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Functional analysis of the p.(Leu15Pro) and p.(Gly20Arg) sequence changes in the signal sequence of LDL receptor

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ABSTRACT

The low density lipoprotein receptor (LDLR) is a transmembrane protein that plays a key role in cholesterol metabolism. It contains 860 amino acids including a 21 amino acid long signal sequence, which directs the protein into the endoplasmic reticulum. Mutations in the *LDLR* gene lead to cholesterol accumulation in the plasma and results in familial hypercholesterolemia (FH). Knowledge of the impact of a mutation on the LDLR protein structure and function is very important for the diagnosis and management of FH. Unfortunately, for a large proportion of mutations this information is still missing. In this study, we focused on the LDLR signal sequence and carried out functional and *in silico* analyses of two sequence changes, p.(Gly20Arg) and p.(Leu15Pro), localized in this part of the LDLR. Our results revealed that the p.(Gly20Arg) change, previously described as disease causing, has no detrimental effect on protein expression or LDL particle binding. *In silico* analysis supports this observation, showing that both the wt and p.(Gly20Arg) signal sequences adopt an expected α-helix structure. In contrast, the mutation p.(Leu15Pro) is not associated with functional protein expression and exhibits a structure with disrupted a α-helical arrangement in the signal sequence, which most likely affects protein folding in the endoplasmic reticulum.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder associated with mutations in the low density lipoprotein receptor gene (*LDLR*) [1]. Phenotypically very similar is hypercholesterolemia due to ligand-defective apoB caused by mutations in the apolipoprotein B gene (*APOB*) [2] and familial hypercholesterolemia 3 associated with the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*) [3]. The clinical manifestation of hypercholesterolemia is mainly caused by a mutation in the *LDLR* or *APOB* gene.

FH is characterized by elevated LDL cholesterol levels, which leads to accelerated atherosclerosis and premature coronary heart disease. FH frequency in most populations is estimated to be 1:200–1:250 [4,5]. The human *LDLR* gene encodes a precursor protein of 860 amino acids comprising a 21 amino acid signal sequence at the N-terminus. This sequence is excised during protein translocation into the endoplasmic reticulum (ER) [6]. LDLR is synthesized on ribosomes of ER, then folded and partially glycosylated within ER and finally matured in the Golgi complex, where glycosylation is completed. Approximately 45 min after synthesis, LDLR appears on the cell surface and mediates the LDL particle uptake by receptor mediated endocytosis. The LDL particle is then released in the endosome, and the receptor recycles, i.e. it returns to the cell membrane. Each cycle takes about 10–15 min [7].

The complex function of the LDL receptor is ensured by its specific functional domains: an N-terminal ligand-binding domain composed of seven ligand binding repeats, an epidermal growth factor precursor homology domain, an O-linked sugar domain, a

Abbreviations: BSA, bovine serum albumin; CHO, chinese hamster ovary; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FH, familiar hypercholesterolemia; LDLR, low density lipoprotein receptor; MD, molecular dynamics.

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transmembrane domain, and a cytoplasmic domain [6]. LDLR mutations occur in all domains and have different effects on receptor function. This has led to the classification of LDLR mutants in five basic classes, class 1: LDLR is not synthesized (null mutations), class 2: LDLR is completely or partly blocked during transport from ER to the Golgi complex, class 3: LDLR does not bind the LDL particle, class 4: LDLR is not able to internalize the LDL particle by endocytosis, class 5: LDLR is not able to release the LDL particle in the endosome and does not return to the cell surface [8].

An almost complete structure of human LDLR has been determined using X-ray crystallography [9] but less is known about the LDLR signal sequence structure. In general, a sequence of signal peptide contains three regions: an N-terminal positively charged region, a hydrophobic region folded into α-helix, and a C-terminal polar region. An example of a signal peptide 3D structure is represented by the SRP54-signal peptide fusion protein, where a α-helical conformation of the peptide is apparent [10]. Apart from experimental methods such as X-ray crystallography and NMR spectroscopy, small protein structures can also be determined computationally using *in silico* methods. Recently, it was demonstrated that implicit solvent molecular dynamic (MD) simulations are instrumental in the study of protein folding [11]. The simulations showed that proteins of 10–90 amino acids can fold correctly on a microsecond time scale.

In this study, we describe the impact of two sequence variants, p.(Gly20Arg) and p.(Leu15Pro), located in the LDLR signal peptide on its protein structure and function using experimental and *in silico* methods. The p.(Gly20Arg) substitution was described as disease causing on the Leiden Open Variation Database (LOVD), http://www.ucl.ac.uk/ldlr/Current/index.php?select_db=LDLR and also on the Human Gene Mutation Database (HGMD), https://portal.biobase-international.com/hgmd/pro/gene.php?gene=LDLR. On the other hand, the frequency of this change is 9/8596 according to http://databases.lovd.nl/whole_genome/genes/LDLR (copied from the Exome Variant Server), which indicates that this change could rather be a benign variant than a causal mutation. The p.(Leu15Pro) substitution is mentioned as causal on www.ucl.ac.uk/ldlr/Current/and www.hgmd.cf.ac.uk/ac/index.php, and not shown on databases.lovd.nl/whole_genome/genes. In addition, we suppose that this change disrupts the expected α-helical arrangement of the signal sequence which could have a pathogenic effect on LDLR protein folding in ER.

2. Materials and methods

2.1. Mutagenesis, cell culture and expression of LDLR

The p.(Leu15Pro) and p.(Gly20Arg) mutations were created using site-directed mutagenesis using the plasmid pcDNA4-LDLR-linker-EYFP (kindly provided from MA Kulseth, Norway) as a template. The EYFP (enhanced yellow fluorescent protein) tag, inserted at the C-terminus of the *LDLR* cDNA and separated by a 10 amino acid linker, was used to facilitate the visualization of the protein in living cells. The EYFP tag does not affect the biosynthesis pathway of LDLR [12]. Sequence variations were introduced using the QuickChange Mutagenesis Lightning kit (Agilent Technologies) according to the manufacturer's instructions and the following oligonucleotides were used. For p.(Gly20Arg), the oligonucleotide 5'-tcgccgcggcgaggactgcagtg-3′ was used to change the codon GGG coding glycine to AGG coding arginine. For p.(Leu15Pro), the mutagenesis was performed using the oligonucleotide 5'-cgtcgccttgcccctcgccgcgg-3′, resulting in the change from CTC (leucine) to CCC (proline). The integrity of all of the constructs was confirmed using DNA sequencing.

The T-Rex CHO cell line was purchased from Life technologies. The tetracycline-regulated expression (T-Rex)-CHO cell line stably expresses the tetracycline repressor. This system allows gene expression induction by adding tetracycline to the cell culture medium. The CHO cells were maintained in Ham's F12 medium containing blasticidin (10 μg/ml, Invitrogen) and supplemented with fetal bovine serum (10%, MP Biomedicals). The CHO cells were transfected with plasmids with the wild-type (wt) or mutated *LDLR* gene by lipid-mediated transfection (Lipofectamine 2000, Invitrogen). The transfected cells were grown in a complete medium supplemented with Zeocin (600 μg/ml; Invitrogen) for 5 weeks to generate stable cell lines.

2.2. Immunocytochemistry

Confocal laser scanning microscopy was used to analyze the LDLR expression and binding of the LDL particles to the LDLR. Cells were seeded onto glass coverslips covered with fibronectin and allowed to adhere overnight. LDLR expression was induced by adding tetracycline (1 μg/ml, Sigma-Aldrich) for 24 h. To study LDL receptor localization, the growing medium was removed and the cells were washed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. The fixed cells were washed 3 times with blocking buffer (PBS with 0.1% bovine serum albumin (BSA)) and then permeabilized by incubating in PBS-0.5% Tween-20 for 10 min at room temperature. The cells were then washed 3 times in PBS-0.1% BSA. The fixed and permeabilized cells were incubated with concanavalin A tetramethylrhodamine conjugates (an ER marker; 1:400; Molecular Probes) for 1 h at room temperature. The cells were then washed 3 times in PBS. The slides were mounted with the SlowFade Anti Fade Kit with DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes) and sealed with nail polish. Fluorescent images were obtained on a Zeiss LSM 780 laser-scanning confocal microscope with a 63 × water objective and analyzed with ZEN software. Image processing was carried out with GIMP (version 2.8.14-fix), which is a freely distributed GNU Image Manipulation Program.

For LDLR binding studies, the cells were incubated in a growing medium containing 20 μg/ml of fluorescently labeled LDL particles conjugated with DiI (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate, Molecular Probes) at 37 °C for 4 h. After incubation, the medium was removed, and the cells were washed 3 times with PBS and fixed as described previously. The slides were mounted with the SlowFade Anti Fade Kit with DAPI and sealed with nail polish. Fluorescent images were obtained on a Zeiss LSM 780 with a 63 × water objective and analyzed using ZEN software. Image processing was carried out with GIMP.

2.3. Modeling signal peptides

MD simulations of LDLR wt signal peptide and two mutants were run using AMBER 14 [13] which is a package of programs for simulations of nucleic acids and proteins. Simulations are usually run using explicit solvent which allows the study of conformational changes of biomolecules on a nanosecond time scale at an atomic level. Alternative is a Generalized Born implicit solvent model where polar solvent is represented as a dielectric continuum. Using this approach larger (microsecond) time scale can be reached, which is useful when studying protein folding. We run Generalized Born implicit solvent simulations and used the same simulation conditions as in the study [11], i.e. force field ff14SBonlysc, mbondi3 intrinsic radii and GB-Neck2 setting for implicit simulation [14]. The LDLR wt signal protein and two mutants p.(Leu15Pro) and p.(Gly20Arg) were built in the Xleap module of AMBER. Their initial conformation was an unstructured chain with a standard N-terminal group (NH_3) while at the C-terminus NME residue was added