Global Defects in the Expression and Function of the Low Density Lipoprotein Receptor (LDLR) Associated with Two Familial Hypercholesterolemia Mutations Resulting in Misfolding of the LDLR Epidermal Growth Factor-AB Pair*

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The low density lipoprotein (LDL) receptor is a modular protein involved in the endocytosis of cholesterol-rich lipoproteins from the circulation. Mutations to the receptor result in familial hypercholesterolemia, and over 60 of these occur in the calcium-binding epidermal growth factor-like domain pair. Two selected mutations in this region (G322S and R329P) were introduced into the domain pair and analyzed by in vitro refolding. Both exhibited differing levels of protein misfolding with R329P being the most pronounced. Solution NMR studies of the mutant domain pairs after purification established that a fraction of protein maintains a native-like fold and that this fraction contains two intact calcium-binding sites. An in vitro analysis of intact receptors containing these binding sites showed significantly reduced cell-surface expression compared with the native LDL receptor levels, again with R329P showing the most severe decrease. The sum of these results suggests that either local changes in structure or domain misfolding may be associated with the mutations. There is also the possibility that the misfolding of the calcium-binding epidermal growth factor-like pair region is propagated to other regions of the intact receptor, resulting in more global defects. Surprisingly, for both mutants, those full-length receptors that fold and reach the cell surface retain the ability to bind LDL and release the ligand upon exposure to low pH. This analysis provides significant insight into the protein defect resulting from each of the two mutations and allows their classification to be 2B (partially transport-defective). The results also highlight a range of misfolding defects that may be associated with familial hypercholesterolemia and may enable the prediction of the consequences of homologous disease-causing mutations to other proteins.

The low density lipoprotein (LDL) receptor mediates the cellular uptake of cholesterol-rich lipoproteins from the circulation by binding to two ligand molecules, apolipoprotein B and apolipoprotein E, of LDL and β-very LDL particles. Receptor-ligand complexes enter the cell through endocytosis at clathrin-coated pits. The bound lipoprotein is released subsequently in the low pH environment of the endosome, and the receptors are recycled back to the cell surface (1).

Mutations to the LDL receptor (LDLR) are associated with familial hypercholesterolemia (FH), an autosomal-dominant inherited disease affecting ~1 in 500 in most populations. FH is characterized clinically by a lifelong elevation of LDL-bound cholesterol in blood and is associated with an increased risk of coronary artery disease and cardiovascular disease (2). Almost 900 LDLR mutations have been associated with FH (3), collated in two online databases, and many have been classified based on their effect on the synthesis (class 1), transport (class 2), ligand binding activity (class 3), internalization (class 4), or recycling (class 5) of the LDLR (4). Despite the wealth of biochemical data available for the LDL receptor and atomic resolution structural data for the extracellular region of the protein (5–10), the molecular defects associated with many of the >400 missense mutations that result in familial hypercholesterolemia remain unclear. Such information is vitally important for attempts to correlate protein defects with protein dysfunction and ultimately with phenotype.

The LDLR consists of a series of discrete extracellular protein modules responsible for the binding and release of lipoprotein ligands. The ligand-binding region is composed of a series of seven LDL receptor-type A (LA) modules followed by a region with a homology to the EGF precursor, an O-glycosylation region, a transmembrane region, and a cytoplasmic tail (11). The EGF precursor-homology region consists of two calcium-binding EGF domains, six YWTD repeats forming a β-propeller domain, and a third EGF domain. Calcium is required for ligand binding (12, 13). We have reported previously (9) the structure of the calcium-saturated EGF-AB pair from the LDL receptor in which the two domains adopt an extended rod-like arrangement. At present, over 60 FH mutations have been localized to the EGF-AB pair. Based on our structure, it was possible to predict the structural consequences of some mis-
Mutated residues are shaded in the LDLR EGF-AB pair showing the positions of the G322S and bond connectivities are shown as jagged lines the consensus calcium-binding sequence is shaded exposure to low pH is not compromised when compared with the ability of the mutant receptors to release bound LDL upon and reach the cell surface retain the ability to bind LDL, and folding assays. Remarkably, the mutant receptors that do fold fraction of native-like folded receptors recovered from receptors detected at the cell surface tightly correlates with the extent of compromise that results from each individual mutation. In addition, these results may enable the prediction of the molecular defects associated with structurally similar disease-causing mutations to calcium-binding EGF-like domains in the LDLR and other proteins associated with human diseases such as human fibrillin-1.

**EXPERIMENTAL PROCEDURES**

Expression Plasmids and Cell Lines—The plasmid construct for bacterial expression of the LDLR EGF-AB pair has been described previously (16). The insert for the full-length native LDL receptor (WT) and the ΔEGFP mutant receptor, which lacks residues 293–693, was cloned into the vector pcDNA3.1D/V5-His-TOPO (Invitrogen) for mammalian cell expression. LDLR-7 cells, which lack endogenous LDL receptors (kindly provided by Dr. Monty Krueger (17)) were transfected with each construct using LipofectAMINE Plus reagent (Invitrogen), and stable transfectants were selected with G418 (concentration: 1 mg/ml). Cell lines were maintained in Ham’s F-12 medium with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/l-glutamine supplemented with 4% (v/v) lipoprotein-deficient bovine serum (Biomedical Technologies Inc., Stoughton, MA) and 1% (v/v) fetal bovine serum (Cambrex Bio Science Inc., Walkersville, WV). Cells were grown at 37°C under 5% CO₂.

Site-directed Mutagenesis—The substitutions G322S and R329P were introduced individually into both the LDLR EGF-AB construct and full-length LDLR construct using overlap extension PCR (18, 19). The flanking primers used were as follows: pQE30, 5′-CGG ATA ACA ATT TCA CAC AGA-3′ and pQE30, 5′-GTT GAG AGC AGG CAC GCC-3′ for LDLR EGF-AB constructs and LDLR-8, 5′-AGT GCA TCC ACT CCA GCC GTC-3′ and LFLR-R, 5′-CTG TCC GCC AGG GAC GAC-3′ for full-length LDLR constructs. The mutagenic primers used were as follows: G322S-F, 5′-TCT GGC GCA AGC TTC-3′; G322S-R, 5′-GAA GCT GTC GGG GCA CAG-3′; G329P-F, 5′-GAG CAG GAC GAC GGCA-3′; G329P-R, 5′-GGA GCT GTC GGG GCA CAG-3′; and R329P-F, 5′-GTC GCC CAG CCA AGA TGC-3′; and R329P-R, 5′-GCA TCT TGG CGG GCC-3′.

The PCR products from the overlap extension PCRs were restricted using HindIII and KpnI for the LDLR-AB construct (pQE30, Qiagen Ltd, West Sussex, United Kingdom) and EcoRI and Bsu36I for the full-length construct (pcDNA3.1D/V5-His-TOPO, Invitrogen) and ligated into the appropriate vectors, replacing the WT sequence. The presence of each point mutation was confirmed by DNA sequence analysis.

Expression and Purification of AB Pairs—The EGF pair from LDLR (EGF-AB) was expressed and purified using a protocol described previously (16) with minor modifications. The protein was expressed as a His tag fusion in Escherichia coli JM109 cells, either in LB media (for unlabeled samples) or in minimal media using 15N-HCl (as the sole nitrogen source for 15N-labeled isotopically enriched samples). The expressed protein was extracted under denaturing conditions (6 M guanidine HCl, pH 7.0) before being applied to a His tag affinity column containing cobalt resin (TALON™ CellThru, BD Biosciences, Oxford, United Kingdom) for initial purification and eluted into 6 M guanidine HCl buffer, pH 7.0, containing 200 mM imidazole. The sample was reduced with 0.1 M dithiothreitol in 0.1 M Tris-HCl, pH 7.4, prior to purification by reverse-phase HPLC. All of the HPLC analysis was performed on a BioCad Int (Applied Biosystems, Foster City, CA). A Poros Perfusion Chromatography R2/10 column (10/100 mm; Applied Biosystems) was used with an elution gradient of 2–60% of a buffer containing 80% acetonitrile, 0.1% trifluoroacetic acid at a rate of 3.9%/min using a flow rate of 8 ml/min prior to lyophilization. The His tag was removed as described previously (16).

Refolding Conditions—The protein was refolded by dialysis against a buffer containing 3 mM l-cysteine and 0.3 mM l-cysteine in 50 mM Tris-HCl, 10 mM CaCl₂, pH 8.3, at a concentration of 0.4 mg/ml. The protein for analysis was purified by two additional rounds of chromatography, anion-exchange HPLC, and reverse-phase HPLC and then analyzed by electrospray mass spectrometry to confirm the presence of a single disulfide-bonded component of the correct mass. The HPLC was performed on a Poros Perfusion Chromatography HQ/M column (4.6/100 mm; Applied Biosystems) run at pH 7.5 and eluted using a gradient of 0–500 mM NaCl at a rate of 100 mM NaCl/min at a flow rate of 5 ml/min. The protein with a native or native-like fold
eluted between 185 and 255 mM NaCl. This material was collected and then purified by RP-HPLC as described above using a Poros Perfusion Chromatography R2/10 column (4.6/100 mm) and an elution gradient of 2–60% of a buffer containing 80% acetonitrile, 0.1% trifluoroacetic acid at a rate of 5.2%/min with a flow rate of 3 ml/min prior to lyophilization.

For refolding trials, equal quantities of protein, normalized spectrophotometrically using a molar extinction coefficient calculated as described previously (20), were refolded simultaneously under the conditions specified above. The level of refolding was analyzed by RP-HPLC using a Poros Perfusion Chromatography R2/10 column (4.6/100 mm) with an elution gradient of 10–60% of a buffer containing 80% acetonitrile, 0.1% trifluoroacetic acid at a rate of 0.5%/min using a flow rate of 3 ml/min. The samples for RP-HPLC analysis were acidified (by the addition of 5% (v/v) acetic acid) to halt disulfide exchange after refolding prior to HPLC. Chromatograms were analyzed by peak integration using the BioCad software (Applied Biosystems) and cross-checked using manual peak integration in Excel (Microsoft Corporation, Redmond, WA).

NMR Spectroscopy—All of the NMR data were acquired using General Electric/home-built instruments with triple resonance triaxial gradient probes operating at 600 MHz. Protein samples were assayed in 95% H2O, 5% 2H2O at pH 6.5 and 30 °C. WT, G322S, and R329P 15N-labeled samples were assayed at 0.18, 0.17, and 0.05 mM, respectively. The spectra recorded at calcium concentrations of 0, 10, and 12 mM demonstrated calcium-dependent displacement of cross-peaks and saturation of both sites at a calcium concentration of 10 mM. Two-dimensional 1H-15N HSQC spectra (21) were acquired for each sample with 2048 by 128 complex points and with acquisition times of 102 ms in F2 and 78 ms in F1. The data were processed using Felix 97 (Accelrys, San Diego, CA), typically using a 70° shifted squared sine-bell window function in F2 and a Kaiser window function with an 8 Kaiser parameter in F1. The spectra were referenced with respect to the H2HO resonance and zero-filled in the F2 dimension to produce spectra with a digital resolution of 4.88 Hz/point. Two-dimensional NOESY spectra (22) were recorded with a mixing time of 150 ms for the comparison of the WT and G322S samples. Each spectrum was acquired with 700 t1 increments of 2048 complex points and with a spectral width of 10,000 MHz. The data were processed using Felix 97 (Accelrys), typically using a 85° shifted sine-bell window function in F2 and using a 70° shifted squared sine-bell window function in F1. The spectra were referenced with respect to the H2HO resonance and zero-filled in the F2 dimension to produce spectra with a digital resolution of 2.44 Hz/point. Minimum chemical shift displacements in the HSQC spectra were measured as the distance between the assigned peak in the WT spectrum and the nearest peak in the spectrum of the mutant. Combined 1H and 15N chemical shift changes were calculated using the Pythagorean theorem with 15N chemical shifts scaled by one-fourth relative to proton shifts.

Chromophoric Chelator Measurement of Binding Constants—The
calcium-binding properties of the EGF-AB pairs were determined using methods described previously (16, 23). Purified protein samples, previously assessed by NMR in the presence of 12 mM calcium, were dialyzed sequentially against EDTA to 20 mM. To remove the EDTA, the samples were transferred into a calcium-free buffer containing 2 mM Tris and 150 mM NaCl, pH 7.5, using successive washes through a Microcon YM-3 centrifugation column (Millipore (UK) Ltd, Watford, United Kingdom) prewashed with the calcium-free buffer. All of the manipulations were carried out using disposable plasticware wherever possible to avoid calcium contamination. Absorbance scans and calcium titrations were performed using a dual-beam Lambda-40 UV-visible spectrophotometer (PerkinElmer Life Sciences). Samples were prepared using the calcium-free buffer solution containing 25 μM 5,5'-Br2-BAPTA (C22H18Br2K4N2O10, Molecular Probes, Eugene, OR) and 12.5 (±3) μM protein in a total volume of 0.9 ml. Protein concentrations were determined by amino acid analysis. The residual calcium concentration was determined to be 0.7 μM on the basis of  

\[ A_{\text{max}} \] and  

\[ A_{\text{off}} \] measurements for samples containing saturating EDTA or Ca2+, respectively. Protein and blank samples were titrated sequentially using 2 μl of the additions of a 2.55 mM CaCl2 solution (assayed by EDTA titrations using the murexide indicator) with a final addition of 2 μl of 1 mM CaCl2 to complete saturation. The dissociation constant of the chelator was determined using the CaLigator software (24) using data from three blank titrations. Protein-binding constants for the two binding sites were determined using the CaLigator software (n = 2 for each sample). Variable parameters in the fits were the binding constants (\( K_1 \) and \( K_2 \)),  

\[ A_{\text{max}} \] and  

\[ A_{\text{off}} \].

Western Blotting—Whole cell extracts of idLA-7 cells transfected with native and mutated LDL receptors were analyzed for expression by

![Fig. 3. Comparison of NMR data for the WT and mutant EGF-AB pairs. A, selected region of one-dimensional spectra of the LDLR EGF-AB pair acquired at 12 mM calcium. The position of the Thr-294 methyl chemical shift is indicated. B, two-dimensional 1H-15N HSQC spectra of calcium-saturated WT, G322S, and R329P EGF-AB pairs acquired at pH 6.5, 30 °C, and 600 MHz. NH resonances are labeled according to residue sequence number for main chain nitrogens and colored according to domain (blue for EGF-A and red for EGF-B). The NH2 side chain cross-peaks are shown boxed or joined by horizontal lines.](image-url)
Western blotting. After SDS-PAGE on 10% gels and transfer to nitrocellulose, receptors were detected either with monoclonal α-LDL receptor antibody IgG-C7 or IgG-4A4 followed by the addition of a secondary α-mouse antibody conjugated to horseradish peroxidase. Western blots were developed by chemiluminescence (SuperSignal West Femto, Pierce, Rockford, IL).

Flow-cytometric Analysis of Receptor Expression, Binding, and Release—Flow cytometry was performed using a Coulter Epics XL-MCL single-laser 4-color flow cytometer (Beckman Coulter, Miami, FL). Flow-cytometry data collected with the program EXPO32 ADC software (Beckman Coulter) were downloaded into the program winMDI (TSRI FACS Core Facility, La Jolla, CA) for analysis.

The level of receptor expression was assessed by labeling with monoclonal α-LDL receptor antibody IgG-C7 followed by the addition of a secondary α-mouse antibody conjugated to fluorescein isothiocyanate. Fluorescein isothiocyanate fluorescence was determined by flow cytometry. To evaluate LDL binding and release, ldLA-7 cells stably expressing native or mutant receptors were detached from dishes and fixed mildly with 0.5% paraformaldehyde at 4 °C for 1 h. The fluorescent ligand DiI-LDL (Molecular Probes) then was added at a concentration of 7.5 µg/ml in pH 7.4 buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂) and was incubated with the cells for 1 h at 4 °C. To test for low pH-induced ligand release, the pH was shifted to 5.3 by replacing the buffer with 25 mM Tris succinate, pH 5.3, containing 150 mM NaCl and 2 mM CaCl₂ followed by additional incubation at 4 °C for 1 h. DiI-LDL fluorescence then was determined by flow cytometry as noted above.

RESULTS

Effects of G322S and R329P Mutations on Expression and Protein Folding—WT, G322S, and R329P samples of the EGF-AB pair were expressed in and purified from E. coli strain JM109 under identical conditions. Although the stability of recombinant protein expressed from E. coli is subject to a
number of factors, both G322S and R329P consistently resulted in reduced expression levels of the EGF-AB pairs compared with WT (52 and 30%, respectively).

The in vitro refolding ability of each EGF-AB pair was analyzed by RP-HPLC (Fig. 2A). Equal quantities (1 mg) of each sample were refolded simultaneously under identical conditions established for the production of the natively folded WT EGF-AB pair. Samples were subjected to RP-HPLC, and the chromatograms were analyzed by peak integration. The percentage of protein for each sample that eluted under the same conditions as the natively folded WT EGF-AB pair corresponding to a retention time of 46–49.5 min (see Fig. 2A, inset) was determined with WT normalized to 100% (Fig. 2B). The integration of AIEX chromatograms obtained after the refolding of samples for NMR and calcium-binding analysis produced equivalent refolding levels for the two mutants (data not shown). The chromatograms obtained from the RP-HPLC analysis visibly display an increase of additional isomers for both G322S and R329P.

**Effects of G322S and R329P Mutations on the EGF-AB Structure by NMR**—The effects of the G322S and R329P mutations on the protein fold were investigated by NMR spectroscopy. All of the mutant samples used purified material eluted from the AIEX HPLC under the equivalent conditions to the natively folded WT EGF-AB pair. Samples were subjected to RP-HPLC, and the chromatograms were analyzed by peak integration. The percentage of protein for each sample that eluted under the same conditions as the natively folded WT EGF-AB pair corresponding to a retention time of 46–49.5 min (see Fig. 2A, inset) was determined with WT normalized to 100% (Fig. 2B). The integration of AIEX chromatograms obtained after the refolding of samples for NMR and calcium-binding analysis produced equivalent refolding levels for the two mutants (data not shown). The chromatograms obtained from the RP-HPLC analysis visibly display an increase of additional isomers for both G322S and R329P.

**TABLE I**  
Chelator analysis  
Calcium affinities for the two sites in the EGF-AB pair were calculated using CaLigator software (24) from two titrations for each sample and using the $K_{eq}$ value calculated for the chelator (5,5'-Br$_2$BAPTA $K_{eq}$ = 1.41 ± 0.16 $\mu$M). Each titration used 25 $\mu$M 5,5'-Br$_2$BAPTA with 12.5 (±3) $\mu$M protein sample.  

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_{eq}$ mean ($\mu$M)</th>
<th>$K_{eq}$ mean ($\mu$M)</th>
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<tbody>
<tr>
<td>WT</td>
<td>15.00 ± 1.27</td>
<td>52.56 ± 1.33</td>
</tr>
<tr>
<td>R329P</td>
<td>14.64 ± 1.57</td>
<td>63.49 ± 1.72</td>
</tr>
<tr>
<td>G322S</td>
<td>14.56 ± 0.99</td>
<td>54.62 ± 2.65</td>
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Each titration used 25 $\mu$M 5,5'-Br$_2$BAPTA with 12.5 (±3) $\mu$M protein sample.

**Fig. 4. Minimum chemical shift displacement analysis of the structural effects of the mutations.** A, combined chemical shift differences for $^1$H and $^{15}$N calculated between each mutant and the WT LDLR EGF-AB pair. B, schematic representation of the tertiary structure of the LDLR EGF-AB pair showing the positions of the G322S and R329P mutations indicated by spheres. Residues affected by the amino acid substitutions with combined $^1$H and $^{15}$N chemical shift differences greater than 0.1 ppm are shown in magenta for G322S and in blue for R329P. Residues shown in black were not assigned in the HSQC spectra. Calcium atoms are denoted by red spheres. The inset denotes the interdomain NOE connectivities determined from the two-dimensional NOESY experiment.
protein eluted as a single peak, corresponding to the native isomer for WT EGF-AB. The mass spectrometric analysis of these samples confirmed the presence of only a single component with all of the disulfide bonds formed. A comparison of the one-dimensional spectra of the calcium-saturated samples shows that the Hγ2+ resonance of Thr-294 for each of the mutants is shifted to the same position as that for the WT (Fig. 3A).

$^1$H $^{15}$N HSQC assignments for the backbone amides of the WT domain pair (9) were compared with cross-peak positions in the spectra of the two mutants (Fig. 3B). Both mutants display a similar dispersion to the WT EGF-AB pair. To spatially localize the extent of structural effects of the mutations, minimum chemical shift displacements were measured between assigned peaks in the WT spectrum to the nearest peak in the mutant spectrum.

The combined $^1$H-$^{15}$N chemical shift differences are displayed in Fig. 4A. Those residues manifesting chemical shift differences greater than 0.1 ppm are highlighted in Fig. 4B. The G322S mutation results in cross-peak displacement
mainly for residues in the immediate vicinity of the mutation site. In contrast, R329P results in chemical shift changes for the majority of the residues in EGF-A. Because line broadening can arise as a consequence of conformational exchange, the backbone positions highlighted in Fig. 4B may differ structurally and/or dynamically in the mutants. Interdomain NOE connectivities conserved between the WT and G322S samples are highlighted in the inset to Fig. 4B, demonstrating a similar EGF domain arrangement. The same analysis for the R329P mutant was not possible because of the low recovery of the isolated product.

The coexistence of multiple isoforms of the protein in solution would be expected to lead to a greater number of HSQC cross-peaks. The total number of HSQC cross-peaks is reduced marginally from the WT number (n = 113) for both G322S (n = 110) and R329P (n = 110), suggesting that both samples are homogeneous within the detection range of the NMR experiment, corresponding to a single disulfide-bonded isomer. All 12 cysteine residues in the G322S EGF-AB pair exhibit chemical shift perturbations of <0.12 ppm, indicative of native disulfide connectivity. The perturbations determined with the R329P mutant for the cysteines of the B-domain and the first disulfide cysteines of the A-domain (connectivity I–III) are <0.1 ppm. The II–IV disulfide cysteines show perturbations between 0.1 and 0.15 ppm, whereas the V–VI disulfide cysteines display perturbations between 0.2 and 0.5 ppm. These results suggest that the B-domain of the R329P mutant retains native disulfide connectivity. Chemical shift changes for the A domain II–IV and V–VI disulfide pairings in the R329P mutant spectrum may result from either localized structural and/or dynamic changes.

**G322S and R329P Calcium Affinity**—Changes in spectroscopic absorbance were monitored for measurement of the calcium dissociation constants of 5,5'-Br2-BAPTA and the EGF-AB pairs. A $K_d$ of 1.41 (± 0.16) μM was derived for 5,5'-Br2-BAPTA in 150 mM NaCl and 2 mM Tris-HCl at pH 7.5, which is in good agreement with previously calculated values of 1.4 μM obtained under the same conditions (16, 25).

Two titrations were analyzed for each of the samples (WT, G322S, and R329P), with the best fits to the data determined...
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for both sites (see Table 1). The values calculated for the WT EGF-AB pair are in good agreement with those values, previously determined at physiological ionic strength (I = 0.15) and pH (7.4–7.5), of −50 μM for EGF-A and 10–20 μM for EGF-B (16). For the B-domain calcium-binding site (K_{Δ}), the values determined for both G322S and R329P are equivalent to the WT constant. The value for the A-domain site (K_{Δ}) is comparable to WT for G322S. The R329P mutant displays a slightly decreased affinity from the WT value.

Cell-surface Expression and LDL Binding of Intact G322S and R329P—To assess the level of cell-surface expression of the native LDLR and the two mutants, a flow-cytometry assay was used to detect receptors with the monoclonal antibody IgG-C7, which only recognizes the first ligand-binding repeat of the receptor when properly folded and in the presence of calcium. By comparison to the native receptor, G322S expression at the cell surface is reduced −2-fold and R329P expression is reduced by almost an order of magnitude (Fig. 5).

To determine whether the receptors that do reach the cell surface retain the ability to bind and release LDL, we performed a binding assay using fluorescein-labeled DiI-LDL and analyzed the amount of bound LDL by flow cytometry. The amount of LDL bound to the mutant receptors correlates tightly with cell-surface expression. Receptors lacking the EGF precursor domain are known not to release ligand at endosomal pH (12), and therefore, the ΔEGFP construct has been used as a negative control for the assay (Fig. 6). Remarkably, the bound LDL is released from the mutant receptors upon exposure to low pH nearly as efficiently as it is released from the native receptor, indicating that the receptors that are properly folded at the cell surface are functional and that the primary defect resulting from these mutations is a misfolding/expression problem.

DISCUSSION

The two mutations selected for investigation (G322S and R329P) have been detected in a number of FH patients (4, 14, 26–29), and both of them result in elevated total cholesterol levels associated with heterozygous FH (7.5–15 mmol/liter). Although for FH patients, the molecular characterization of the LDLR gene is a valuable diagnosis tool, the techniques available for determining the number of functional LDLR molecules are difficult and expensive (30). Our previous research on the LDLR EGF-AB pair has provided a basis for the investigation of the effects of FH-associated mutations. A methodology for the bacterial expression, refolding, and purification of the LDLR EGF-AB pair, containing six disulfide bonds, was established (16). In addition, NMR was used to determine the structure of pair (9) and the dissociation constants of the two calcium-binding sites (16). In this research, we have characterized the consequences of the G322S and R329P mutations in vitro based on the analysis of refolding, structure, and calcium-binding properties of the EGF-AB pair and we have compared these results with an in vivo expression of the analysis and function of the same mutations in the full-length receptor.

The results demonstrate that both mutations result in misfolding of the EGF-AB pair but to a varying degree. The only different populations in the refolding mixture that are resolvable using analytical RP-HPLC should be multimers or disulfide-bonded isomers. The isomers may include partially folded species that are blocked by the refolding potential and cis/trans-Pro, which could result from R329P mutation. Based on the analysis of RP-HPLC chromatograms, both mutants form an increased number of conformers upon in vitro refolding of the pair. Consequently, for each mutant construct, the amount of the native-like material recovered was reduced with R329P displaying the most pronounced decrease. This decrease was confirmed by analysis of AIEX chromatograms obtained after refolding. Non-reducing gel electrophoresis of the wild type and mutant-refolding mixtures did not detect the presence of multimeric forms. Because of the very small amounts of each conformer produced during the refolding, it was not possible to study the differences of the various forms in further detail.

A number of sporadic and genetic diseases result from protein misfolding caused either by mutations that change the amino acid sequence of a protein or from misfolded wild-type proteins (31). Disturbances to the folding process in general are characterized by either the appearance of aggregating protein or rapid degradation of impaired polypeptides, utilizing the protein quality control mechanism that occurs in the endoplasmic reticulum, dependent primarily on ER chaperones (32, 33). An analysis of the in vivo expression data of the full-length receptors shows the presence of protein on the cell surface for both mutants but at a level much reduced from WT. R329P again shows the most acute effect qualitatively corresponding to the level of misfolding determined by HPLC analysis.

Additionally, R329P also consistently displayed the greatest decrease in the expression levels of the EGF-AB pair in bacteria, in line with the in vivo expression. The stability of recombinant protein in bacteria can be influenced by a number of elements within the cell. However, a comprehensive analysis of the steady-state levels of a series of mutants using one expression system under standardized conditions does allow the ordering of the variants with respect to residual levels of mutant protein accumulated under these conditions (32).

If the mutant receptors do arrive at the cell surface, flow-cytometry data for both G322S and R329P demonstrate that these proteins are functional in both the binding and release of LDL. These results are consistent with LDLR studies on a heterozygous FH subject with the G322S mutation that showed reduced receptor-mediated uptake of LDL (29). The observation that functional receptors for both mutants are able to reach the cell surface is suggestive of a native-like fold in the proteins and that any structural perturbation due to the mutation does not extend significantly into the ligand-binding domains or β-propeller domain.

The observation of an increase in the number of isomers for both G322S and R329P after refolding is consistent with the mutations resulting in domain misfolding, although a fraction elutes at the same retention time as wild type. This fraction was isolated for both mutants under conditions identical to those used for the natively folded WT EGF-AB pair and was found to correspond to a single disulfide-bonded isomer according to mass spectrometry, analytical RP-HPLC, and solution NMR data.

Solution NMR data were used on this material to localize the structural changes to the EGF-AB pair resulting from the mutations. The extent of chemical shift perturbation shows good agreement with the degree of misfolding associated with each of the mutations, again with the effects of the R329P mutation propagating more extensively through the A-domain than with those of the G322S mutation. These results show that, for a proportion of the refolded material, the mutations result in local changes in structure.

Calcium is known to play an important role in ligand binding to LDLR (12, 13, 34) and is known to stabilize the fold of the EGF-AB pair (16). The observation of the same calcium-dependent chemical shift for the H2α resonance of Thr-294 for G322S and R329P as for the wild EGF-AB pair indicates the presence of a functional calcium-binding site and therefore a correctly folded EGF-A domain for all three samples (16). Calcium binding to both sites was also investigated using chroomophoric chelator measurements of the binding constants. The
values determined for both mutant samples are similar to those determined previously for the WT EGF-AB pair (16), providing further evidence for both sites remaining intact and for a native-like fold.

The sum of our results show that both mutations result in the misfolding of the domain pair with R329P displaying the more severe effect. In both cases, although there is a major proportion of material produced, which misfolds, a fraction maintains a native-like fold and is able to bind calcium to both domains, suggesting that function is preserved. Within the intact receptor, there is also the possibility that the scrambling of disulfide bonds in one domain results in more global misfolding as suggested by the observation that LDLR folding in the cell involves transient long range non-native disulfide bonds that are isomerized into native short range cysteine pairs (35). Global misfolding would be consistent with the low level surface expression of the receptors that we have detected. Receptors might be delivered to the cell surface more readily if the face expression of the receptors that we have detected. Receptors that are isomerized into native short range cysteine pairs (35).

Prior to this research, neither of these mutations had been unambiguously characterized in terms of effect or FH mutation class. Based on the results presented here and the available clinical data, we propose that both mutations should be classified as 2B. This is the more common form of class 2, having a variable portion of the newly synthesized receptor transported at a reduced rate to the Golgi and then to the cell surface. Class 2A and 2B alleles are known to contain missense mutations or short in-frame deletions that partially or completely disrupt the folding of the receptor (2). A few studies have indicated that lipid levels and cardiovascular disease may be influenced by the type of mutation that causes FH (36, 37). From the available clinical data for the two mutations, there is not a significant difference in the levels of total cholesterol measured, whereas in comparison, the data shown here demonstrate a markedly more severe effect for R329P on in vitro refolding and structure and on expression in ldl-A7 cells. These observations may be explained by multifactorial influences on blood cholesterol levels. It is known that the concentration of blood cholesterol and the severity of coronary atherosclerosis can vary among individuals with homozygous FH, even among those with the same mutation. Especially for heterozygotes, the clinical manifestations may be modified by environmental factors such as diet (2).

In addition to the ubiquitous ER-resident proteins involved in protein quality control, a few molecular chaperones (BOCA, MESD, and receptor-associated protein) have been determined to be specific for the LDLR family of proteins. It is thought that BOCA and MESD must probably interact with the EGF precursor region and, specifically, the β-propeller, whereas receptor-associated protein interacts with the ligand-binding LA repeats of LDLR receptor family proteins. Indeed, the enforced coexpression of receptor-associated protein can partially correct the misfolding of several class 2 FH mutants that alter residues in the LA repeats of the LDLR (38, 39). The optimization of refolding conditions in vitro can control the level of protein aggregation to some extent, especially through dilution of protein concentration. This option is not available in vitro because of molecular crowding, and molecular chaperones are required to minimize the aggregative effects and enhance correct folding, although the sum of our in vitro data and the clinical data available for R329P and G322S suggest that the effects of these mutations cannot be corrected by the specific chaperones for the receptor.

The data for G322S and R329P contribute to a growing body of knowledge regarding the structural consequences of disease-associated mutations to calcium-binding EGF domains that substitute more conformationally restricted residues in the loop regions. A Gly to Ser mutation in the calcium-binding EGF domain 13 of fibrillin-1 is associated with ascending aortic aneurysm (40, 41), and the corresponding mutation in factor IX results in mild hemophilia B. The fibrillin-1 G1127S substitution occurs in turn ahead of the major double-stranded β sheet and has been shown to result in defective refolding of the isolated domain in vitro. The G60S mutation in the factor IX calcium-binding EGF in the identical position also displayed the misfolding of the isolated domain, but unlike the fibrillin domain, a proportion of the G60S material had a calcium-dependent conformation, similar to the wild-type domain (42). Taken together, these results suggest that such mutations may generally result in folding defects.

REFERENCES

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G322S and R329P Mutations to the LDLR EGF-AB Pair

Global Defects in the Expression and Function of the Low Density Lipoprotein Receptor (LDLR) Associated with Two Familial Hypercholesterolemia Mutations Resulting in Misfolding of the LDLR Epidermal Growth Factor-AB Pair
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