Low-Density Lipoprotein Receptor Point Mutation Results in Expression of both Active and Inactive Surface Forms of the Same Mutant Receptor†

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Received June 11, 1992; Revised Manuscript Received September 22, 1992

ABSTRACT: LDL receptors, expressed in cultured fibroblasts from patients homozygous for the FH Afrikaner-1 (FH1) mutation (Asp^{206} to Glu), are transported from the endoplasmic reticulum (ER) to the Golgi apparatus more slowly than in normal cells. In the present study, binding characteristics of FH1 cells for lipoprotein ligands (LDL and βVLDL) and for receptor-specific monoclonal antibodies pointed to the existence of two surface forms of the same mutant receptor. One of these forms bound lipoproteins with normal high affinity whereas another did not. Binding studies of transfected hamster cells expressing only the mutant human gene confirmed the single-gene origin of the different forms. The existence of functionally distinct forms of the receptor protein was supported by the observation that only lipoprotein-binding receptor molecules were trapped intracellularly and degraded following ammonium chloride treatment of cells in the presence of ligand. The lipoprotein-binding receptor population was indistinguishable from normal receptors with respect to its affinity for LDL and βVLDL, uptake and degradation of lipoprotein, and receptor recycling. Ligand blotting versus immunoblotting of receptors revealed normal-sized mutant receptors that were not recognized by lipoprotein ligand. Despite these differences, both mutant forms of the receptor were degraded at rates similar to those of normal receptors. We propose that the single amino acid substitution in this receptor interferes with the folding and/or posttranslational processing of precursor molecules in such a way that receptors adopt alternative stable structures.

The low-density lipoprotein (LDL)[1] receptor is a recycling, transmembrane glycoprotein which internalizes LDL by receptor-mediated endocytosis (Goldstein et al., 1985). Like other plasma membrane proteins, the LDL receptor is synthesized in the rough endoplasmic reticulum and undergoes posttranslational modification and processing during its passage via the Golgi apparatus to the plasma membrane. On the basis of structural and functional studies (Brown & Goldstein, 1986; Hobbs et al., 1990), the mature LDL receptor molecule can be divided into several domains. The ligand-binding domain of the LDL receptor contains 7 homologous repeats of a 40-amino acid cysteine-rich sequence. The ligands which bind to the receptor are apolipoprotein B-100 (the sole apolipoprotein of LDL) and apolipoprotein-E (found in several lipoprotein classes, including βVLDL). Mutational analysis of the ligand-binding domain has revealed that the repeats are not functionally equivalent and that binding of the two different ligands requires different combinations of the repeats (Esser et al., 1988; Russell et al., 1989): LDL binding requires repeats 3–7, whereas βVLDL binding appears to depend primarily on the presence of integrity of repeat 5.

A variety of naturally-occurring and artificial mutations in the LDL receptor have been described which interfere with receptor expression by affecting one or more properties of the receptor, such as receptor synthesis or degradation, precursor transport and processing, ligand binding, and ligand internalization (Hobbs et al., 1990). One of the common "founder gene" mutations in Afrikaners, the FH Afrikaner-1 (FH1) mutation, results in a single conservative amino acid substitution (Asp^{206} to Glu) in the consensus binding sequence in the fifth binding repeat (Leitersdorf et al., 1989). The mutant receptors show retarded posttranslational processing and transport to the cell surface (Fourie et al., 1988; Leitersdorf et al., 1989). Characterization of receptor activity on the basis of ligand and antibody binding suggested that the mutant receptors are also functionally heterogeneous (Fourie et al., 1988).

In this study, we have sought to confirm the existence of functionally distinct forms of the mutant receptor both in fibroblasts and in transfected CHO cells. Evidence for functional heterogeneity was obtained in both types of cells by means of binding studies at low temperature, in which various antibodies and lipoproteins were used as ligands, and in the transfected CHO cells, also by ligand blotting and immunoblotting. We also characterized the functional behavior of the different forms of the receptor in fibroblasts at 37 °C and found that, in contrast to normal receptors, a subpopulation of mutant receptors was resistant to trapping and degradation when ligand was internalized in the presence of ammonium chloride. However, the normal turnover rates of the receptor subpopulations were not different. Alternative stable conformations are proposed to explain the formation of functionally distinct forms of this mutant receptor.

EXPERIMENTAL PROCEDURES

Materials. LDL (density = 1.019–1.063 g/mL) and lipoprotein-deficient serum (LPDS) (density >1.25 g/mL) were prepared from human plasma, and the LDL was iodinated

† Supported by the South African Medical Research Council, the University of Cape Town, and the Heart Foundation of Southern Africa.

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[1] Abbreviations: LDL, low-density lipoprotein; βVLDL, β-migrating very low density lipoprotein; LPDS, lipoprotein-deficient serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified essential medium; FH, familial hypercholesterolemia.

0006-2960/92/0431-12754$03.00/0

by the iodine monochloride method, all as described previously (Aulinskas et al., 1983). Rabbit βVLDL was prepared and iodinated as described (Kovanen et al., 1981). The monoclonal anti-LDL receptor antibody IgG-C7 was prepared from hybridoma cells obtained from the American Type Culture Collection (CRL/691) as previously described (Beisiegel et al., 1981). Anti-LDL receptor monoclonal antibody, IgG-HL1, was a generous gift from M. S. Brown and J. L. Goldstein (Dallas, TX). Goat anti-mouse IgG (GAM-IgG) was purchased from Cappel Laboratories (Malvern, PA). Antibodies were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (Beisiegel et al., 1981). $Na^{125}I$ was from Amersham (Buckinghamshire, England), and Trans^{35}S label (ICN Radiochemicals, Irvine, CA) was the source of [^{35}S]methionine. Dulbecco's modified Eagle's minimum essential medium (DMEM) and methionine-free Eagle's minimum essential medium (MEM) were from Flow Laboratories (Ayrshire, Scotland). Ham's F-12 medium was provided by Highveld Biologicals (Kelvin, South Africa).

Cells. Human skin fibroblasts were from LDL receptor-normal subjects (Normal) and from FH patients homozygous for the FH Afrikaner-1 (FH1a, FH8a, FH12a, JG, ES) LDL receptor mutation. The fibroblast cell line FH1a was the same as that used by Leitersdorf et al. (1989) to determine the FH1 mutation. Cells were cultured and LDL receptors up-regulated for 48 h in LPDS-containing medium all as described before (Grant et al., 1990). In some cases, cells were down-regulated by a 48-h treatment with DMEM/LPDS containing 1 μg/mL 25-hydroxycholesterol and 12 μg/mL cholesterol. Chinese hamster ovary cell lines TR715, TR1163, and TR1178 contained transfected plasmids encoding the normal LDL receptor, the FH Afrikaner-1 (FH1) mutant receptor, and the FH Afrikaner-2 (FH2) mutant receptor (Val^{408} to Met), respectively, and were established and grown as described (Leitersdorf et al., 1989).

LDL Receptor Assays. For surface binding at 4 °C of ^{125}I-ligands (LDL or βVLDL) and of ^{125}I-antireceptor antibodies (IgG-C7 or IgG-HL1), cells were incubated for 2 h in DMEM [buffered with 20 mM Hepes (pH 7.4) and supplemented with LPDS at a concentration of 2.5 mg of protein/mL (DMEM/LPDS)] containing the amounts of ^{125}I-ligands or ^{125}I-antibodies indicated in the figures and tables. For the measurement of ^{125}I-IgG-C7 binding following the exposure of cells to LDL or βVLDL, 4 °C binding was preceded by incubation with 10 mM suramin for 40 min at 4 °C to remove any surface-bound lipoproteins (Davis et al., 1987). The cell-associated content and rate of degradation of ^{125}I-LDL at 37 °C were determined as described (Goldstein et al., 1983). Nonlinear regression analyses using the program Enzfitter (Elsevier/Biosoft, Cambridge, U.K.) were used to calculate K_d values and to obtain high-affinity components of ligand concentration curves, assuming ligand binding to a single class of high-affinity sites.

For ^{35}S labeling and analysis of LDL receptors, cells were incubated with [^{35}S]methionine (50 μCi/mL) in methionine-free MEM/LPDS and chased in DMEM/LPDS containing 200 μM unlabeled methionine, as detailed in the figure legends and previously described (Tolleshaug et al., 1982). LDL receptors were solubilized, immunoprecipitated using IgG-C7/GAM-IgG immunocomplexes, separated by SDS–PAGE, and visualized by fluorography, all as previously described (Tolleshaug et al., 1982).

Ligand blotting and immunoblotting of the LDL receptors from transfected CHO cells were performed as described (Daniel et al., 1983; Van Driel et al., 1987), using ^{125}I-βVLDL and IgG-C7 (visualized with ^{125}I-GAM-IgG), respectively.

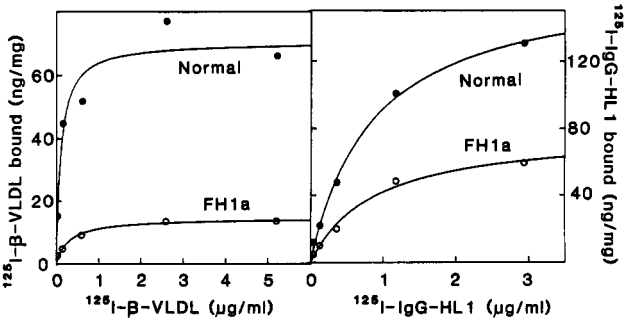

FIGURE 1: Surface binding at 4 °C of ^{125}I-labeled βVLDL and ^{125}I-labeled IgG-HL1 to normal and FH1a fibroblasts. Cells from a normal subject (●) and FH1a (○) were analyzed for cell-surface binding at 4 °C of ^{125}I-labeled βVLDL (1581 cpm/ng) (left) of ^{125}I-labeled IgG-HL1 (2929 cpm/ng) (right) as described under Experimental Procedures. The data points (averages of duplicates) represent high-affinity binding values calculated by nonlinear regression analysis of total binding curves. Maximum high-affinity binding of ^{125}I-labeled βVLDL in normal and FH1a cells was 70 and 15 (21% of normal) ng/mg, respectively. The K_d values for ^{125}I-labeled βVLDL in normal and FH1a cells were 0.1 and 0.3 μg/mL, respectively. Maximum high-affinity binding of ^{125}I-labeled IgG-HL1 was 170 and 79 (46% of normal) ng/mg, respectively. The K_d values for ^{125}I-IgG-HL1 binding were 0.85 μg/mL for normal cells and 0.88 μg/mL for FH1a cells.

RESULTS

Comparison of Ligand and Antibody Binding. LDL receptors in fibroblasts from subject FH1a (homozygous for the FH Afrikaner-1 mutation) were characterized using as ligands βVLDL and a monoclonal antibody, IgG-HL1 (Figure 1). FH1a cells bound ^{125}I-βVLDL and ^{125}I-IgG-HL1 at 4 °C with high affinities, similar to the corresponding affinities in normal cells (see legend to Figure 1). However, maximum high-affinity binding of ^{125}I-IgG-HL1 (Figure 1, right panel) to FL1a cells revealed a relatively greater number of receptors (approximately 50% of normal) than was detected on the basis of maximal ^{125}I-βVLDL binding (approximately 20% of normal) (Figure 1, left panel). The interesting feature of these observations was that the low lipoprotein-binding activity in FH1a cells could not simply be ascribed either to a reduced number of receptor molecules or to a decreased affinity for lipoproteins. Similar findings were previously reported for the same cell strain when LDL and IgG-C7 were used as alternative ligand and monoclonal antibody, respectively (Fourie et al., 1988).

The efficiencies of LDL and βVLDL binding were assessed in relation to the binding of IgG-HL1 (Table I). In agreement with the results of Figure 1, maximal ligand binding relative to antibody binding showed that only about half of the mutant FH1a receptors were functional in binding lipoprotein ligands whereas the others were not. LDL and βVLDL binding values expressed in terms of IgG-HL1 binding were 38% and 49% of the values for receptor-normal cells, respectively. The specificity of antibody binding was demonstrated by the fact that maximum antibody binding to LDL receptor-negative fibroblasts or sterol-down-regulated normal cells was always less than 10% of the values for normal up-regulated cells (data not shown). Our interpretation of the results is that distinct forms of the mutant receptor molecule exist in FH1a cells; some molecules bind lipoprotein ligands (and anti-receptor antibody) with normal high affinity, whereas other receptor molecules recognize only anti-receptor antibody and not lipoproteins.

The expression of the mutant LDL receptor was analyzed in receptor-deficient CHO cells transfected with a plasmid encoding the FH1 mutant receptor (TR1163). These cells expressed numbers of surface receptors, assessed by mono-

Table I: Surface Binding at 4 °C of ^{125}I-LDL and ^{125}I-βVLDL to FH1a Fibroblasts[a]

cells	expt no.	^{125}I-IgG HL1, ng/mg	^{125}I-LDL bound ng/mg of protein	^{125}I-LDL bound ng/ng of IgG	% functional receptors (LDL binding)	^{125}I-βVLDL bound ng/mg of protein	^{125}I-βVLDL bound ng/ng of IgG	% functional receptors (βVLDL binding)
normal	1–4	53 ± 34	218 ± 87	4.5 ± 3.1	100	59 ± 18	1.5 ± 0.2	100
FH1a	1	46	66	1.43	39	29	0.63	53
FH1a	2	19	54	2.84	36	17	0.89	54
FH1a	3	44	36	0.81	40			
FH1a	4	20				12	0.60	40
				mean (% normal value)	38 ± 2			49 ± 8

[a] After incubation for 48 h in lipoprotein-deficient medium, normal and FH1a fibroblasts were incubated for 2 h at 4 °C in medium containing ^{125}I-LDL (7–10 μg/mL), ^{125}I-βVLDL (2 μg/mL), or ^{125}I-IgG-HL1 (2 μg/mL). Bound values (±SD) represent high-affinity binding calculated after correction for nonspecific binding values, obtained in sterol-down-regulated cells. Nonspecific binding of ligands and antibody to FH1a cells represented 20–30% of total binding. The percent of receptors which bind LDL and βVLDL (% functional receptors) was calculated from the amount of ligand bound relative to IgG bound and expressed relative to the corresponding normal (100%) value in the same experiment.

Table II: ^{125}I-LDL and ^{125}I-IgG-C7 Binding at 4 °C to Transfected CHO Cells Expressing Normal and Mutant LDL Receptors[a]

cell type	^{125}I-IgG-C7 bound, ng/mg	^{125}I-LDL bound ng/mg of protein	^{125}I-LDL bound ng/ng of IgG	% functional receptors
TR715 ("normal")	56 ± 6	212 ± 12	3.7	100
TR1163 ("FH1")	41 ± 20	28 ± 13	0.8	20
TR1178 ("FH2")	6 ± 4	18 ± 7	4.0	108

[a] LDL receptor-deficient CHO cells (ldlA-7), transfected with normal or mutant FH1 LDL receptors, were grown for 6 days and then analyzed for cell-surface binding at 4 °C of ^{125}I-LDL (7–10 μg/mL) and ^{125}I-IgG-C7 (1 μg/mL). Values (±SD) are averages of duplicate incubations in three separate experiments and represent high-affinity binding values calculated after correction for nonspecific values obtained in the presence of excess unlabeled LDL (200–350 μg/mL) or IgG-C7 (50 μg/mL). The percent of receptors which bind LDL (% functional receptors) was calculated from the amount of ligand bound relative to IgG bound and expressed relative to the corresponding normal (100%) value in the same experiment.

clonal IgG-C7 binding, quite similar to those in cells transfected with the normal human receptor (TR715) (Table II). As in fibroblasts, however, only a fraction (20%) of the mutant receptors in the transfected CHO cells bound LDL. In contrast, cells expressing low levels of a rapidly-degraded mutant receptor (TR1178) bound extremely low amounts of IgG-C7 as well as LDL. The affinities of LDL binding at 4 °C to the normal and FH1 mutant receptors in the CHO cells, determined from binding curves (not shown), were 1.1 ± 0.2 and 0.4 ± 0.1 μg/mL, respectively. These K_d values for LDL are similar to each other and to the values previously obtained for the normal and FH1 fibroblast receptors (Fourie et al., 1988).

Ligand Uptake and Degradation at 37 °C. FH1a cells also exhibited high-affinity uptake and degradation of ^{125}I-LDL at 37 °C. These processes occurred at rates approximately 4–5 times lower than in normal cells (Figure 2; Van der Westhuyzen et al., 1984), consistent with the lower binding activity of the mutant receptors at 4 °C. At 37 °C, mutant receptors exhibited apparently normal affinity for LDL as judged by cellular association and degradation of LDL (Figure 2). Therefore, those mutant receptors that bound LDL also mediated internalization and degradation and, presumably, recycled normally.

Ligand-Induced Loss of Surface Receptors. Incubation of normal fibroblasts with LDL or βVLDL in the presence of ammonium chloride leads to a rapid decrease in the level of surface LDL receptors (Grant et al., 1990). This is believed to be due to intracellular trapping of receptors through inhibition of acid-dependent ligand dissociation. Trapped receptors are degraded with a half-life of approximately 2 h (Grant et al., 1990). Since these effects depend on ligand binding, it was of interest to determine what fraction of the

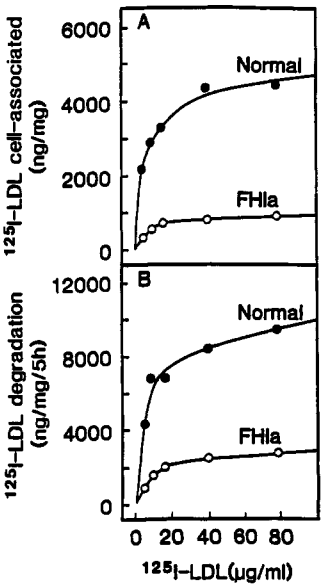

FIGURE 2: Cell association and degradation of ^{125}I-LDL at 37 °C in normal and FH1a fibroblasts. After incubation for 48 h in lipoprotein-deficient medium, each cell monolayer received 2 mL of medium containing the indicated concentrations of ^{125}I-LDL. After incubation for 5 h at 37 °C, the amounts of cell-associated (surface-bound plus intracellular) (A) and degraded (B) ^{125}I-LDL were determined. The data are averages of duplicate determinations from a typical experiment and represent high-affinity values calculated by subtracting nonspecific values, determined from cells which had been down-regulated by incubation for 48 h in the presence of 1 μg/mL 25-hydroxycholesterol and 12 μg/mL cholesterol, from total values. The half-maximal values (calculated by nonlinear regression) for cell-associated and degraded ^{125}I-LDL were 6.2 and 5.4 μg/mL, respectively, for normal cells and 8.4 and 8.9 μg/mL, respectively, for FH1a cells. The maximum levels of cell-associated and degraded ^{125}I-LDL for FH1a cells were 21% and 31% of normal values, respectively.

mutant receptors would be affected by this treatment. As was shown previously, incubation with ammonium chloride and βVLDL led to the disappearance of 90% of the IgG-detectable LDL receptors in normal cells (Figure 3). FH1 cells exhibited maximal trapping at a similar low βVLDL concentration (implying a high-affinity process), but in these cells only 40% of surface receptors were trapped even at the highest βVLDL concentrations used. Studies in which LDL was used in place of βVLDL yielded similar results (data not shown). These results indicate that a significant proportion of mutant receptors did not bind lipoproteins.

The effect of ammonium chloride and LDL on the turnover of ^{35}S-labeled receptors in normal and FH1 cells was also determined (Figure 4). Incubation of normal cells for 3 h with LDL and ammonium chloride caused a dramatic increase in the degradation of immunoprecipitable receptors (88%

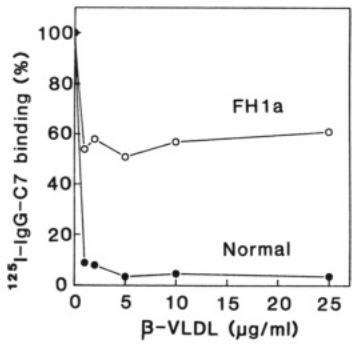

FIGURE 3: Ligand (βVLDL)-dependent loss of surface LDL receptors in normal and FH1a fibroblasts in the presence of ammonium chloride. After incubation for 48 h in lipoprotein-deficient medium, normal and FH1a fibroblasts were preincubated at 37 °C for 30 min in medium containing the indicated concentrations of unlabeled βVLDL. Ammonium chloride (final concentration 10 mM) was then added to all dishes containing βVLDL, and the incubation was continued for 90 min. The cells were then incubated at 4 °C for 40 min with 10 mM suramin [in 50 mM NaCl, 3 mM $CaCl_2$, and 10 mM Hepes (pH 7.4)] to release bound, unlabeled βVLDL, after which surface receptors were assayed by ^{125}I-IgG-C7 (216 cpm/ng, 1 μg/mL) binding in the presence or absence of 50 μg/mL unlabeled IgG-C7. The data points represent high-affinity binding values and are averages of duplicate determinations.

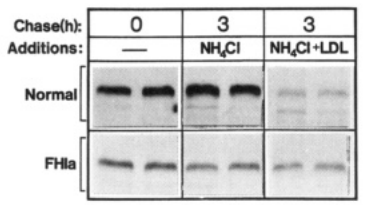

FIGURE 4: Effect of ammonium chloride and LDL on ^{35}S-labeled LDL receptors in normal and FH1a fibroblasts. After 24-h incubation in lipoprotein-deficient medium, cells from a normal subject and FH1a were pulsed-labeled for 2.5 h at 37 °C with [^{35}S]methionine (30 μCi/mL) in methionine-free MEM and then chased for 13 h in medium containing 200 μM unlabeled methionine, to ensure that all the labeled receptors in FH1a had been processed to the mature form. The medium was then changed to one containing 10 mM ammonium chloride plus or minus 40 μg/mL LDL, and the cells were incubated for a further 3 h at 37 °C. The cells were then washed and solubilized, and LDL receptors were immunoprecipitated and subjected to SDS–polyacrylamide gel electrophoresis (7% acrylamide).

degraded in 3 h) compared to the insignificant degradation observed with ammonium chloride alone (Figure 4) or LDL alone (Grant et al., 1990). However, in FH1 cells, degradation of only 40% of the mutant receptors occurred during the incubation with LDL and ammonium chloride. At the end of the incubation, the ^{35}S label in the FH1 receptor band was actually significantly greater than that in the normal receptor band. In another experiment (not shown), incubation periods of 3 and 6 h with LDL and ammonium chloride were used to exclude the possibility that the mutation in FH1 cells merely retarded degradation or promoted dissociation in the endosome, allowing receptors to return to the surface. Whereas the level of ^{35}S-labeled receptors in normal cells decreased even further after 6 h of incubation, no significant further decrease in receptors in FH1 cells occurred. These results support the concept that a major fraction of FH1 receptors was unable to bind LDL and, therefore, to be subsequently trapped and degraded.

Ligand Blotting and Immunoblotting. The relative levels of ligand- and antibody-binding receptors in cells were estimated by ligand blotting and immunoblotting of equal amounts of protein from TR715 (normal) and TR1163 (FH1) CHO cells. The monoclonal IgG-C7 bound both to normal and to FH1 receptors (Figure 5). The additional band

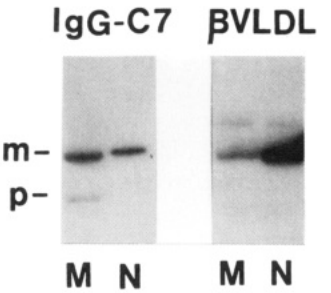

FIGURE 5: Immunoblotting and ligand blotting of LDL receptors of CHO cells transfected with mutant FH1 and normal LDL receptor genes. CHO cells transfected with either normal (N) or mutant FH1 LDL receptor (M) gene were lysed (one 100-mm diameter dish per sample), and proteins were separated by SDS–PAGE under nonreducing conditions and then transferred to nitrocellulose membranes. The membranes were incubated with either IgG-C7 (5 μg/mL) followed by ^{125}I-labeled GAM-IgG or ^{125}I-labeled βVLDL (5 μg/mL) and then dried and exposed to X-ray film. The positions of precursor (p) and mature (m) LDL receptors are shown. Equivalent protein amounts were used for the two blotting procedures.

migrating faster than mature mutant receptors was assumed to represent receptor precursors accumulated as a result of the slow transport and processing of receptors in these cells (Leitersdorf et al., 1989). When amounts of cell extracts identical to those used for the immunoblots were probed in the same experiment with saturating levels of ^{125}I-βVLDL, the mutant receptor band bound significantly less ligand than that derived from normal cells, despite the presence of more receptors as indicated by the IgG-C7 blot. The mutant FH1 receptor, separated by SDS–PAGE, therefore showed a deficiency in ligand binding even though certain FH1 receptors have a normal affinity for βVLDL (Figure 1, left panel). Our interpretation was that the FH1 mutation totally disrupts ligand binding in a fraction of the mutant receptors.

Turnover of FH1 Mutant LDL Receptors. It was previously shown that LDL receptors in FH1 cells are degraded at normal rates (Fourie et al., 1988). We accordingly determined whether both the LDL-binding form and the abnormal form which recognized only IgG-C7 were turned over with normal half-lives. This was carried out by measuring the decline in surface-binding activity for ^{125}I-LDL and ^{125}I-IgG-C7 following the suppression of LDL receptor gene expression by the addition of LDL to the medium (Figure 6). The kinetics of down-regulation of both binding activities, in both normal and FH1 cells, were virtually identical. Surface-binding activity for both LDL and IgG-C7 decreased with a half-life of approximately 11 h, consistent with previously determined half-lives of the LDL receptor in fibroblasts (Casciola et al., 1988). Thus, both the LDL- and IgG-C7-binding forms of the mutant receptor were degraded at the same rates, suggesting that while their conformational differences are sufficient to result in functional differences, they must be subtle enough not to be distinguishable by the unknown system responsible for degradation of the receptor.

DISCUSSION

The major conclusion of this study is that LDL receptor molecules, coded by a particular gene bearing a single pointmutation, exist in more than one functionally distinct stable form in cultured cells. Whereas some molecules exhibit normal receptor activity (lipoprotein ligand binding, internalization, and recycling behavior), others appear to be completely unable to bind ligand. Our findings suggest that specific alterations in the primary structure of receptor molecules may affect their ability to pursue a single and correct folding routine.

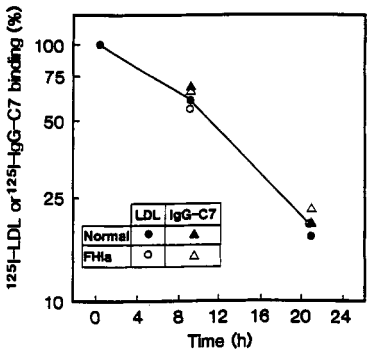

FIGURE 6: Down-regulation kinetics of IgG-C7 and LDL-binding populations in normal fibroblasts. After incubation for 48 h in lipoprotein-deficient medium, each monolayer received 2 mL of medium containing 40 μg/mL LDL. At zero time and after 8.7- and 20.7-h incubation at 37 °C, the cells were analyzed at 4 °C for cell-surface binding of ^{125}I-LDL (407 cpm/ng, 5.5 μg/mL) and ^{125}I-IgG-C7 (2812 cpm/ng, 0.8 μg/mL). The data shown from a single experiment represent high-affinity values calculated by subtracting nonspecific values (means of duplicates), obtained in the presence of excess unlabeled LDL (300 μg/mL) or IgG-C7 (50 μg/mL), from total values (means of triplicates) and are expressed as percentages of the values at zero time. The line joins the mean percentage values at each time point.

We have confirmed that this particular mutant receptor is present in more than one functionally distinct form also in transfected CHO cells. Furthermore, in addition to showing decreased levels of ligand (LDL and βVLDL) versus antibody (IgG-C7 and -HL1) binding, we have shown in the present study that (i) the ligand-binding population mediates lipoprotein uptake and degradation normally and can be seletively trapped and degraded intracellularly in the presence of ligand and ammonium chloride, (ii) only a fraction of the mutant LDL receptors detectable by immunoblotting can bind βVLDL on ligand blots, and (iii) despite their functional and, presumably, conformational differences, both the ligand- and antibody-binding forms of the mutant receptors are degraded at rates similar to normal receptors.

The substitution of Asp^{206} by Glu in the FH1 mutant receptor is in the fifth cysteine-rich binding repeat, within a highly-conserved amino acid triad (Ser-Asp-Glu) (Leitersdorf et al., 1989). The observation that a subpopulation of the mutant receptors is normally able to recognize lipoprotein ligands may be ascribed to the extremely conservative nature of the substitution and indicates that this amino acid substitution does not necessarily interfere with the binding capability of the receptor. The results obtained for certain artificial LDL receptor mutations in transfected COS cells (Esser et al., 1988; Russell et al., 1989) can be similarly interpreted. The replacement of Asp^{206} by Tyr or Asn markedly decreased ligand binding relative to antibody binding. Although it was difficult to distinguish between effects on affinity and on the number of binding sites in the transfected COS cells, these authors concluded that the major effect of these mutations was on the number of ligand-binding sites (Russell et al., 1989); the implication was that only some (but not all) molecules of a particular mutant protein attain ligand-binding competency.

FH1a cells showed a marked delay in the processing and transport of newly-synthesized LDL receptor precursors to the Golgi (Fourie et al., 1988). The criterion for normal transport of newly synthesized proteins from the ER to the Golgi appears to be a correct native protein conformation (Gething et al., 1986; Rothman et al., 1987; Lodish et al., 1988), and the observation that the receptors in FH1a cells are retarded in the ER thus seems highly suggestive for conformational abnormality. Immunoblots showed that FH1 mutant receptors migrated with normal mobility on SDS–PAGE under nonreducing conditions, but this technique would not necessarily detect a conformational abnormality. Interestingly, the retarded processing applied both to the active and also to the inactive forms of the receptor, since no rapidly processed fraction of receptors was detected that could account for the active receptor population (Fourie et al., 1988). Therefore, even for those receptors that achieve an active conformation, processing requires a longer than normal time in the ER. It is possible that the processing and transport times of the two receptor forms are nevertheless not identical, but we have been unable to detect any difference in the rates at which the two newly-synthesized forms of the receptor reach the cell surface (unpublished observations). While alternative receptor conformations may be the molecular explanation for the inactivity of certain FH1 receptor sites, other factors such as receptor orientation on the cell surface, posttranslational modification, and receptor oligomerization should be considered.

Davis et al. (1987) found that artificial mutant receptors lacking the EGF precursor-like domain were unable to bind LDL when on the cell surface, but recognized LDL on ligand blots, suggesting that the mutation interfered with receptor orientation on the cell surface. Membrane-free FH1 receptors, on the other hand, showed the same decreased ligand binding compared with antibody binding when studied by ligand blotting and immunoblotting as was seen in intact cells, suggesting that mutant receptors are exposed normally on the cell surface and are not partly hidden in some way, for instance, by the cell glycocalyx.

The best-characterized modifications of the LDL receptor are N- and O-linked glycosylation, although their structural and functional significance remains unclear. Deletion in vitro of the "O-linked sugar" domain has no apparent effect on receptor function and stability (Davis et al., 1986). A lack of O-linked sugars outside this domain was reported to reduce the binding affinity for LDL (Yoshimura et al., 1987), while Kozarsky et al. (1988) found that receptors lacking all O-linked sugars were unstable due to enhanced proteolytic cleavage. While posttranslational processing and glycosylation in FH1 cells are clearly delayed, mutant precursors are nevertheless glycosylated to apparently normal-sized (120 kDa) precursors and mature forms (160 kDa) that are eventually transported to the cell surface. Although N-linked glycosylation of mutant receptors in FH1a cells appears to be normal (unpublished results), abnormal O-linked glycosylation of the stalled precursors has not been excluded and may be involved in the generation of abnormal receptor conformations, perhaps by stabilizing inappropriate folding intermediates. It is noteworthy that accumulation of other transport-deficient LDL receptors occurs in cellular compartments where O-linked sugar addition is proposed to take place (Pathak et al., 1988). Alternatively, abnormal glycosylation may affect ligand binding directly. In the case of plasminogen, the difference of one N-linked carbohydrate chain caused a 10-fold difference in the affinity of binding to plasminogen receptors (Gonzales-Gronow et al., 1989).

Evidence was suggested that LDL receptors function as oligomers, possibly tetramers (Mahley & Innerarity, 1983; Grant et al., 1990; Innerarity, 1990). It has been proposed that in the binding of LDL, the four receptor molecules of a tetramer each bind one molecule of ligand, whereas in the binding of βVLDL, the multiple apoE molecules on a single particle simultaneously saturate all the receptors of the oligomeric structure (Mahley & Innerarity, 1983). Cross-linking studies have demonstrated that LDL receptors are at

least partially aggregated on the cell surface (Van Driel et al., 1987). The observation that FH1a cells had the same affinity for βVLDL as normal cells suggests that receptors were normally oligomerized in FH1a cells. Further, the results of ligand blotting and immunoblotting of mutant FH1a receptors, separated by SDS–PAGE before immobilization, also argue against differential receptor aggregation or oligomerization as the cause for the observed receptor heterogeneity. Certainly, the trapping and degradation of only a fraction of the receptors in FH1a cells in the presence of ammonium chloride indicate that the binding results for FH1a cells cannot be simply explained by postulating receptor oligomers with cryptic ligand-binding sites and show that FH1a receptor forms exist that are functionally distinct.

We believe that functionally distinct forms of the mutant FH1 receptor are most likely correlated with distinct stable receptor conformations and suggest the following model to explain these forms. During the retarded processing of the mutant receptors in the ER, some molecules undergo aberrant folding, perhaps involving incorrect disulfide bond formation and/or abnormal O-linked glycosylation. As a result, mutant receptor precursors are slowly processed into more than one stable conformation; some molecules are in a functionally inactive form, while others successfully adopt a fully-functional conformation. The membrane glycoprotein (gp55) encoded by Friend spleen focus-forming virus has been reported to fold into heterogeneous forms (Gliniak et al., 1991). These forms were associated with inefficient and imprecise disulfide bonding in the ER. In this case, however, only a small fraction of gp55 was competent for export as a disulfide-bonded homodimer.

It is possible to extend our model for the FH1 receptor to incorporate the concepts of both alternative receptor conformations and abnormal receptor oligomerization. An abnormal conformation may be unfavorable for oligomerization, resulting in a proportion of the mutant surface receptors being monomeric. If ligand binding specifically requires oligomerized receptors, the observed decrease in ligand binding would be explained for FH1 receptors. Kadowaki et al. (1991) recently discussed a similar situation for a point mutation (His^{204} to Arg) in the insulin receptor. This mutation impairs receptor dimerization and transport to the cell surface of the majority of the mutant receptors, but a 10% subset of the receptors oligomerize, reach the cell surface, and bind insulin with normal affinity. The authors suggest that the point mutation reduces the probability that the mutant insulin receptor will fold into its normal conformation, but does not preclude the possibility. The point mutation in the FH1 LDL receptor may have a similar effect on the folding of the receptor protein.

The main feature of our model for the FH1 receptor, which might also be applicable to other membrane or secretory proteins, is that a single protein may be processed into alternative stable structures, including a fully functional form. Such a model, if correct, may be relevant to the potential effects that other alterations in the LDL receptor may have on receptor expression. Furthermore, the model raises the interesting possibility that the activity of such mutant receptors, and even possibly normal receptors, may be modulated under different conditions and in different cell types, depending on the efficiency of posttranslational folding and processing.

ACKNOWLEDGMENT

D. W. Russell and L. Thilo are thanked for helpful discussions and Sheena Jones and Hajira Karjiker for excellent technical assistance.

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Registry No. Asp, 56-84-8; Glu, 56-86-0; ammonium chloride, 12125-02-9.