European Journal of Human Genetics (2001) 9, 815–822


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ARTICLE

LDL receptor-GFP fusion proteins: new tools for the characterisation of disease-causing mutations in the LDL receptor gene

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The function of a series of LDL receptor GFP fusion proteins with different, flexible, unstructured spacer regions was analysed. An optimised version of the fusion protein was used to analyse the effect of an LDL receptor mutation (W556S) found in FH patients and characterised as transport defective. In cultured liver cells this mutation was found to inhibit the transport of LDL receptor GFP fusion protein to the cell surface, thus leading to impaired internalisation of fluorescent labelled LDL. Co-localisation studies confirmed the retention of the mutant protein in the endoplasmic reticulum. Wild type (WT) and W556S LDL receptor GFP fusion proteins were expressed in mouse liver by means of hydrodynamic delivery of naked DNA. Two days after injection liver samples were analysed for GFP fluorescence. The WT LDL receptor GFP protein was located on the cell surface whereas the W556S LDL receptor GFP protein was retained in intracellular compartments. Thus, the GFP-tagged LDL receptor protein allows both detailed time lapse analysis and evaluations in animals for the physiological modelling of mutations. This method should be generally applicable in functional testing of gene products for aberrant processing. *European Journal of Human Genetics* (2001) **9**, 815–822.

Keywords: *In vivo* assay; tail vein injection; Familial Hypercholesterolaemia; confocal microscopy; time lapse

Introduction

Familial Hypercholesterolaemia (FH) is caused by mutations in the gene encoding the low density lipoprotein (LDL) receptor. These mutations are classified into five different groups, each group containing mutations that interfere with a particular step in the complex life cycle of the LDL receptor.[1] Like other membrane proteins, the LDL receptor has a signal peptide that is cleaved from the pre-protein during translocation into the endoplasmic reticulum (ER) which is followed by N-glycosylation and transport to the cell membrane. In the cell membrane the receptor binds LDL-particles and undergoes endocytosis. After release of the ligand, the receptors are recycled to the cell membrane.

Much of the present knowledge of the LDL receptor comes from the analysis of the plethora of naturally occurring mutations in the LDL receptor gene identified by efficient screening techniques.[2,3] Functional testing of these gene variants by overexpression in tissue culture has been used extensively in order to investigate the disease causing nature of the mutations.[4–10]

Cellular studies have shown that the 238 amino acid Green Fluorescent Protein (GFP) may be used as an *in vivo* reporter of gene expression and protein localisation.[11–14] Direct visualisation of GFP tagged proteins in living cells allows studies of protein dynamics.[15,16] To further extend our studies on LDL receptor mutations we have constructed fusion proteins

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Received 2 July 2001; revised 8 August 2001; accepted 15 August 2001

between LDL receptor (WT or mutant) and GFP. After having verified that the GFP-tag does not affect intracellular traffic or receptor function, we have used these fusion proteins to develop methods for characterising LDL receptor mutations in living cells and in liver *in vivo*. Here, we describe the properties of such fusion proteins and show that gene transfer to mice liver *in vivo* by intravenous injection of LDL receptor GFP DNA using a hydrodynamics-based procedure[17,18] can be used as a functional assay of LDL receptor mutations.

Materials and methods

Constructs

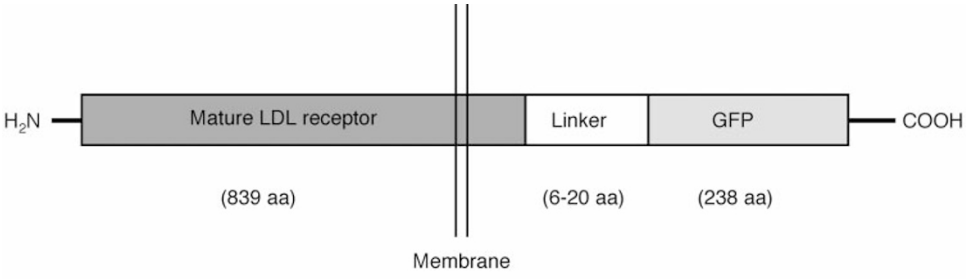
Fusions between coding sequences encoding WT LDL receptor and Enhanced Green Fluorescent Protein (EGFP) driven by the CMV promoter were generated in the pEGFP-N1 vector (Clontech Laboratories Inc., Palo Alto, CA, USA) by standard cloning procedures.[19] Linkers between LDL receptor and GFP were constructed by inserting one or two copies of the sequence 5′-AAGCTGCAGCAGCTGCGGCCG-3′ in the unique *Sma*I-site between the LDL receptor and GFP sequences. In this way we created LDLR-13-GFP (one copy of the linker), LDLR-19-GFP (two copies of the linker) and LDLR-20-GFP (two copies of the linker). The differences in the amino acid sequence of the linkers in these constructs corresponds to different reading frames (see Figure 1). For comparison a vector without the above mentioned linker was constructed (LDLR-6-GFP). Fusions containing W556S or C660X mutant LDL receptor sequences were generated by replacing the 1275 bp *Acc*B7I-fragment of the LDL receptor cDNA sequence with the corresponding fragment of previously described plasmids containing mutant LDL receptor cDNAs.[20] The W556S missense mutation is a G to C transversion at cDNA position 1730 in exon 12, causing the tryptophane to serine substitution. The C660X mutation is a C to A transversion at position 2043 in exon 14, creating a premature termination codon. The truncated protein retains only two domains: a complete ligand-binding region (residues 1–292) and a partial epidermal growth factor precursor homology region (residues 293–659). All constructs were verified by DNA sequencing. In some experiments plasmids encoding WT or W556S LDL receptor cDNA driven by the CMV promoter[10] were used for comparisons.

Cell culture and transfection

Chang cells, a human liver cell line (ATCC, CCL-13) were cultivated in RPMI 1640 (In Vitro, Fredensborg, Denmark) containing 10% foetal calf serum (FCS) (Life Technologies Ltd., Paisley, UK), 100 units/ml penicillin, 0.1 mg/ml streptomycin (both Leo Pharmaceutical Products Ltd., A/S, Ballerup, Denmark), in a 5% CO_2 and 95% air atmosphere at 37°C. Cells were seeded in chambered coverglass or slide-flasks for microscopy (Nunc A/S, Roskilde, Denmark) or 6-well plates for flow cytometry (TPP®, Trasadingen, Switzerland) 24 h before transfection. Transfection was performed using FuGene 6™ (Roche Diagnostics GmbH, Mannheim, Germany) according to suppliers' recommendations.

Fluorescence microscopy

Confocal laser scanning microscopy (CLSM) was used to determine the cellular localisation of the LDL receptor GFP fusion proteins in the transfected cells. Cells cultivated in slideflasks were washed 48 h post-transfection in PBS and fixed in 4% (w/v) paraformaldehyde as previously described.[10] For immunostaining of the LDL receptor proteins, the cells were permeabilised in 0.01% Triton X-100 and



GFP-series	Amino acid sequence and length of linker
LDLR-6-GFP	RVPVAT (6 aa)
LDLR-13-GFP	QAAAAAAGVPVAT (13 aa)
LDLR-19-GFP	KLQQLRPKLQQLRPVPVAT (19 aa)
LDLR-20-GFP	PAAAAAASAAAAAAWVPVAT (20 aa)

Figure 1 LDL receptor GFP fusion proteins. A series of different LDL receptor (LDLR) GFP fusion constructs were generated by cloning LDL receptor cDNA into the N-terminal end of the GFP gene in the expression vector pEGFP-N1 (Clontech). The LDL receptor is separated from GFP by four different linkers as indicated.

incubated with 2.5 μg/ml monoclonal anti-LDL receptor antibody immunoglobulin IgG-C7 (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England) for 30 min, washed in PBS and incubated with secondary goat anti-mouse antibody conjugated with Alexa 568 or Alexa 488 (Molecular Probes Inc., Eugene, OR, USA) diluted 1 : 400 in PBS containing 5% FCS (Life Technologies Ltd.) for 30 min. For nuclear staining the slides were submerged in PBS containing 1 μg/ml Höechst 33258 (bisbenzimid; Sigma-Aldrich Denmark A/S, Vallensbaek Strand, Denmark) for 1 min.

Binding of ligand to the LDL receptor was studied by removing the culture medium from the chambered coverglass and adding pre-warmed growth medium containing 3 μg/ml DiI-conjugated LDL[21] (Molecular Probes) for 15 min at 37°C. Then the cells were washed in complete growth medium and analysed. In order to selectively label the ER cells were incubated with 500 nM ER-Tracker™ (Molecular Probes) for 15 min at 37°C prior to wash and analysis. The liver samples (approximately 5 mm in thickness), obtained from mice, were placed between two coverslips and analysed for the GFP fluorescence immediately after excision.

Samples were analysed in an upright or inverted Leica TCS confocal laser scanning microscope (Leica Microsystems GmbH, Heidelberg, Germany). Image analysis was performed using IP-Lab Spectrum P 3.1a (Signal Analytics Corp.) and Adobe® Photoshop® 6.0 (Adobe Systems Inc.). The immunofluorescence stained cells shown in Figures 2, 4, 5 and 6 are representative of several microscopic fields examined.

Flow cytometry

For LDL receptor activity measurements transfected cells were cultivated for 5 h in growth medium at 37°C containing 3 μg/

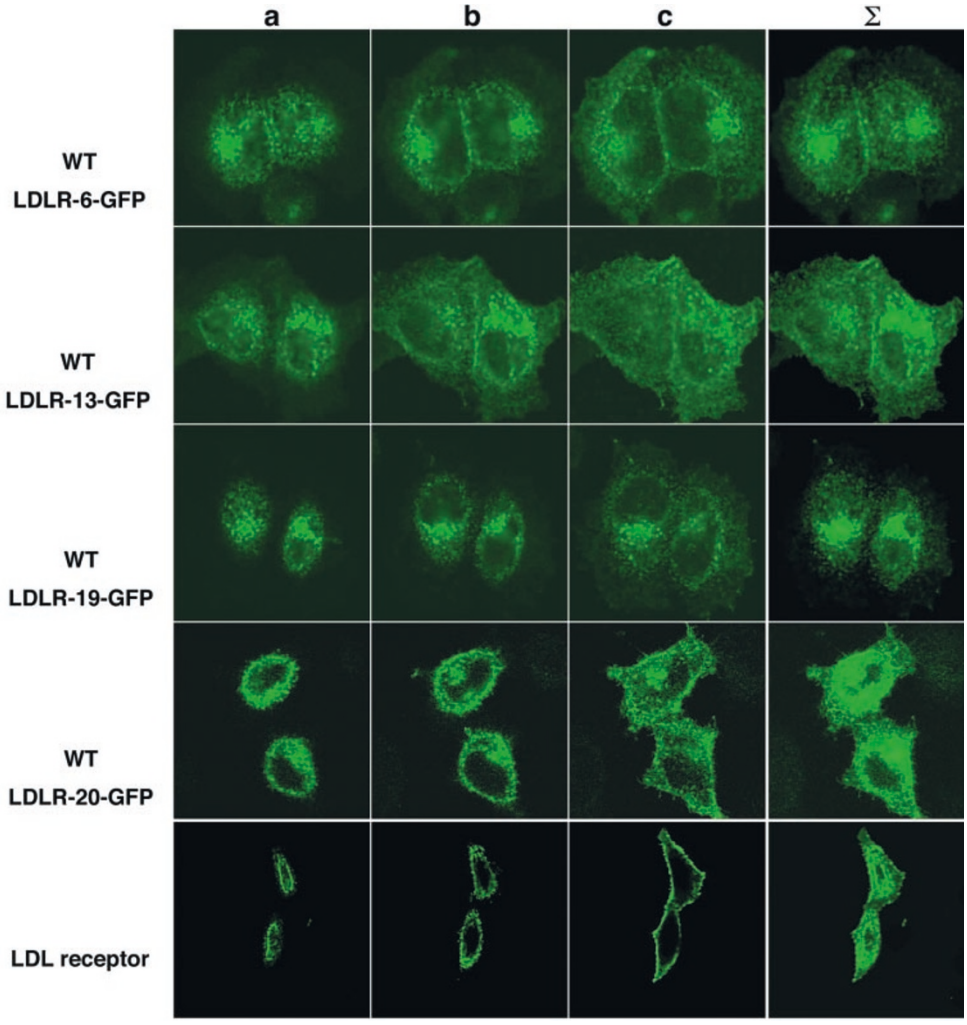


Figure 2 GFP fluorescence from various LDL receptor GFP fusion proteins. Fluorescence of the various LDL receptor GFP fusion proteins (top four panels) or immunostained LDL receptor (lower panel) expressed in transfected Chang liver cells. Using CLSM analysis three optical sections for each construct (**a, b,** and **c**) were obtained. The sum of these sections (Σ) is also shown. Original magnification 630 ×.

ml DiI-conjugated LDL (Molecular Probes). Cells were harvested by incubation in PBS containing 0.6 mM EDTA followed by incubation in PBS containing 0.6 mM EDTA and 0.01% trypsin (Life Technologies Ltd.), washed in growth medium and PBS and analysed on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For surface staining cells were harvested 48 h after transfection as described above. Cells were stained with 2.5 μg/ml monoclonal anti-LDL receptor antibody immunoglobulin IgG-C7 (Amersham Pharmacia Biotech UK Ltd.) for 30 min and secondary R-phycoerythrin (RPE) conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1 : 100 for 30 min at 4°C. Flow cytometry was performed using a 488 nm argon laser, and data were acquired using the FL1 (GFP) and FL2 (RPE and DiI) detectors. Forward scatter (FSC) and side scatter (SSC) gates were established to exclude dead cells and cell debris from the data analysis. 5×10^4 cells were analysed in each sample.

Tail vein injection

LDL receptor−/− mice (B6.129S7-*Ldlr*^{tm1Her}) were obtained from M&B, Ry, Denmark.[22] Animals were 8–11 weeks of age with a body weight of 20–25 g when used for experiments. To limit variation only male mice were used. Before the injection procedure, animals were kept at a high ambient temperature to dilate the tail veins. Anaesthesia was carried out in a chamber with 4% (v/v) halothane air until digital reflex was absent. Naked DNA was administered to the animals by injecting 16.67 μg/ml of plasmid DNA contained in sterile 147 mM NaCl, 4 mM KCl, 1.13 mM $CaCl_2$ (Ringer solution) into the tail vein as previously described.[23] We used a weight volume of Ringer solution corresponding to 8% of the bodyweight, i.e., 1.6–2 ml. Injection was performed within 5–7 s. Following injection, the animals were allowed to recover while raising ambient temperature to approximately 28°C. Animals were sacrificed 2 days after the injections. Liver samples were excised immediately after

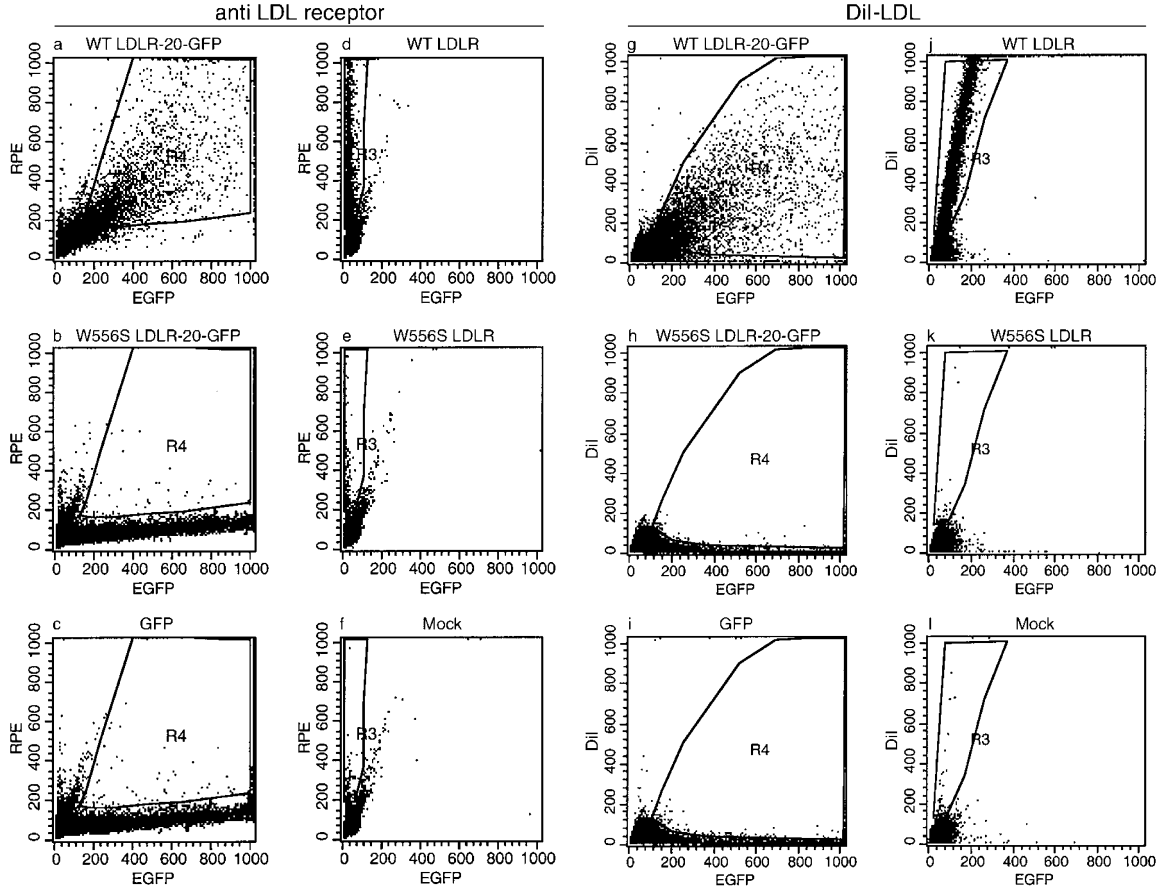


Figure 3 Flow cytometry of LDL receptor overexpressed in transfected Chang cells. Cells were stained with C7/RPE for immunofluorescence detection of LDL receptor protein on the cell surface, or incubated at 37°C with DiI-LDL for measurement of LDL receptor activity. The regions R3 and R4 define the area above background (defined as a region containing 0.1% of the mock (R3) or GFP (R4) transfected cells). Left panels (**a**–**f**): LDL receptor protein on the cell surface, detected by the antibody C7. Right panels (**g**–**l**): DiI-LDL uptake. Representative dot plots of cells expressing LDLR-20-GFP (**a**,**g**), WT LDLR (**d**,**j**), W556S LDLR-20-GFP (**b**,**h**), W556S LDLR (**e**,**k**), GFP (**c**,**i**), or empty vector (mock) (**f**,**l**) are shown.

sacrificing the animals. The experiments were approved by The Danish Animal Experiments Inspectorate.

Results

In order to study the intracellular processing/trafficking of the LDL receptor, we developed expression plasmids containing the LDL receptor gene fused to a GFP gene. To avoid disturbance of the ligand binding domain the GFP was placed at the intracellular C-terminal end of the LDL receptor. Since the cytoplasmic tail is important for proper endocytosis,[24] a spacer region was inserted between the C-terminus of the LDL receptor and the N-terminus of the GFP allowing individual folding of the two proteins. We constructed a series of LDL receptor GFP chimeric plasmids with different, flexible, unstructured spacer regions (Figure 1). The presence of a flexible region between GFP and the fusion partner has previously been shown to improve the cellular transport and function of fusion proteins.[12]

After transfection of Chang cells (a human liver cell line) with the various constructs, GFP fluorescence from the fusion proteins was analysed (Figure 2). Confocal Laser Scanning Microscopy (CLSM) showed that all four constructs gave rise to membrane associated proteins similar to the native LDL receptor (compare top four panels with lower panel in Figure 2). Intracellularly localised GFP signals were also identified, but the fusion protein with the longest spacer region (WT LDLR-20-GFP) resulted in the lowest amounts. On the basis of these localisation studies this construct was chosen for further studies.

Using flow cytometry we then investigated whether the GFP-tagged LDL receptor was functional (Figure 3). The amounts of LDL receptor protein present on the cell surface were measured as immunostaining of viable cells with the C7 antibody recognising an extracellular epitope.[25] Transfection of plasmids encoding wild type (WT) LDL receptor and WT LDLR-20-GFP resulted in comparable levels of surface localised protein (Figure 3a,d, and Table 1). Likewise, the activity measured by the uptake of the fluorescently labelled LDL (DiI-LDL) via these two proteins was at the same level (Figure 3g,j, and Table 1).

Having established that the WT LDL receptor GFP protein is normally localised and display activity comparable to the native LDL receptor protein, we analysed two disease-causing LDL receptor mutations, W556S and C660X. The C660X mutation containing a premature stop mutation was constructed to exclude the presence of a putative intrinsic promotor in the LDL receptor gene resulting in GFP fluorescence. The W556S mutation, a common LDL receptor

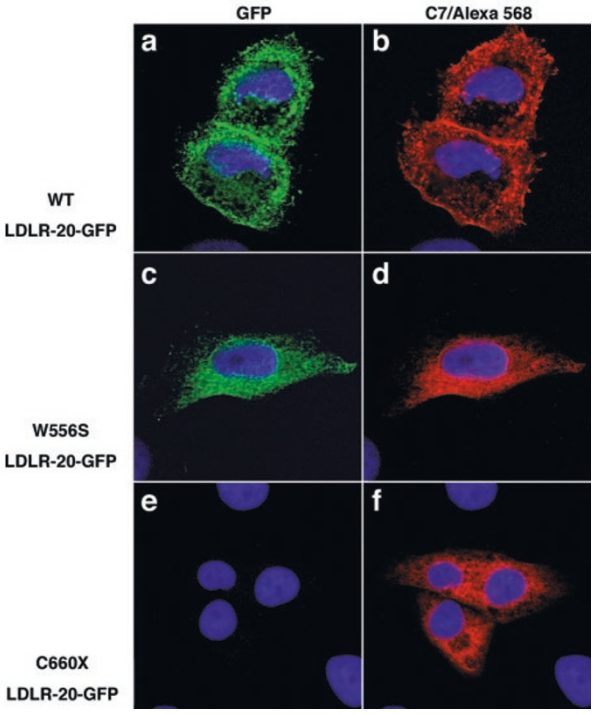


Figure 4 Comparison of GFP fluorescence and LDL receptor immunostaining. Chang cells were transfected with WT LDLR-20-GFP (**a**,**b**), W556S LDLR-20-GFP (**c**,**d**), and C660X LDLR-20-GFP (**e**,**f**) expression vectors and analysed by CLSM simultaneously for GFP fluorescence (green) and immunofluorescence staining using a monoclonal primary antibody, C7, detected by an Alexa 568-conjugated secondary antibody (red). Original magnification 630×.

Table 1 Quantitative data from flow cytometry of LDL receptors overexpressed in transfected cells

Plasmid	*C7/R-PE Mean % of cells above background*	*DiI 37°C Mean % of cells above background*	*C7/R-PE Mean fluorescence above background*	*DiI 37°C Mean fluorescence above background*
WT LDL receptor	2.76±0.73	3.72±0.57	298±23	170±14
WT LDLR-20-GFP	2.39±0.64	3.47±0.59	276±35	156±19
W556S LDL receptor	0.14±0.06	0.06±0.04		
W556S LDLR-20-GFP	0.06±0.02	0.07±0.05		
Vector	0.1	0.1		
Vector pEGFP-N1	0.1	0.1		

The results (mean±SEM) are based on double determinations of three independent transfections (six measurements). Left panels: Percentage of cells above background. Right panels: Fluorescence of cells above background for cells transfected with the two wild-type constructs allowing comparison of the LDL receptor with and without the GFP tag. The background is defined as described in Figure 3.

mutation in the Danish population, and the C660X mutation were previously characterised as class 2 mutations leading to impaired transport of the protein to the cell surface.[10,26] Fluorescence microscopy of cells expressing the WT or the two mutant LDL receptor fusion proteins was performed. Immunofluorescence staining using the C7 antibody showed that the WT LDLR-20-GFP protein was localised on the cell surface (Figure 4b) whereas the two mutant proteins were localised intracellularly, resembling an ER pattern (Figure 4d,f). This is in agreement with previous studies.[10,20] Similar to the C7 staining, GFP fluorescence from the WT LDLR-20-GFP protein was localised to the cell surface. Correspondingly GFP fluorescence from cells expressing the W556S fusion protein resembled the intracellular, ER like, C7 staining (Figure 4c). No GFP fluorescence could be observed with C660X LDLR-20-GFP containing a stop codon before GFP in the normal reading frame (Figure 4e). This shows that the GFP fluorescence is not caused by a putative intrinsic promotor region in the LDL receptor cDNA but indeed represents the LDL receptor GFP fusion protein. This finding was further corroborated by immunoprecipitation of proteins from cells transfected with WT-LDLR-20-GFP showing that only fusion proteins of the expected molecular weight were precipitated using either LDL receptor or GFP antibodies (data not shown). Flow cytometry confirmed that the W556S mutation inhibited transport of the protein to the cell surface (Figure 3b) and uptake of DiI-LDL (Figure 3h and Table 1). Thus the GFP fluorescence is a valid measure for the localisation of the fusion protein.

The function of the LDL receptor variants was analysed in individual living transfected Chang cells by CLSM. The GFP fluorescence from WT and W556S LDL fusion proteins resembled the pattern obtained by staining fixed cells with the antibody C7 (compare Figure 5b and e with Figure 4b and d). Co-localisation studies using an *in vivo* staining dye (ER-Tracker^{TM}) further established that the W556S mutant fusion protein is retained in ER (Figure 5c,f).

Transfected cells identified by GFP fluorescence were incubated with DiI-LDL at 37°C to examine the ligand uptake. As shown in Figure 5, the WT LDLR-20-GFP protein internalised DiI-LDL whereas the mutant W556S LDLR-20-GFP was inactive (Figure 5a,d). This result is in agreement with the functional analysis performed by flow cytometry (Figure 3 and Table 1).

WT and W556S LDLR-20-GFP constructs were tested in mice by use of the newly developed hydrodynamic transfection technique leading to expression exclusively in liver.[17,18,23] Two days after DNA injection, animals were sacrificed and fresh unfixed liver tissue visualised using a fluorescent microscope (Figure 6). Approximately 1% of the liver cells expressed the GFP fusion proteins (Figure 6b,d). When using CLSM to obtain thin optical sections WT LDLR-20-GFP was located in a ring-shaped structure representing the cell surface, whereas mutant fusion proteins containing the LDL receptor W556S mutation accumulated intracellularly (Figure 6a,c). For comparison the expression of the control plasmid pEGFP-N1 is shown in Figure 6e,f.

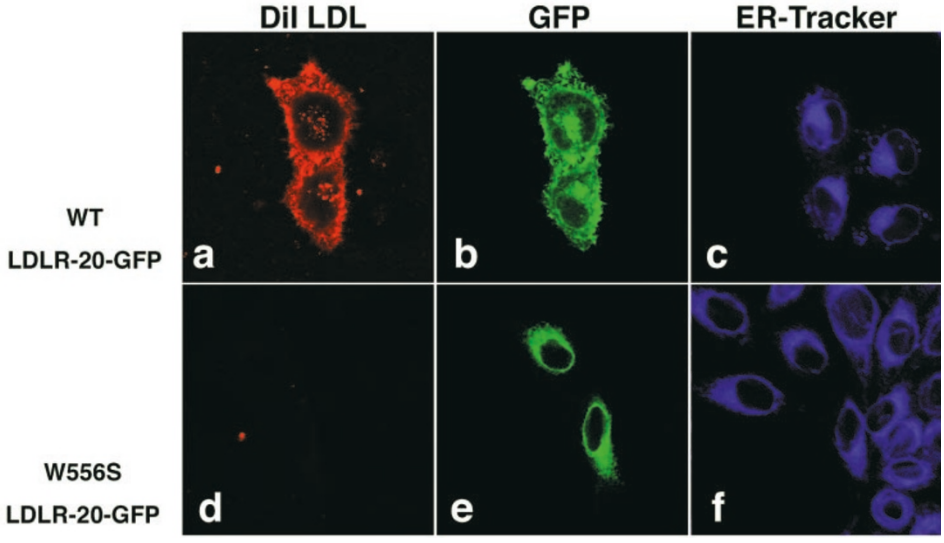


Figure 5 Simultaneous detection of DiI-LDL and LDL receptor fusion proteins in viable transfected cells. Forty-eight hours after transfection with WT LDLR-20-GFP (**a,b,c**) and W556S LDLR-20-GFP (**d,e,f**) expression vectors, cells were incubated with DiI-LDL (**a,d**) and ER-tracker (**c,f**) for 15 min at 37°C. After the incubation period the slides were washed and fresh medium was added allowing for studies of living cells on an inverted CLSM equipped with a heated stage. Optical section shows the distribution of DiI-LDL (**a,d**), LDL receptor GFP fusion protein (**b,e**), and ER staining (**c,f**) for WT and W556S LDLR-20-GFP. Original magnification 400×.

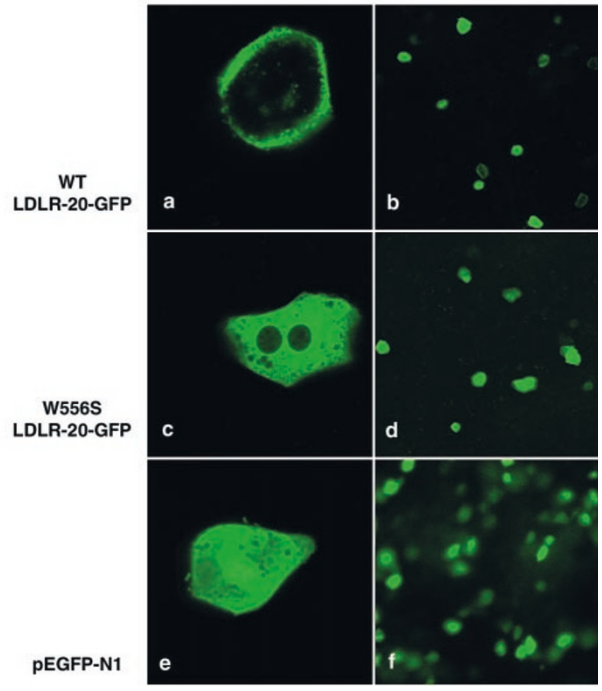


Figure 6 *In vivo* localisation of LDL receptor GFP fusion proteins in mouse liver after hydrodynamic DNA transfer. LDL receptor−/− knock out mice were injected with expression vectors containing WT LDLR-20-GFP cDNA, W556S LDLR-20-GFP cDNA, or pEGFP-N1. Forty-eight hours after injection the animals were sacrificed and liver tissue samples were removed and immediately analysed using an inverted CLSM. (**a**) WT LDLR-20-GFP, (**c**) W556S LDLR-20-GFP, (**e**) control plasmid pEGFP-N1. (**b**,**d**,**f**) Low magnification pictures with low optical resolution in depth of tissue expressing WT LDLR-20-GFP, W556S LDLR-20-GFP, and GFP, respectively. Original magnification 400× or 50×.

Discussion

In this study, we report the development of a functional LDL receptor GFP fusion protein and document that the protein can be used in the functional study of a disease-causing mutation in the LDL receptor gene *ex vivo* as well as *in vivo*.

Due to the high prevalence of mutations in the LDL receptor gene, it is important to have efficient assays of their functional effect. Since most FH patients are heterozygous, these assays are often performed as expression studies of the individual alleles in tissue cultured mammalian cells. The function of the protein is then measured using immunostaining or by uptake of fluorescent- or radioactive labelled ligand.

In this context the hybrid LDL receptor GFP protein gives several advantages. First of all, the GFP tag makes it possible to study the protein in living cells. Secondly, for the detection of the protein it is not necessary to use anti-LDL receptor antibodies. This is important since mutations may disturb the epitope recognised by the antibody used. Furthermore, the hybrid protein can be studied in mouse liver *in vivo* offering a natural cellular environment for such studies. Development of transgenic mice carrying LDL receptor mutations is an alternative approach to the *in vivo* analysis of transgenes in mouse liver, but this is much more costly and time-consuming.

In tissue cultured liver cells we found that the wild type protein with the longest linker to GFP resulted in excellent membrane display and low amount of intracellular protein and that this fusion protein was fully functional as measured by uptake of DiI-labelled LDL (Figure 3). CLSM studies indicate that the GFP signal from the fusion protein co-localises with the labelled LDL suggesting that the intracellular protein is accumulating in endosomes (Figure 5). Another possibility might be that the protein is localised in aggresomes due to the impaired rate of biosynthesis,[27,28] but our co-localisation studies do not support this idea. Expression of the two mutant LDL receptor GFP fusion proteins revealed that (1) the two mutation proteins were retained in ER, (2) the GFP fluorescence from the W556S fusion protein was identical to the intracellular C7 staining, (3) no GFP fluorescence from the C660X mutant protein could be detected and (4) none of the mutant fusion proteins were functional. Thus, fusion proteins consisting of GFP and LDL receptor can be a valuable tool in the characterisation of potentially disease-causing mutations in the LDL receptor gene expressed in tissue culture. The LDL receptor GFP fusion protein seems most suited for the characterisation of mutations that disturb the transport of the protein to the cell surface (class 2 mutations). These mutations account for more than 50% of point mutations in the LDL receptor gene.[1]

The fusion protein was also analysed in mouse liver *in vivo* after hydrodynamic gene transfer. From these experiments we conclude that the WT LDL receptor GFP fusion protein localise at the cell surface whereas the W556S LDL receptor GFP fusion protein localise in intracellular compartments. Thus, the handling of fusion proteins in mouse liver *in vivo* resembles the situation in a liver cell line. It has been previously observed that the effect of disease-causing mutations in the LDL receptor gene may be tissue specific.[29] Whether liver expression *in vivo* may lead to a better prediction of the severity of LDL receptor mutations remains to be determined.

The mechanism behind the efficient gene transfer to liver *in vivo* is not known, but it has been suggested that the rapid injection of a large volume causes a transient right-sided congestive heart failure and back-pressure to the liver vessels.[17,18] We observed no injury in the liver or other organs following the hydrodynamic gene transfer, and the health of the animals appeared generally unaffected. Other reports found that the hydrodynamic gene transfer influenced the liver function only mildly, causing a transient elevation in the levels of serum alanine transferase.[17,18]

In conclusion, our data show that the GFP-tagged LDL receptor proteins allows both detailed time lapse studies in cell cultures and analysis in mice under physiological

conditions. Our data show that hydrodynamic gene transfer is an attractive supplement to expression studies in tissue culture for the analysis of potentially disease-causing mutations in the LDL receptor gene. Moreover, utilisation of DNA delivery of GFP tagged protein constructs to animals should be generally applicable in the analysis of many genetic diseases other than Familial Hypercholesterolaemia.

Acknowledgements

We thank Mrs Anne Keblovszki and Mrs Bodil Schmidt for excellent technical support. This study was supported by grants from the Karen Elise Jensens Fond and the Danish Heart Foundation. We thank Dr Henrik K Jensen for critical reading of the manuscript.

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