appeared in the model: Age = 38.52

To investigate whether the frequencies of the alleles -390(G)7, -390(G)8, or -390(G)9 were different among heterozygous FH patients and the Danish population in general, 58 Danish control individuals were genotyped with respect to the -390(G)7-9 polymorphism, and the allele frequencies were compared with the allele frequencies of 75 apparently unrelated FH patients (index patients) (Table 5). Pearson’s $\chi^2$–test showed no statistical difference in the distribution of the alleles between the FH population and the Danish control population (p = 0.931). This is in accordance with the results expected. All the FH patients included in this study were genetically diagnosed and known to harbour a disease causing mutation in their LDL receptor gene (W66G, W556S or W23X). So in this specific population the main genetic cause of the hypercholesterolemia was mutations in the LDL receptor gene. Therefore, it is unlikely that the polymorphism in the GRP78 promoter region, localized on another chromosome, would show different allele distribution compared to the control population.

Table 5. Distribution of the allele and genotype frequency of the G-variant in heterozygous FH patients and control subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Allele Frequency</th>
<th>Number of subjects with the specific genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G=7 (88)</td>
<td>7/7 (28) 7/8 (29) 8/8 (14) 7/9 (3) 8/9 (1) 9/9 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G=8 (58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G=9 (4)</td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>75</td>
<td>58.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7%</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58</td>
<td>58.6%</td>
<td>7/7 (20) 7/8 (24) 8/8 (10) 7/9 (4) 8/9 (0) 9/9 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.9%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.4%</td>
<td></td>
</tr>
</tbody>
</table>

Number of alleles is given in parentheses.

We hypothesized that the -390(G)7-9 polymorphism could be a modifying factor of increased plasma cholesterol levels. However, in this study population the mutation of the LDL receptor gene caused the main increase of the cholesterol levels in the heterozygous FH patients. In order to detect a small effect caused by a modifying factor it is necessary to obtain precise and repeated measurements of the plasma lipid levels and to obtain a large sample size. None of these criteria were fulfilled in the described material. Further to this, it may be more informative to study a possible effect of the GRP78 polymorphism in a non-FH material. Most FH cases are caused by...
mutations in the LDL receptor gene and these mutations have a tremendous effect on the plasma lipid levels. Hence minor effects caused by modifying polymorphisms may be undetectable when combined with the effect of the LDL receptor mutations. In conclusion, no detectable association between the -390(G)7-9 polymorphism and the plasma cholesterol levels was detected. Nevertheless, we can not totally rule out that the -390(G)7-9 polymorphism has a minor effect on the cholesterol level. It might be that the polymorphism possesses little influence on cholesterol level when considered individually, but when inherited in combination of deleterious alleles confers susceptibility to dyslipidemia.

Investigations of combined effects of more lipoprotein metabolism related polymorphisms require very large sample sizes. The regulation of the plasma cholesterol system is a complex system, influenced by environmental and genetic factors as well. Many proteins are involved in the metabolism of lipids, including the regulation of intracellular synthesis and up-take of cholesterol and those involved in metabolizing the different lipoproteins in the plasma, for instance LPL, CEPT and LCAT. Polymorphisms and mutations in some of these factors have been identified and shown to influence plasma lipid levels (reviewed in [152][153]. However, the current knowledge does not alone account for the observed variation of the cholesterol phenotype. In the general population a combination of polymorphisms in the genes encoding these proteins, may act together to determine the heritable variation in plasma lipid concentrations. According to the complexity of the lipoprotein metabolism, and the fact that many of the involved proteins have not been screened systematically for sequence variants, it is difficult to identify new sequence variants contributing to a complex trait like elevated cholesterol levels. This was indeed concluded in a large study, which systematically identified sequence variations in 10 genes involved in the synthesis and up-take of cholesterol [154]. No evidence was found that the newly identified polymorphisms in any of the 10 genes contributed to dyslipidemia.

We hypothesized that polymorphisms in the regulatory part of Grp78 may influence plasma lipid levels. However, recent results showed that Grp78 is tightly regulated at the translational level, indicating that a polymorphism like -390(G)7-9 with a minor effect on the transcription level, may not alter the protein level of Grp78. Functional polymorphisms in the coding region of the GRP78 may be better candidates for influencing the regulation of the LDL receptor and apo B. At present the coding region of GRP78 is not systematically screened for polymorphisms. No known mutations in the ER chaperone proteins have to my knowledge been published except for the protein specific chaperone MTP. Considering the broad function of the ER chaperones it is likely that they are so essential to the cell that mutations which alter the function are detrimental to the cells.

Loss of MTP function decreases the amount of triglyceride availability for VLDL assembly and results in a concomitant increase in apo B degradation [155]. Most interestingly, recent results
showed that the LDL receptor mediates presecretory degradation of apo B, and it was suggested that the LDL receptor bind to apo B during the period of lipid loading of apo B [156](Fig. 25). The proportion of apo B that escapes degradation within the secretory pathway primarily determines the rate of VLDL secretion. The amount of folded LDL receptor protein in the ER lumen may be involved in the regulation of apo B secretion. Hence loss of functional LDL receptors may result in both disturbed up-take of LDL and disrupted regulation of the secretion of VLDL. In fact, increased secretion of VLDL has been observed in some FH patients [157, 158, 159]. Furthermore, it was shown that statin treatment of liver cells increased degradation of apo B [160], an effect which may be due to an increased amount of LDL receptor protein. Exactly how

Figure 25. Proposed model for the quality control of the LDL receptor. The LDL receptor is synthesized as a precursor protein, and this study showed that Grp78 associates with the newly synthesized LDL receptor and may be involved in retention of mutant LDL receptors in the ER. Calnexin was co-immunoprecipitated with the LDL receptor, suggesting an interaction between the newly LDL receptor precursor protein and calnexin. Other studies showed that RAP may facilitate the folding of the LDL receptor [106]. Additionally, it was shown that the LDL receptor associates with its ligand apo B, and thereby contributing to the complex post-transcriptional regulation of apo B. At present, the degradation pathway of the LDL receptor is unknown. However, it is likely that, similar to other ER proteins, the LDL receptor is retro-translocated out of the ER and degraded by the ERAD system. Finally, accumulation of mutant LDL receptors in the ER may induce the UPR pathway.
the LDL receptor influences the degradation of apo B is uncertain. Nevertheless, efficient quality control of newly synthesized LDL receptor and apo B may be obligatory to keep balanced plasma lipid levels. Genetic variations in the genes encoding the LDL receptor, apo B and the chaperones Grp78, calnexin, MTP, and RAP may disturb the complex regulation of apo B secretion and LDL receptor mediated up-take and thereby influence the plasma cholesterol levels.

Concluding Remarks

The main purpose of this study was to identify chaperones and folding enzymes involved in the folding and maturation of the wild-type LDL receptor and potential candidates for retaining class 2 LDL receptors in the ER. It was shown that the ER chaperones Grp78 and calnexin co-immunoprecipitated with wild-type and ER retained mutant LDL receptors. The association between Grp78 and two mutant class 2 (W556S and C646Y) LDL receptor precursors persisted for more than four hours, while the interaction between wild-type LDL receptors was no longer detectable after 2½ hours indicating the Grp78 dissociated from the wild-type LDL receptor. This indicates that Grp78 may be involved in retention of mutant LDL receptors in the ER. About five times more Grp78 was co-immunoprecipitated with W556S and C646Y-mutant LDL receptors showing that Grp78 is associated with the accumulated mutant LDL receptor protein. Grp78 is linked to more functions during protein synthesis and folding in the ER, including import, assisting protein folding and retrograde translocation. In the steady-state situation about five-times more Grp78 co-immunoprecipitated with the W556S and C646Y-mutant LDL receptors compared to cells over-expressing the wild-type receptor. Since the amount of newly synthesized LDL receptor protein is comparable, considering the cell expressing wild-type or mutant protein, it is unlikely that Grp78 is only involved in import of receptor protein in the ER. However, Grp78 may act as a chaperone for the LDL receptor and may be involved in a possible retrograde translocation of LDL receptors out of the ER for potential degradation by the ERAD system (Fig. 15). Further to this, recent results indicated that induction of the UPR was mediated by dissociation of Grp78 from ATF6 and IRE1. The dissociation is thought to be caused by competitive binding between the accumulating misfolded proteins and ATF6 and IRE1 [127, 161]. Indeed over-expression of the W556S- and C646Y- mutant LDL receptors induced the transcription of the Grp78 gene 2-3 times compared to over-expression of the wild-type LDL receptor. However, more investigations must be performed in order to conclude, if accumulation of mutant LDL receptors induces the UPR pathway.

Grp78 and calnexin are both components involved in the quality control of the LDL receptor and apo B [99, 162]. The understanding of the complex regulation of apo B secretion and LDL uptake taking place in the ER may lead to a better understanding of the inherited variation in plasma cholesterol levels and may ultimately lead to alternative treatment strategies of FH patients.
Materials & Methods

The following section contains a description of the methods used for generation of not yet published results and methods which have not been described in the section “Experimental Procedures” in Appendix 3.

Construction of Grp78 Reporter Plasmids

A 587 bp fragment of the GRP78 promoter region (-587 to -1; numbers indicate the nucleotide position relative to the translation start site) (Appendix 2) was amplified by the polymerase chain reaction (PCR) from Genomic DNA. The gene specific primers were designed with a Sac I site in the forward primer: 5’CCCGAGCTCCTCCTGCTGGACCTACTC3’ and a Hind III site in the reverse primer: 5’GGGAGAGCTTAAGCTTGCCAGCCAGTGG3’. Templates were chosen in order to obtain fragments with various length of the G-stretch representing the polymorphism -390(G)7-9 [163], with -390 representing the first G-nucleotide, which is repeated 7 to 9 times in the population. The PCR conditions are given below.

The PCR products were purified using the Concert™ Rapid PCR Purification System (Life Technologies), digested with Sac I and Hind III, and purified using the Concert™ Rapid Gel Extraction system (Life Technologies). Finally, the PCR products were ligated with a Sac I/Hind III digested, alkaline phosphatase-treated, purified pGL3-Basic vector (Promega).

The ligation mixtures were transformed into Epicural Coli XL-2 Blue ultracompetente cells (Stratagene). The resulting colonies were screened directly by PCR using GRP78 promoter specific primers. Positive clones were isolated. To verify that the purified plasmids contained different numbers of Gs, representing the -390(G)7-9 polymorphism, plasmids were subjected to sequence analyses. Bacterial clones comprising the correct construct were selected for large scale plasmid purification using QIAGEN®Plasmid Midi kit (Qiagen).

PCR conditions for generation of GRP78 promoter fragment.

<table>
<thead>
<tr>
<th>PCR reaction</th>
<th>PCR conditions (DNA Thermal cycler 480)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP (0.2 mM)</td>
<td>96°C, 2 min</td>
</tr>
<tr>
<td>Forward primer (0.1 μM)</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Reverse primer (0.1μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10xPfuTurbo buffer*</td>
<td>96°C, 0.5 min</td>
</tr>
<tr>
<td>Template</td>
<td>65°C, 1 min</td>
</tr>
<tr>
<td>PfuTurbo DNA polymerase* (2.5w/μl)</td>
<td>72°C, 1.5 min</td>
</tr>
<tr>
<td>H2O to 50 μl</td>
<td>72°C, 10 min</td>
</tr>
<tr>
<td>*Stratagene</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

50
Generation of Calnexin Expression Construct

The calnexin cDNA was generated using “1st Strand™” cDNA kit (CLONTECH Laboratories) according to supplier’s recommendations. Calnexin cDNA was amplified using two primers flanking the 5’ and 3’ ends of the coding region of the calnexin gene (sense primer: 5’-GCGGTCGACGAGGAGGTAGAG; antisense primer: 5’-AAATCACAGCTCGAGCTCTTAAGATTG-3’). The PCR product was purified, cut with the endonucleases Sal I and Xho I, and cloned into the Xho I site of the pcDNA3.1(+) vector. Correct orientation and sequence of the pcDNA3.1 construct was confirmed by DNA sequencing.

Metabolic Labelling and Chemical Cross-linking of Proteins

Chang cells (ATCC, CCL-13) were cultivated in RPMI 1640 (In Vitro, Denmark) containing 10% heat-inactivated foetal calf serum (FCS) (Life Technologies, Inc.), 100 unit/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 seeded in T-75 flasks (TPP®, Switzerland) 24 hours before transfection. On the day of transfection, cells were inspected microscopically in order to check that they had reached 50% confluency. Transfection was performed using FuGENE™6 transfection reagent (Roche Diagnostics Corporation) according to suppliers’ recommendations. 16 hours after transfection the cells were seeded in 12.5 cm² culture dishes. The transfected cells were incubated in methionine- and cysteine-free RPMI 1640 (In Vitro, Denmark), containing 5% dialyzed FCS for 30 minutes to deplete intracellular pools of methionine and cysteine. The depletion medium was replaced with methionine- and cysteine-free RPMI 1640, containing 0.1 mCi/ml Promix (³⁵S-methionine and ³⁵S-cysteine) (Amersham Pharmacia Biotech), the cells were labelled for 2 hours and washed 3 times in 4°C PBS. The cells were cross-linked while still attached to the culture flask. The cells were incubated on ice for 30 minutes in PBS with 2 mM DSP from a freshly prepared 10X stock in DMSO. Non-reacted cross-linking reagent was blocked for 10 minutes by adjusting the PBS-cross-linking mixture to 50 mM Tris/HCl. The cells were then scraped, washed, and lysed as described in the section “Experimental Procedures” in appendix 3.

Immunoprecipitation of Cross-linked Proteins.

The immunoprecipitation was performed with polyclonal and monoclonal anti-LDL receptor antibodies [137, 164], monoclonal anti-KDEL antibodies (5 μg/ml, SPA-827, StressGen Biotechnologies Corp.), polyclonal anti-calnexin antibodies (1:200, SPA-860, StressGen Biotechnologies Corp.) and protein A-sequensose. The immunoprecipitation was performed overnight at 4°C with gentle shaking. The immunocomplexes were washed 4 times in wash buffer (50 mM Tris/HCl (pH 8.6), 150 mM NaCl, 1% Triton X-100, 0.5% SDS). The immunocomplexes were eluted from the beads by incubation in Laemmli sample buffer, for 5 minutes at 95°C and analysed by 4-15 % gradient SDS-polyacrylamide gel electrophoresis. The gel was dried and analysed using the Phosphor Imager (STORM 840, Molecular Dynamics) and the Imagequant software (Molecular Dynamics).
Transfection Experiments for Transient Expression of Reporter Plasmids

Twenty-four hours before transfection Chang cells (ATCC, CCL-13) were cultivated in RPMI 1640 (In vitro) containing 5% heat-inactivated foetal Calf serum (Life Technologies), 100 unit/ml penicillin (Leo) and 0.1 mg/ml streptomycin (Leo) in a 5% CO₂/95% air atmosphere at 37°C. The cells were seeded in 12-well culture dishes (TPP®). On the day of transfection the cells were inspected microscopically in order to check that they had reached 50% confluency. The cells were transfected with 75 ng reporter plasmid and 150 ng CMV-β-galactosidase reference plasmid using FuGene 6™ transfection reagent according to suppliers’ recommendations. When indicated cells were co-transfected with 150 ng AAV-based, pMP6 expression vector harbouring WT-, W556S-, C646Y-, W66G-, or E119K LDL receptor cDNA, generated as described previously [137]. For induction of UPR cells were treated with 5 µg/ml Tunicamycin (Sigma) for 16 hours prior to harvesting. Forty-eight hours after transfection, cells were washed twice in 4°C PBS and lysed in 75 µl lysis buffer (100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100), and harvested with a plastic scraper. Firefly luciferase activities were measured with 15 µl of cell lysate mixed with 90 µl of freshly prepared assay-buffer (25 mM glycylglycin, 15 mM K₃PO₄, 15 mM MgSO₄, 4 mM EGTA, 2 mM ATP, 1 mM DTE). The luciferase activity was determined by automatic injecting 50 µl luciferin stock (0.2 mM, Luciferin, 25 mM glycylglycin, 1mM DTE) to the samples. β-galactosidase activities were measured with 3 µl of cell lysate using the Galacto-Light Plus assay (Tropix) according to suppliers’ recommendations. A Berthold Microlumat LB96P luminometer was used at a linear range. “Relative activity” is defined as the ratio of firefly luciferase activity to β-galactosidase activity and was calculated simply by dividing luminescence intensity obtained with the assay for firefly luciferase by that for β-galactosidase. “Fold induction” is defined as the ratio of induced to basal levels of reporter activity and was calculated by dividing average value of relative activity in lysate of ER-stressed cells by that of unstressed cells (each value determined from two or four independent transfections).

Northern Blotting

Total RNA was isolated from transfected Chang cells or HepG2 cells stably overexpressing wildtype or mutant LDL receptors (kindly provided by Marie S. Andersen M.Sc. and Associate Professor Thomas G. Jensen M.D., D.M.Sc., Department of Human Genetics, University of Aarhus). The RNA was isolated using the TRIZol® reagent (Invitrogen). Twenty micrograms of RNA (each lane) were subjected to electrophoresis in a 1% agarose formaldehyde gel according to standard procedure and blotted to a nylon membrane (Zeta-Probe) (Biorad). A GRP78 specific probe (cDNA position 602-1185, 583 bp) and a glyceraldehydes-3-phosphate dehydrogenase (GAPDH) specific probe (cDNA position 53-417, 364 bp) were amplified by PCR. Probes (35 ng) were labelled with [α-32P]dCTP by random priming using Prime-It II Random priming Primer labelling Kit (Stratagene) and purified with a ProbeQuant G-50 column (Amersham Biosciences). The membranes were pre-hybridized for 4 hours at 42°C in hybridization solution (50% deionized formamide, 1x Denhardt solution, 5x SSPE, 5% Dextran sulphate, 1% SDS, 0.2
mg/ml herring Sperm DNA). Hybridization was carried out at 42°C for 20 hours. Blots were subsequently washed once in 2 x SSPE, 0.05% SDS at 20°C for 20 minutes, and three times in 0.1 x SSPE, 0.1% SDS at 50°C for 20 minutes. The radioactive bands were visualized by phosphor-imaging using a PhosphorImager (Storm 840, Molecular Dynamics).

**Western Blotting**

Transfected Chang cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 2% Chaps, 2 mM CaCl2, 2.5 mM MgCl2, 2.2 % DMSO, 1 mM PMSF, 0.5 mM leupeptin, 1 µg/ml aprotinin, 10 mM N-ethylmaleimide (NEM)) for 30 minutes at 4°C, prior to centrifugation at 10,000xg for 30 minutes. When indicated the lysates were subjected to immunoprecipitation with anti-LDL receptor antibodies and protein A-Sepharose as described in appendix 4. The protein concentrations of the resulting supernatants were determined using the Bio-Rad Protein assay (Bio-Rad) based on the method of Bradford. 20 µg of total protein was mixed with Laemmli samplebuffer, incubated at 95°C for 5 minutes and analyzed by 4-15% gradient SDS-polyacrylamide gel electrophoresis. The separated proteins were subsequently transferred to a PVDF membrane by semi-dry electroblotting. The blotted membranes were blocked in PBS, 5% skim milk powder, 0.05% Tween 20, and incubated overnight with a monoclonal anti-KDEL antibody (1:2000, SPA-827, StressGen Biotechnologies Corp.) or monoclonal anti-calnexin antibody (1:5000, SPA-866, StressGen Biotechnologies Corp.) at room temperature. The membranes were washed and incubated for 2 hours with a horseradish peroxidase conjugated goat-anti-mouse secondary antibody (1:25,000, DAKO). Both antibodies were diluted in PBS, 5% skim milk powder, 0.05% Tween 20. Chemiluminescence detection was performed with the ECL Plus Western Blotting Detection system (Amersham Biosciences) prior to analysis using the Phosphor Imager and the ImageQuant software.

**PCR Amplification of the GRP78 Promoter and Genescan Analysis of the -390(G)7-9 Polymorphism**

Genomic DNA from heterozygous FH patients was kindly provided by: Lillian G. Jensen, Ph.D., Chief Physician Mogens L. Larsen, M.D., D.M.Sc. Chief Physician Ole Færgeman, M.D. D.M.Sc., and Physician Henrik K. Jensen, Ph.D., M.D.Sc, Department of Clinical Biochemistry, Aarhus Amtssygehus. DNA from Danish control individuals was obtained from the cell bank repository at the Panum Institute, Copenhagen, Denmark. Using genomic DNA as template, PCR amplification of a 180 bp fragment of the GRP78 promoter (-486 to -306) was performed with the primers: Forward primer: 5´-GGATGGGGCGGATGTTATCTACC-3´, Reverse primer: 5´-TCGTTGGAGGCCGTTCATTGG-3´. The reverse primer was 5´-labelled with 6-Fam (Applied Biosystems). The PCR conditions are given below.

The PCR products were sized using Genescan®-350[TAMRA] Size Standard. (Applied Biosystems). 1µl diluted PCR product was mixed with 3 µl deionised formamid, 0.5 µl PE loading buffer, and 0.8 µl TAMRA 35-350 bp Size Standard. The mixture was denaturated for 2
minutes at 95°C. 2 µl was loaded onto a 4.75% polyacrylamid, 6M urea gel on an ABI 377 automated fragment analyzer. To reduce risk of sizing errors, samples with known sizes, confirmed by direct sequencing, were included at each gel. Alleles were determined by the GeneScan® software.

**PCR conditions for generation of a Fam-labelled GRP78 promoter fragment.**

<table>
<thead>
<tr>
<th>PCR reaction</th>
<th>PCR conditions (GeneAmp® PCR System 9700)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP (0.2 mM)</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>Forward primer (10 pmol/µl)</td>
<td>1.5 µl 96°C, 12 min 1 cycle</td>
</tr>
<tr>
<td>Reverse primer (Fam) (10 pmol/µl)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>10xPCR buffer*</td>
<td>2.5 µl 94°C, 45 sec</td>
</tr>
<tr>
<td>Template</td>
<td>0.8 µl 59°C, 5 sec 30 cycles</td>
</tr>
<tr>
<td>Amplitaq gold DNA polymerase* (5 u/µl)</td>
<td>0.5 µl 72°C, 30 sec</td>
</tr>
<tr>
<td>H2O to 25 µl</td>
<td></td>
</tr>
</tbody>
</table>

*Products obtained from Applied Biosystems.

**Statistical Methods**

We used a general linear model with GRP78 genotype (5 categories), LDL receptor mutation (3 categories), index case (yes, no), gender (male, female), and age as independent variables. Total plasma cholesterol was the dependent variable. We estimated the effects of the GRP78 genotype, and tested for statistically significant differences in mean cholesterol between genotypes. Data analysis was performed using SPSS version 10.0 (SPSS Inc., USA). A value of P<0.05 was considered statistically significant. *The design and calculations were performed by Chief Physician Ulrik Gerdes, M.D., D.M.Sc., Department of Clinical Biochemistry, Aarhus Amtssygehus.*
Summary

Elevated blood-cholesterol level, atherosclerosis and increased risk of premature coronary heart disease characterize Familial Hypercholesterolemia (FH). FH is an autosomal dominant inherited disorder of lipoprotein metabolism. Heterozygous FH is a common disorder with an estimated frequency of about 1 in 500, and it is mainly caused by mutations in the low density lipoprotein (LDL) receptor gene. Although FH is considered being a monogenic disease, a remarkable inter-individual variation exists in the degree of hypercholesterolemia, even within families carrying the same LDL receptor mutation. Environmental factors play an important part in this variation, but genetic factors other than mutations in the LDL receptor may also influence the clinical phenotype. It could be genetic variations in the genes encoding specific chaperones localized in the endoplasmic reticulum (ER). The chaperones involved in folding and maturation of newly synthesized LDL receptors may possess the ability to influence the number of functional LDL receptors at the cells surface. In fact, about 50% of the characterized mutations in the LDL receptor gene results in a mutant receptor protein that is retained in the ER. However, when this project was initiated only limited information was available about which factors were involved in the folding, retention and degradation of newly synthesized LDL receptors. During this Ph.D. study it was shown that the molecular chaperone Grp78 and calnexin co-immunoprecipitated both with wild-type and ER retained mutant LDL receptors in lysates obtained from human epithelial cells over-expressing wild-type or mutant LDL receptors. A pulse-chase study showed that Grp78 showed prolonged association with the mutant receptors when compared to the wild-type LDL receptor. Furthermore, about five times more Grp78 protein 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. A similar effect was not observed during over-expression of calnexin or during co-immunoprecipitation of calnexin, leaving it an open question whether calnexin is involved in the quality control of the newly synthesized LDL receptor.

The chaperone Grp78 is a key component of the “unfolded protein response” (UPR) pathway. This pathway helps the cells to respond to changing conditions and needs during cellular stress situations, such as accumulation of mutant proteins in the ER. Simplified, the cells respond by an increased synthesis of chaperone proteins and a decrease in the general protein synthesis. Alternatively, if the stress situation becomes too violent for the cells they will go into apoptosis. This Ph.D. study showed that over-expression of mutant ER retained LDL receptors resulted in induction of the GRP78 promoter when compared to over-expression of wild-type and non-ER retained mutant LDL receptors. This indicated that accumulation of mutant LDL receptors in the ER induced the UPR pathway and indicated that the UPR system may contribute to prevent damages to the cell caused by accumulation of mutant LDL receptors in the ER.

We hypothesised that polymorphisms in the promoter region or coding region of the GRP78 gene could influence the expression of functional LDL receptors at the cell surface, and thereby
influence the cholesterol level in FH patients. A luciferase-assay showed that a newly identified polymorphism in the GRP78 promoter decreased the activity of the promoter. However, in a population of Danish heterozygous FH patients no association between the polymorphism and total plasma cholesterol levels was detected.
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* Indicates that Malene M. Jørgensen has contributed to the publication.


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Appendix 1

LDL receptor precursor (Swissprot accession: P01130)

1  MGPGWKLRTVALLAAAGTAVGDRCERNPQCQDGKCISYWKVDGSAECQDGSDS
61  ETCLSTVCSDGFSCCGVRNCPQFWRCGVQVDCDNGSDEQGCPKTCSQDEFRCHDGK
121  CISRQVCDSRDCLDGSDAESCPVTGPAFQNSSTCIPQLWACDNPDCEGSGDSEW
181  PQRGLYVFQDSPPCSAFEFHCGLSGCIIHSSWRCDGGPDCDKDSDEENCAVATCRPDE
241  FQCDGNCIHSQRCDREDYSDKMSDEVGCNVTCEPGFNSKCHSGCITLDKVCN
301  DCRDWSDEPIKECGTNECLDNSGCSHVCNDLKIYECLCPDFQQLVAQRRCEDIDE
361  PDTCSQLCVNLGGYKCQCEGFGQLDPHTKCAKAVGSIAYLFNTNRHEVRKMTLDREY
421  SLIFNLRVVALTVASRNIIYWSLSQRMICSTQLDRAHVGVSSYDTVIRSDIQAPDLG
481  VDWIHSNIVWTSLGTVSVAVTKGVRKTLFIRENGSIPRAIVVDVPFHMAYWTDGT
541  KIKKGGLNGBDIYSLVTENIQWPNGITDLDSGRLYWVDKLSHSISSIDVGNNRTILE
601  DEKRLAPFLVFDKVFWTDINEAIFSANRTGSVDNLLAENLSPEDMVFLHINLTV
661  PRGVNWCERTLSNGCQYLCLPAQPINHPFSKFTCACPDDLLARDMRILEAEAAVA
721  QETSTVRLKVSSTAVRTQHTTRTPVPDTSRLPGATPGLTVIATMSHSQALGDVAGRGN
781  EKFPSSVRALSVLPIVVLLVFLCLGVFLLWKNWRKINSINFDNPVQKTEDEVICH
841  NQDGYSYPSQMVSDDDVA

Amino acid sequence of the LDL receptor precursor protein. The mutations utilised in this study (W66G, E119K, C646Y, W556S, and C660X) is shown in blue. Note the numbers specifying the location of the mutation refer to the amino acid number of the mature protein. Above is shown the sequence of the total sequence including the 21 aa ER localisation signal, which is cleaved from the mature protein. The two N-glycosylation sites are shown in red. The signal sequence is shown in italics, the 7-ligand binding repeats is underlined, the 3 EGF-precursor like repeats is highlighted, the 6 YWTD repeats is underlined with a dash, the O-linked sugar domain is double underlined, and finally the transmembrane domain in shown in bold.
Appendix 2

Nucleotide sequence of the human GRP78 promoter (X59969)

1  cccgggggtcactcctgtgagctactccgaccccccctaggccggagtgaggcggac
61  ttgtgccggttaccagccgaatgcctcggggtcagagatgctcaggagatagacagctg
121  ctaagcggaccagggatgggggctggttatctaccaatttggtgacagttagaaa
181  cgaatagcagcggaccagcggctggggggcggagctagtgacgttttataggagggg
241  cccgcttcgaatccggcggccagtctgggtgcctgaggccagcttggcctggccaattgcgag
301  cagggccttacgaagcgctgacgcaggtgaggtcgacgccggccaagacagcacagacagattgacctattggggtg
361  taggcgacggttagctgacccggccaaaagcacacagacagattgacctattgggttg
421  ttcgctgagtgtgaggggaacgagccggcctgtattttgctagcctgcccttcgcctgg
481  ttcgctgagtgtgaggggaacgagccggcctgtattttgctagcctgcccttcgcctgg
541  cctgtgccctagctgtgggctctgtgactgtgctgtgctgtgcccaactggtgcaagatggggg
601  CTCTCCCTGTTGGCCCGATGTCTGCTGCTGCTGCTCGCGCCCGCCGGGGAGGAGGAGGAC
661  AAGAAGGAGGA

The promoter region cloned in the pGL3 basic vector and used in the luciferase assays is shown in black and the coding sequence is capitalized. The TATA box is boxed and the translation initiation start site is indicated by the arrow. The putative SP1 sites are highlighted (TESS). The CCAAT-like motifs are underlined and shown in blue. Finally the three ER stress response elements (ERSE) are highlighted.
Appendix 3

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


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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 2½ 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 (reviewed in Refs. 3 and 4). Missense mutations and small in frame deletions or insertions rarely affect the function of a given protein directly. Mostly these disease-causing mutations affect the ability of the proteins to fold into a correct conformation, and they often give rise to premature degradation or aggregation of the mutant proteins. Diseases caused by this kind of molecular pathological mechanism are termed “conformational diseases” (5, 6). Yet, few diseases have been experimentally proven to be conformational diseases. Prominent examples related to ER are Alzheimer’s disease, Creutzfeld-Jakob disease, α1-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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§ The abbreviations used are: LDL, low density lipoprotein; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; FCS, fetal calf serum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser-desorption ionization-\textit{time-of-flight}; DII, 1,1-di-octadecy-3,3,3′,3′-tetramethyloindocarbocyanine perchlorate; PBS, phosphate-buffered saline; 6-AN, 6-aminonicotinamide.
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 Mg$^{2+}$-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 33862 (GGC-39-UGG). The second was a missense mutation causing a cysteine to tyrosine substitution at amino acid position 646 (C646Y) (15).

**EXPERIMENTAL PROCEDURES**

**Generation of Constructs**—Constructs expressing mutant LDL receptor were generated by site-directed mutagenesis and cloned in the pMP6 expression vector as described previously (14). The Grp78 cDNA was generated using “The 1st-Strand”® cDNA kit (CLONTECH Laboratories) as recommended by the supplier. Grp78 cDNA was amplified using two primers flanking the 5 ’ and 3 ’ ends of the coding region of the Grp78 gene (sense primer, 5 ’-CCGGCTCCGCCCTGCTGCTGCTGCTGCTGCTGCTGCTGGC-3 ’; antisense primer, 5’-GAGTCAGGTTCAATATATGAGCAGC-3’). The polymerase chain reaction product was purified, cut with the endonuclease SalI, and cloned into the XhoI site of the pcDNA3.1 (+) vector (Invitrogen). Correct orientation and sequence of the Grp78 construct was confirmed by DNA sequencing.

**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.), 5% dialyzed penicillin (Leo, Denmark), 0.1 mg/ml streptomycin (Leo, Denmark), and inactivated fetal calf serum (FCS) (Life Technologies, Inc.), 100 units/ml aprotinin, 10 nM N-ethylmaleimide, and harvested with a plastic scraper. For co-immunoprecipitation experiments, the LDL receptor was immunoprecipitated using a polyclonal anti-LDL receptor antibody (14) and protein A-Sepharose (Amersham Pharmacia Biotech). Alternatively, for purification of the LDL receptor, immunoprecipitation was performed under denaturing conditions. The cell extracts were supplemented with SDS to a final concentration of 1% and incubated at 95 °C for 5 min. The extracts were diluted with HBS buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100) to a final concentration of 0.1% SDS and 0.9% Triton X-100. The LDL receptor was immunoprecipitated overnight, at 4 °C, using the polyclonal anti-LDL receptor antibody (14), or a monoclonal anti-LDL receptor antibody (C7) (16), and protein A-Sepharose. The immunocomplexes were eluted from the beads by incubation in Laemmli sample buffer, for 5 min, at 95 °C and analyzed by 4–15% gradient SDS-polyacrylamide gel electrophoresis. Quantitation of gel bands was performed using a PhosphorImager (STORM 840, Molecular Dynamics) and the ImageQuant software (Molecular Dynamics).

**Two-Dimensional Gel Electrophoresis**—For two-dimensional gel electrophoresis the immunocomplexes were dissolved and solubilized by incubating in 50 μl of two-dimensional lysis buffer (8 μm urea, 2% CHAPS, 0.5% IPG-buffer 3–10NL (Amersham Pharmacia Biotech), 0.3% dithiothreitol, 1.25 mm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mm leupeptin). Chang cells 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 3–10NL, 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, 8.8 μm urea, 30% glycerol, 1% SDS) supplemented with 16 mM dithiothreitol and then run in the first incubation and 0.25 mM iodoacetamide 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 PhosphorImager and the ImageQuant software (Molecular Dynamics, CA) as described above.

**Protein Digestion**—Tryptic digestion of protein in excised two-dimensional gel spots was performed as described previously (17, 18). In brief, the excised gel plugs were washed in 100 mM NH$_4$HCO$_3$/acetoniocitrate (1:1). The protein was reduced and S-alkylated with iodoacetamide, and the gel plugs were dried by vacuum centrifugation. Modified porcine trypsin (12 ng/μl) (Promega, sequencing grade) in digestion buffer (50 mM NH$_4$HCO$_3$, 5 mM CaCl$_2$) 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 matrix samples of α-cyano-4-hydroxycinnamic acid and nitrocellulose were prepared by the fast evaporation method (17,18). An aliquot of peptide solution (0.3–1.0 μg) was mixed into 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).
**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-LDLr, or C646Y-LDL receptor were pulse-labeled with [35S]methionine and [35S]cysteine for 30 min and chased 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 × 10^6 cells were analyzed in each sample.

**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-diiodoacetyl-3,3,3,3-tetramethyldiethylcarbocyanine perchlorate (DiI-conjugated LDL (21) (Molecular Probes). Cells were harvested in PBS containing 0.6 μM 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 μM EDTA followed by a short incubation in PBS containing 0.6 μM EDTA and 0.01% trypsin. The cells were washed 2 times with PBS, and 0.6 μM EDTA was added to the sample, 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 × 10^6 cells were analyzed in each sample.

**Immunofluorescence Detection—**was performed with ECL Plus (Amersham Pharmacia Biotech) and direct image analysis using the Molecular Dynamics Storm blot imaging system.

**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 polyvinyliden difluoride membrane, blocked, and immunoblotted with anti-Grp78 antibody (PharMingen, Canada). The antibody was diluted 1:10,000 in PBS-Tween (0.15 X NaCl, 0.05% phosphate buffer, pH 7.2, 0.05% Tween20) 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 (Amersham Pharmacia Biotech) and direct image analysis using the Molecular Dynamics Storm blot imaging system.

**Grp78 Is Involved in ER Retention of Mutant LDL Receptors**

**Identification of the 78-kDa Protein Interacting with the LDL Receptor as Grp78**—To identify the 78-kDa protein co-immunoprecipitating with the LDL receptor, we used two-dimensional gel electrophoresis to purify and MALDI-TOF mass spectrometry to identify the protein.

Cells expressing wild type LDL receptor, W556S-LDL receptor, or C646Y-LDL receptor were continuously labeled for 2 h with [35S]methionine and [35S]cysteine and subjected to immunoprecipitation of wild type or mutant LDL receptor proteins (120 kDa, non-mature LDL receptor protein, and 160 kDa, mature LDL receptor protein) to immunoprecipitate a 78-kDa protein. The intensity of the bands representing the non-mature LDL receptor protein (120 kDa) and the band representing Grp78 (78 kDa) were quantified using a PhosphorImager. The band representing Grp78 in each lane was normalized to the band representing the non-mature receptor protein, is shown with time (a wild type LDLr, W556S-LDLr, C646Y-LDLr).

**Fig. 1.** Grp78 interacts transiently with the wild type LDL receptor and displays prolonged interaction with two mutant LDL receptors. Chang cells were transfected with plasmids expressing wild type (WT-LDLr) or mutant (W556S-LDLr or C646Y-LDLr) LDL receptors. The cells were pulse-labeled for 30 min and chased in complete medium containing 5 mM methionine, 5 mM cysteine, and 1 mM cycloheximide. The cells were lysed, and the extracts were subjected to immunoprecipitation with polyclonal anti-LDL receptor antibodies under non-denaturing conditions. A, immunoprecipitation of wild type and mutant LDL receptor proteins (120 kDa, non-mature LDL receptor protein, and 160 kDa, mature LDL receptor protein) co-immunoprecipitated a 78-kDa protein. B, the intensity of the bands representing the non-mature LDL receptor protein (120 kDa) and the band representing Grp78 (78 kDa) were quantified using a PhosphorImager. The intensity of the band representing Grp78, relative to each band representing the non-mature receptor protein, is shown with time (a, wild type LDLr, W556S-LDLr, C646Y-LDLr).

**dka protein and the band representing mutant LDL receptors is about 1/5 and constant during the 4-h chase, whereas it decreases from 1/10 to undetectable for the wild type receptor (Fig. 1B). Taken together, these results indicate that the 78-kDa protein interacts transiently with the wild type LDL receptor and is involved in retention of mutant LDL receptors in the ER.**
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 Grp78 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 glucolysis (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 cultivation of cells, in complete medium, for 4 h after 6-AN treatment allows NAD(P) and poly(ADP-ribose) polymerase activity to become normal, whereas Grp78 levels remain elevated (24). To verify that Grp78 can be induced also in our system, Chang cells, transfected with a plasmid expressing wild type (WT-LDLr) or mutant (W556S-LDLr or C646Y-LDLr) LDL receptors or were mock-transfected (pMP6). The cells were lysed and subjected to immunoprecipitation under non-denaturing conditions with polyclonal anti-LDL receptor antibodies (lanes 1–4). Immunoprecipitated complexes were analyzed by Western blotting with an antibody recognizing Grp78 and Grp94. As control aliquots (1/50) of the total extracts were isolated before immunoprecipitation and subjected to immunoblotting (lanes 5–8).

**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...
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 DiI-conjugated LDL at 37 °C (Fig. 7, B and C). 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 DiI-LDL incubation (p ≤ 0.00055). The DiI-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 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 NH$_2$ 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 NH$_2$ 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

Figure 7. Characterization of expressed cell surface-located LDL receptor protein in transfected Chang cells using flow cytometric measurements. Chang cells were transfected with plasmids expressing the wild type LDL receptor (1–3) or were mock-transfected (4–6). A subset of these cells were co-transfected with plasmids expressing Grp78 (2 and 5) or were treated with 0.2 mM 6-AN for 36 h (3 and 6). A, dot plots of representative flow cytometric analyses of transfected Chang cells incubated DII-LDL at 37 °C for 5 h before harvesting. Side angle light scatter is given on the x axis, and the fluorescence of individual cells in arbitrary units is given on the y axis. 99.75% of the mock-transfected cells has fluorescence below the horizontal background line. B, histogram showing median fluorescence above background, as defined under A, representing relative LDL receptor amounts on the surface of transiently transfected Chang cells, as measured by immunofluorescence staining with C7 antibody at 4 °C. Reported results are mean values (n = 3). C, histogram showing median fluorescence above background defined as the fluorescence value below which 99.75% of the mock-transfected cells was found. The results represent the activity of the cell surface-located LDL receptors, measured by take up of DII-LDL at 37 °C. Reported results are mean values (n = 3).
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 in 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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Grp78 Is Involved in ER Retention of Mutant LDL Receptors