Receptor-Associated Protein Facilitates Proper Folding and Maturation of the Low-Density Lipoprotein Receptor and Its Class 2 Mutants†

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ABSTRACT: Familial hypercholesterolemia is the consequence of various mutations in the low-density lipoprotein receptor (LDLR). In the current study, we show that a specialized molecular chaperone, the receptor-associated protein (RAP), promotes proper folding and subsequent exocytic trafficking of the wild-type LDLR and several of its class 2 mutants. Co-immunoprecipitation with anti-RAP antibody demonstrates that RAP interacts with the LDLR. Kinetic analyses of LDLR posttranslational folding and maturation in the absence or presence of RAP coexpression show that RAP prevents aggregation and promotes the maturation of the LDLR. Additionally, depletion of Ca^{2+} in intact cells impairs LDLR folding, and coexpression of RAP partially corrects this misfolding. Finally, we show that the increased mature cell surface LDLR in the presence of RAP coexpression is functional in its ability to endocytose and degrade ^{125}I-LDL. Taken together, our results show that the folding, trafficking, and maturation of the LDLR and its class 2 mutants are promoted by RAP.

Heterozygous familial hypercholesterolemia (FH)[1] is a common disorder with an estimated frequency of about 1 in 500. It is characterized by elevated levels of low-density lipoprotein (LDL) in plasma and deposition of LDL-derived cholesterol in tendons, in skin (xanthomas), and within arteries (atheromas) (*1, 2*). The primary defects in FH patients are mutations in the gene encoding the LDL receptor (LDLR). This receptor, located on the surface of cells in the liver and other organs, binds plasma LDL and mediates its cellular uptake via receptor-mediated endocytosis and its delivery to lysosomes, where the LDL is degraded and its cholesterol is released for metabolic use. When LDLR is deficient or defective, the rate of removal of LDL from plasma is diminished. At present, more than 600 different mutations have been identified. Among them, approximately 50% are class 2 mutations which are defined as mutations that cause abnormal transport from the endoplasmic reticulum (ER) to the Golgi complex (*1, 2*).

LDLR is composed of five structural and functional domains: a ligand-binding domain that contains seven complement-type ligand-binding repeats; an epidermal growth factor (EGF) precursor homologous domain; an O-linked glycosylation domain; a membrane-spanning domain; and a cytoplasmic tail. Each of the ligand-binding repeats, which are also present in other members of the LDLR family, contains about 40 amino acids that include 6 cysteine residues forming 3 disulfide bonds (*3*). The EGF precursor homology domain of LDLR consists of two cysteine-rich EGF repeats followed by six YWTD repeats and a third EGF repeat. Mutational analysis has showed that this domain is necessary for the dissociation of ligands from the receptor in endosomes (*4, 5*). The cytoplasmic tail of LDLR contains a NPXY motif that serves as a signal for receptor endocytosis through coated pits (*6*).

LDLR serves as a prototype for an expanding family of cell surface receptors. These receptors, which include the very low-density lipoprotein receptor (VLDLR), apoE receptor 2 (apoER2), LDLR-related protein (LRP), LRP1B, and megalin, share a modular domain organization with that of the LDLR and play a variety of roles in cellular protein uptake and signal transduction (*7, 8*). The 39 kDa receptor-associated protein (RAP) is a unique receptor antagonist for members of the LDLR family. RAP, which binds with high affinity to LRP, megalin, VLDLR, and apoER2, and with a lower affinity to the LDLR, is able to inhibit the binding of all currently known ligands of LDLR family members (*9*).

Newly synthesized proteins in the secretory pathway undergo posttranslational modification, folding, and oligomerization in the ER wherein the proper folding is assisted by molecular chaperones. Misfolded or unassembled proteins are often retained in the ER and targeted for degradation (*10*). Using LRP and the VLDLR as target receptors, RAP has been defined as a specialized chaperone for members of the LDLR family, and functions in the promotion of proper

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[1] Abbreviations: EGF, epidermal growth factor; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; LDL, low-density lipoprotein; LDLR, LDL receptor; LRP, LDLR-related protein; RAP, receptor-associated protein; VLDLR, very low-density lipoprotein receptor.

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folding and trafficking of these receptors along the early secretory pathway (*11*−*17*). As the affinity for binding of RAP to cell surface LDLR is significantly lower than that to cell surface LRP and the VLDLR (*18*−*21*), the potential role of RAP in LDLR folding and maturation is not clear. A recent study in RAP knockout mice indicates that RAP may play a role in the biogenesis of the LDLR, since the level of the LDLR was downregulated in brain of homozygous RAP-deficient mice (*22*). The objective of the current study was to investigate whether RAP serves as a molecular chaperone during the folding and trafficking of the LDLR. We found that RAP is associated with the LDLR and plays an important role in the folding and maturation of the LDLR and several of its class 2 mutants.

EXPERIMENTAL PROCEDURES

Construction of cDNAs. A PCR fragment encoding the full-length human LDLR was generated and cloned into the expression vector pcDNA3 by using the same strategies as described previously for soluble LRP minireceptors and the VLDLR (*12*, *15*). To facilitate immunoprecipitation and Western blotting, an HA epitope was included near the amino terminus of the LDLR. Plasmid pLDLR-2 with the cDNA of the full-length LDLR was obtained from ATCC, and used as the PCR template. Site-directed mutagenesis was carried out using the QuickChange Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. All oligonucleotides were synthesized at the Washington University School of Medicine Protein Chemistry Laboratory. The integrity of all the constructs was confirmed by DNA sequencing. The cDNA construct of the full-length human RAP has been described previously (*12*).

Cell Culture and Transfection. Human glioblastoma U87 cells were cultured and transiently transfected with various plasmids as described before (*11*, *23*). The efficiency of transient transfection in these studies was consistently about 20−30% as assessed by immunofluorescent staining of expressed proteins (*24*). LDLR-deficient Chinese hamster ovary (CHO) cell line *ldl*A7 (kindly provided by Dr. Monty Krieger, Massachusetts Institute of Technology, Cambridge, MA) was cultured in Ham's F-12 medium supplemented with 5% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. *ldl*A7 cells were plated into 10 cm dishes, and transfected with 20 μg of various plasmids using Mirus TransIT-LT1 (PanVera, WI) according to the manufacturer's instructions.

Metabolic and Pulse−*Chase Labeling.* Metabolic labeling with [^{35}S]cysteine was performed essentially as described before (*11*). For pulse−chase experiments, cells were pulse-labeled for 0.5 h and chased with serum-containing medium for the indicated periods as detailed in each figure. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by 6% SDS−PAGE under either nonreducing or reducing conditions. The gels were scanned by a phosphoimager, and the intensity of individual bands was directly quantified by ImageQualNT.

Immunoprecipitation and SDS−*PAGE.* Polyclonal anti-RAP and monoclonal anti-HA antibodies have been described before (*11*, *24*). Immunoprecipitations were carried out essentially as described before (*11*). The washing buffer for monoclonal anti-HA antibody was PBS containing 0.1% SDS and 1% Triton X-100, while the washing buffer for polyclonal anti-RAP was PBS containing 1% SDS and 1% Triton X-100. Preliminary experiments were performed to ensure that the primary antibody used in each immunoprecipitation was in excess. Protein A−agarose beads were used to precipitate protein−IgG complexes. The immunoprecipitated material was released from the beads by boiling each sample for 5 min in Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol]. If the immunoprecipitated material was analyzed under reducing conditions, 5% (v/v) β-mercaptoethanol was included in the Laemmli sample buffer. The percentage of SDS−polyacrylamide gels is indicated in each figure legend. Rainbow molecular weight markers (Bio-Rad) were used as the molecular weight standards.

Western Blotting. Transfected U87 cells were lysed with 0.5 mL of lysis buffer (PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Equal quantities of protein were subjected to SDS−PAGE under reducing or nonreducing conditions. Following transfer to a poly(vinylidene difluoride) membrane, successive incubations with anti-HA antibody and horseradish peroxidase-conjugated goat anti-mouse IgG were carried out for 60 min at room temperature. The immunoreactive proteins were then detected using the ECL system.

LDL Degradation Assay. Human plasma LDL was kindly provided by Dr. Monty Krieger, and was iodinated using the method described previously (*25*, *26*). For degradation assays, *ldl*A7 cells were transfected with 20 μg of various plasmids in 10 cm dishes. On day 2, the cells were split into 12-well plates. On day 4, cells were washed twice with PBS and switched to Dulbecco's modified Eagle's medium (DMEM, minus glutamine) containing 6 mg/mL fatty acid-free bovine serum albumin and 4 μg/mL ^{125}I-LDL (specific activity 700 cpm/ng). After incubation at 37 °C for 4 h, the media were harvested, and specific LDL degradation was measured by trichloroacetic acid precipitation.

RESULTS

RAP Interacts with the LDLR. Previous studies have shown that recombinant RAP binds to LDLR on the cell surface (*21*). To test whether RAP is associated with the LDLR intracellularly, we examined whether the LDLR co-immunoprecipitates with RAP. U87 cells were transiently transfected with cDNA for the human LDLR (HA-tagged) with cotransfection of either vector pcDNA3 (−RAP) or cDNA for RAP. Following metabolic labeling with [^{35}S]cysteine for 4 h, cell lysates were immunoprecipitated with anti-HA, or anti-RAP antibodies, and analyzed via SDS−PAGE under reducing conditions. As expected, anti-HA antibody precipitated both the ER form (120 kDa) and the mature form (160 kDa) of the LDLR (Figure 1, lanes 1 and 2). The conversion from the 120 kDa ER form of LDLR to the 160 kDa mature form arises from O-linked and possible N-linked glycosylation, and reflects that the receptor has been correctly folded and exited the ER. A relative increase of the mature form of the LDLR was observed when cells were cotransfected with cDNA for RAP. In the absence of RAP cotransfection, only the two subunits of LRP (515 and 85 kDa), but not the LDLR, were co-immunoprecipitated with the endogenous RAP (Figure 1, lane 3). However, when the cells were

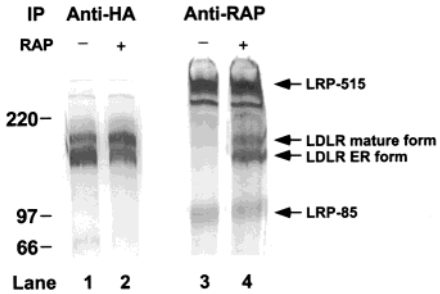

FIGURE 1: Co-immunoprecipitation of LDLR with RAP. U87 cells were transiently transfected with cDNA for LDLR with cotransfection of either vector pcDNA alone (−) or pcDNA-RAP (+). Cells were then metabolically labeled with [^{35}S]cysteine and immunoprecipitated with anti-HA antibody or anti-RAP antibody. The immunoprecipitated material was then analyzed by 6% SDS−PAGE gel under reducing conditions. The positions of co-immunoprecipitated LRP and LDLR are marked. The molecular mass standards in this figure and subsequent figures are given in kDa.

cotransfected with cDNAs for the LDLR and RAP, in addition to the two LRP subunits, anti-RAP antibody immunoprecipitated both the ER form and the mature form of the LDLR (Figure 1, lane 4). Thus, these results indicate that within the intact cell RAP is able to interact with the LDLR.

We next evaluated a potential role for RAP on LDLR folding. U87 cells were transiently transfected with cDNA for the LDLR with cotransfection of either pcDNA3 (−RAP) or cDNA for RAP (+RAP). Transfected cells were then metabolically pulse-labeled with [^{35}S]cysteine for 30 min and chased for 0, 30, 60, or 120 min. After each chase period, cells were lysed and quantitatively immunoprecipitated with anti-HA antibody and analyzed via SDS−PAGE under either nonreducing or reducing conditions. Initially, after 30 min of labeling, a single band of labeled protein was observed in the absence of RAP coexpression (Figure 2A, lane 1). After 120 min of chase, about 40% of the labeled protein appeared as a slower migrating band, representing the glycosylated mature form of the LDLR (Figure 2A, lane 4; Figure 3). Figure 2B shows that coexpression of RAP significantly increased the rate of maturation of the LDLR. After 120 min of chase, over 75% of labeled LDLR had been processed into the mature form (Figure 2B, lane 8; Figure 3). When samples from the same experiment were analyzed under nonreducing conditions, we found that in the absence of RAP coexpression, significant amounts of the LDLR migrated at the top of the stacking and separating gels (Figure 2A). These "aggregates" represent misfolded LDLR and likely resulted from mislinked intermolecular disulfide bonds since they were reduced to monomeric receptor species under reducing conditions (Figure 2A). When RAP was coexpressed with the LDLR, the amount of these "aggregates" was significantly decreased (Figure 2B). These results together suggest that RAP promotes LDLR folding by preventing the formation of "nonproductive" intermolecular disulfide bonds and aggregation of the LDLR.

Ca^{2+} Is Required for LDLR Folding. Calcium-binding motifs play an important structural role in the ligand-binding repeats of LDLR (*27*, *28*). Calcium is needed to guide the formation of native disulfide bonds in refolding reactions performed in vitro with ligand-binding repeats and is required to maintain their structural integrity (*27*−*32*). To examine whether Ca^{2+} is required for LDLR folding in vivo, we analyzed the state of LDLR folding upon Ca^{2+} depletion. Herein, we used thapsigargin, an ATPase inhibitor, to deplete ER Ca^{2+} as we have done previously with LRP (*33*). U87

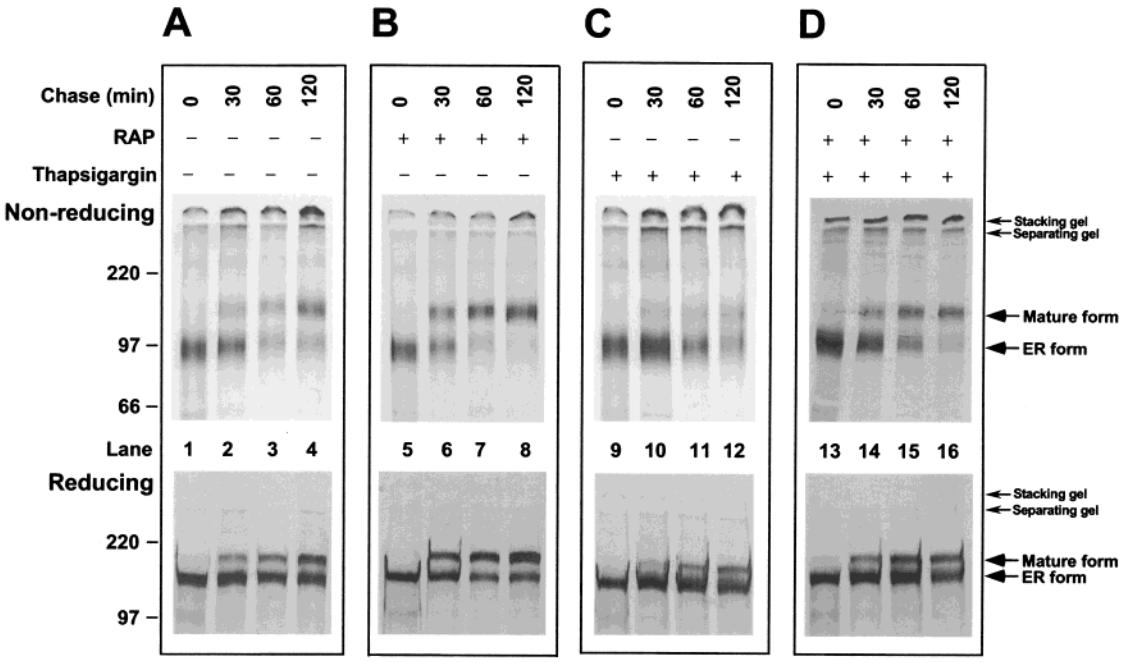

FIGURE 2: RAP facilitates folding and maturation of the LDLR. U87 cells were transiently transfected with cDNA for LDLR, with the cotransfection of either vector pcDNA3 (−RAP) or vector pcDNA-RAP (+RAP). The transfected cells were metabolically pulse-labeled with [^{35}S]cysteine for 30 min and chased for the indicated times in the absence or presence of 100 nM thapsigargin. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by 6% SDS−PAGE under either nonreducing or reducing conditions. The tops of the stacking and separating gels are marked. The ER and mature forms of the LDLR are indicated. (A) Pulse−chase analysis of LDLR folding and maturation in the absence of RAP coexpression. (B) RAP facilitates folding and maturation of the LDLR. (C) Depletion of Ca^{2+} results in LDLR misfolding. (D) Coexpression of RAP partially corrected LDLR misfolding due to Ca^{2+}depletion.

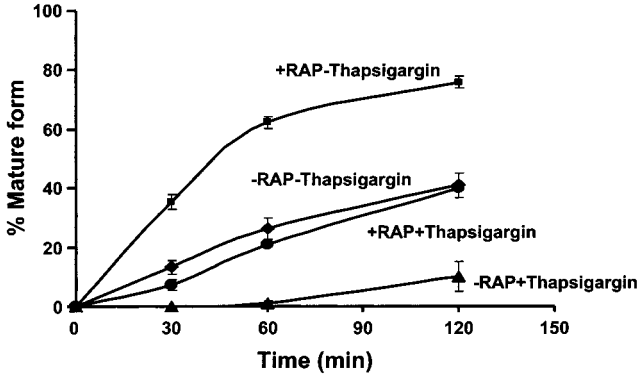

FIGURE 3: RAP increases the maturation rate of the LDLR. Pulse−chase analyses of LDLR folding and trafficking were carried out as described in the Figure 2 legend. The percentages of the mature forms observed in reducing gels are plotted against chase time. Values are the average of three experiments with the SE indicated by error bars.

cells were transiently transfected with cDNA for the LDLR with cotransfection of either pcDNA3 (−RAP) or pcDNA-RAP (+RAP), metabolically pulse-labeled with [^{35}S]cysteine for 30 min, and chased in the presence of 100 nM thapsigargin for 0, 30, 60, or 120 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed by SDS−PAGE. Figure 2C show that depletion of Ca^{2+} caused misfolding of LDLR with the appearance of significant amounts of LDLR "aggregates" in the nonreducing gel. Consequently, less LDLR is converted into the mature form. After 120 min of chase, only about 10% of the labeled protein appeared as the slower migrating band (Figure 2C, lane 12; Figure 3). On the other hand, in the presence of RAP coexpression, more LDLR was proper folded and converted into the mature form (Figure 2D). After 120 min of chase, approximately 40% of the labeled protein appeared as the mature form (Figure 2D, lane 16; Figure 3). However, even in the presence of RAP coexpression, depletion of Ca^{2+} still resulted in significant LDLR misfolding. Taken together, these results indicate that depletion of Ca^{2+} impairs LDLR folding, and that coexpression of RAP can partially correct this misfolding.

Effects of RAP on the Folding of LDLR Class 2 Mutants. The class 2 mutants of the LDLR are characterized by the retention of receptor molecules within the ER. However, the molecular basis for ER-retention for individual mutants is largely unknown. To examine a potential role for RAP in the proper folding of these class 2 mutants, we generated six class 2 LDLR mutants, S156L, C176Y, D200G, E207K, D283N, and C646Y, using site-directed mutagenesis techniques. These are all naturally occurring mutations found in FH patients (*1*, *2*). The S156L mutation occurs within ligand-binding repeat 4 of the LDLR; C176Y, D200G, and E207K within ligand-binding repeat 5; D283N within ligand-binding repeat 6; and C646Y within EGF precursor repeat 3.

We first examined whether ER-retention of these mutants is due to receptor misfolding. Indeed, in the absence of RAP coexpression, all six mutants were misfolded, and retained in ER (Figure 4, lanes 2, 6, 10, 14, 18, and 22). After 60 min of chase, only 5−12% of the receptors were converted to the mature form for the mutants S156L, C176Y, D200G, or D283N, and no conversion was seen for the mutants E207K or C646Y (Figure 5). In contrast, approximately 24% of the wild-type LDLR had converted to the mature form after 60 min of chase (Figure 5). The absence of the mature form for mutants E207K and C646Y after 60 min of chase suggests that the effects of these mutations on receptor folding are more severe than those of the other mutations. More interestingly, when samples from identical experiments were analyzed under nonreducing conditions, all six mutants exhibited significant amounts of receptor "aggregates" migrating at the top of the stacking gel and separating gel (Figure 4). Similar to wild-type LDLR, these misfolded LDLR "aggregates" were reduced to monomeric receptor species under reducing conditions, suggesting that these LDLR "aggregates" were likely the results of mislinked intermolecular disulfide bonds during folding.

We next examined the effect of RAP coexpression on the proper folding and maturation of these LDLR mutants. Figure 4 shows that coexpression of RAP significantly enhanced the conversion of the receptor ER forms to their mature forms for some of these class 2 mutants. After 60 min of chase

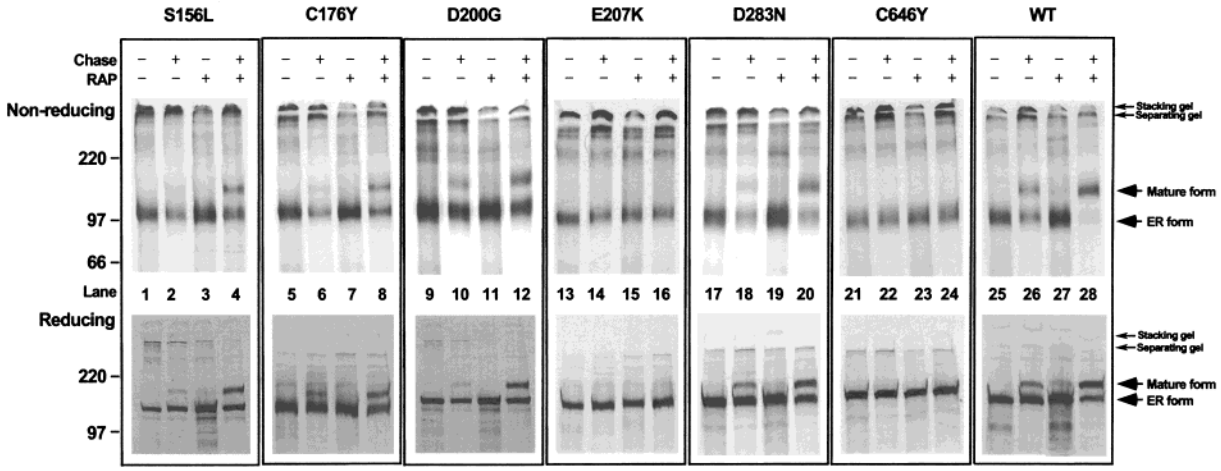

FIGURE 4: RAP facilitates folding and maturation of LDLR class 2 mutants. U87 cells were transiently transfected with cDNA for the wild-type LDLR or its various class 2 mutants, with the cotransfection of either vector pcDNA3 (−RAP) or vector pcDNA-RAP (+RAP). The transfected cells were metabolically pulse-labeled with [^{35}S]cysteine for 30 min and chased for 0 or 60 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed by 6% SDS−PAGE under either nonreducing or reducing conditions. The tops of the stacking and separating gels are marked. The ER and mature forms of LDLR are indicated.

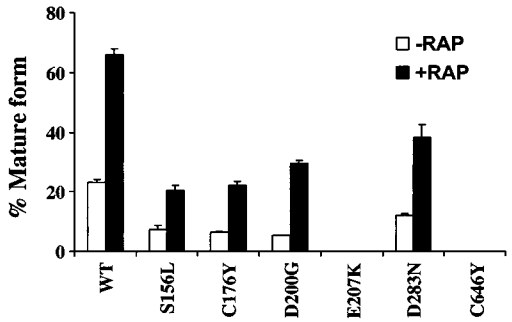

FIGURE 5: RAP increases maturation rates of LDLR class 2 mutants. Pulse−chase analyses of folding and maturation of the wild-type LDLR and its class 2 mutants were carried out as described in the Figure 4 legend. The percentages of mature forms observed in reducing gels after 60 min of chase for each receptor form, in the absence or presence of RAP coexpression, are plotted. Values are the average of three experiments with the SE indicated by error bars.

and coexpression of RAP, the percentages of the labeled mutants S156L, C176Y, D200G, and D283N that had been processed into the mature form were 20%, 22%, 29%, and 38%, respectively (Figure 5). When samples from identical experiments were analyzed under nonreducing conditions, it was noted that in the presence of RAP coexpression, the amounts of the LDLR "aggregates" were decreased (Figure 4). No effect was observed for RAP in folding and maturation of mutants E207K and C646Y (Figure 4). Taken together, these results clearly demonstrate that RAP can prevent the formation of nonproductive intermolecular disulfide bonds during folding, and thus promote the proper folding and maturation of some of the class 2 LDLR mutants.

Because RAP coexpression increased the rate of maturation of the LDLR and several of its mutants, we reasoned that coexpression of RAP might also influence the steady-state levels of these receptors. U87 cells were transiently transfected with cDNAs for the LDLR or its mutants with cotransfection of either pcDNA3 (−RAP) or pcDNA-RAP (+RAP). The steady-state levels of the receptors were analyzed by Western blotting with the anti-HA antibody (Figure 6). As seen in the figure, three forms of the receptors, i.e., ER form, mature form, and receptor "aggregates", were seen for the wild-type LDLR and mutants S156L, C176Y, D200G, and D283N under nonreducing conditions (Figure 6). The amounts of these "aggregates" were variably decreased in the presence of RAP coexpression. Again, the receptor "aggregates" were reduced to monomeric receptor species under reducing conditions. No mature form was observed for mutants E207K and C646Y. In the presence of RAP coexpression, the amounts of the mature forms of the LDLR and mutant S156L, C176Y, D200G, and D283N were increased as observed under reducing conditions (Figure 6). Thus, coexpression of RAP promotes the folding and increases the steady-state levels of mature receptor.

Having established that RAP coexpression increases the amount of mature receptor at steady-state, we investigated effect of RAP coexpression on the cellular physiology of LDLR function, i.e., the uptake and degradation of LDL. To eliminate the influence of endogenous LDLR on ligand degradation, we used LDLR-deficient *ldl*A7 cells to analyze receptor-mediated ^{125}I-LDL degradation via the wild-type LDLR and the D283N mutant. Similar to that seen in U87 cells, in the presence of RAP coexpression, the amounts of mature forms of LDLR and D283N mutant were markedly increased in *ldl*A7 cells (data not shown). *ldl*A7 cells transfected with LDLR cDNA exhibited ^{125}I-LDL degradation at a level of 318 fmol/mg of cell protein after 4 h incubation at 37 °C, whereas *ldl*A7 cells transfected with the pcDNA3 vector alone exhibited little if any LDL degradation (Figure 7). *ldl*A7 cells transfected with LDLR mutant D283N exhibited a reduced level of ^{125}I-LDL degradation (23% when compared to that of the wild-type

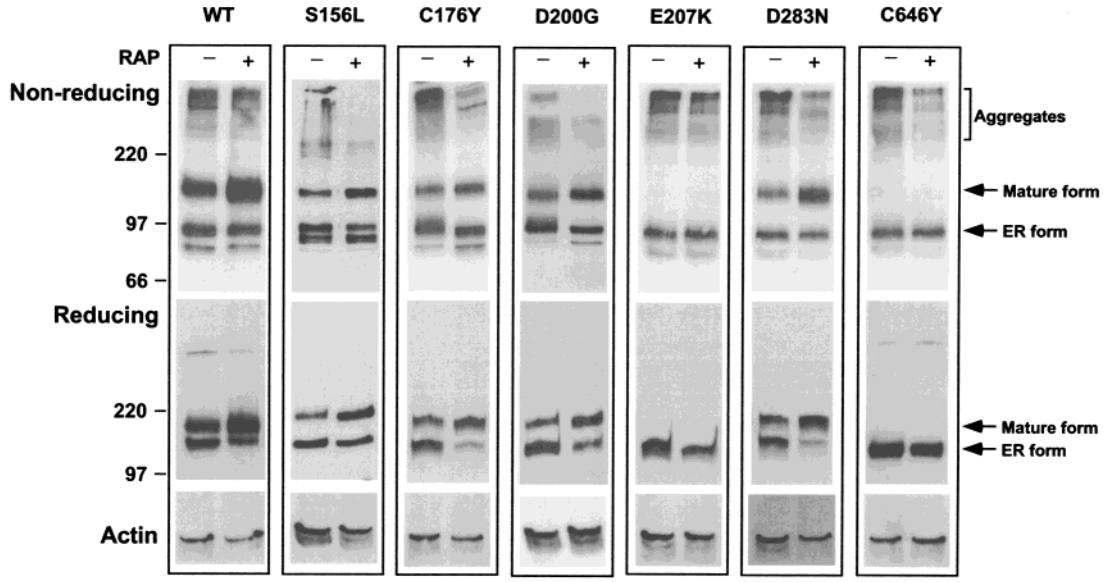

FIGURE 6: Effects of RAP coexpression on the steady-state levels of the wild-type LDLR and its class 2 mutants. U87 cells were transiently transfected with cDNAs for the wild-type LDLR or its mutants with cotransfection of either pcDNA3 (−RAP) or cDNA for RAP (+RAP). Two days following transfection, the steady-state levels of the receptors were analyzed by Western blotting with the anti-HA antibody under either nonreducing or reducing conditions. The LDLR "aggregates" migrated in the separating gel are marked. The ER and mature forms of the LDLR and the receptor "aggregates" are indicated. Lower panel: the same amounts of lysates were analyzed by Western blotting with the anti-actin antibody under reducing conditions, indicating that the same amounts of protein were loaded under various conditions for each receptor.

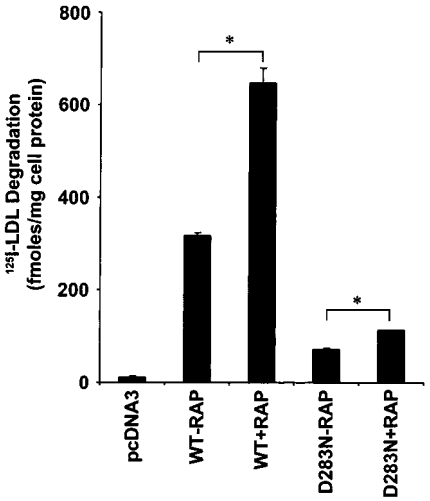

FIGURE 7: Effects of RAP coexpression on LDLR function. *ldl*A7 cells were transiently transfected with cDNAs for the wild-type LDLR or its mutant D283N, with the cotransfection of either vector pcDNA3 (−RAP) or vector pcDNA-RAP (+RAP). *ldl*A7 cells transfected with vector pcDNA3 alone serve as the background control. Cells were incubated with DMEM containing 6 mg/mL fatty acid-free bovine serum albumin and 4 μg/mL ^{125}I-LDL. After incubation at 37 °C for 4 h, the media were harvested, and specific LDL degradation was measured. (*) $P < 0.01$ when degradation was compared in the presence or absence of RAP coexpression.

LDLR; see Figure 7). However, ^{125}I-LDL degradation by LDLR and the D283N mutant was significantly increased 2.0-fold and 1.6-fold, respectively, in the presence of RAP coexpression (Figure 7).

DISCUSSION

Protein folding within the ER is promoted by molecular chaperones, which minimize irreversible aggregation and increase the efficiency of folding. At present, the specific factors and molecular mechanisms responsible for LDLR folding and maturation are largely unclear. Jorgensen et al. have shown that the general ER chaperone BiP binds LDLR and is involved in the ER-retention of misfolded LDLR mutants (*34*). More recently, Garcia et al. demonstrated that mutations in a putative LDLR adaptor protein are responsible for defective LDLR activity, and cause an autosomal recessive form of hypercholesterolemia (*35*). Our current study demonstrates that a specialized ER chaperone, RAP, promotes LDLR folding and maturation. Using anti-RAP antibody and co-immunoprecipitation, we demonstrated that RAP is associated with the LDLR in vivo under conditions of receptor and RAP overexpression. The analyses of LDLR folding and maturation in the absence or presence of RAP coexpression showed that RAP promotes the receptor folding and maturation by preventing the formation of nonproductive intermolecular disulfide bonds and aggregation of LDLR and several of its class 2 mutants.

RAP is one of the "specialized" ER chaperones that has been described and characterized within the past few years (*9*). Unlike the general ER chaperones (e.g., BiP and calnexin), which bind protein substrates nonselectively, RAP binds only members of the LDLR family (*9, 36*). It has been demonstrated previously that RAP promotes proper folding and trafficking of LRP and the VLDLR by preventing the formation of intermolecular disulfide bonds and premature ligand binding within the early secretory pathway (*11, 12, 14*−*16*). Because RAP binds to LDLR at the cell surface with lower affinity when compared to LRP and the VLDLR (*18*−*21*), it was not clear whether RAP plays a similar role in the folding of LDLR as it does for LRP and the VLDLR. Results herein indicate that when coexpressed with the LDLR, RAP can promote LDLR folding and maturation. This role for RAP in LDLR folding may be important under certain physiological and pathophysiological conditions. In RAP-deficient mice, the level of LDLR expression is downregulated in brain (*22*), but is normal in liver (*14*), suggesting that RAP may play differential roles in the folding and biogenesis of the LDLR in different cells and tissues. It is possible that other LDLR chaperones such as BiP, or yet unidentified chaperones, are able to compensate for RAP in hepatic LDLR folding in homozygous RAP-deficient mice. Generally in mammalian cells, if one chaperone fails to interact with an incompletely folded protein, another chaperone likely will (*37*).

Calcium-binding motifs play an important structural role in ligand-binding repeats of LDLR (*27*−*32*). Examination of the crystal structure of a ligand-binding repeat from the LDLR reveals that each repeat contains a single Ca^{2+} ion trapped in an octahedral cage formed primarily by four conserved acidic residues (*28*). Interactions between the Ca^{2+} ion and these acidic residues appear to be important for stabilizing and maintenance of the receptor in its native conformation. In the current study, we demonstrated that Ca^{2+} ion is required for proper folding of the LDLR, likely by serving as a "nucleation" center for each ligand-binding repeat during receptor folding. We also showed that coexpression of RAP can partially correct this misfolding event, further strengthening our conclusion that RAP is a chaperone for LDLR.

Our results suggest that RAP functions as a chaperone to promote correct folding of the LDLR, and that calcium may function independently during receptor folding by stabilizing the calcium-binding conformation and allowing correct disulfide formation (*33*). Alternatively, it is possible that both RAP and calcium preferentially bind to LDLR ligand-binding domains once the receptor has folded correctly. By doing so, they could shift the equilibrium between the unfolded/misfloded form and the correctly folded form toward the correctly folded form, and at the same time lower the concentration of species on which disulfide isomerases would have to act to bring about correct folding. In this regard, it is significant that, with only endogenous RAP expressed, RAP is associated exclusively with the higher affinity, but related, ligand LRP (Figure 1). Despite the failure of RAP to associate with LDLR under these conditions, there is considerable processing of LDLR to the mature, correctly folded form (Figure 1). These results also suggest that RAP is not critical for LDLR folding under physiological conditions in some types of cells. However, when RAP is overexpressed at nonphysiological concentrations, there is sufficient RAP to bind to LDLR (Figure 1), and to promote the folding of the receptor. Thus, by concurrent overexpression of RAP, it is possible in some cases to rescue partial failure of native and mutant LDLRs to mature form. This may have application for FH patients in the future as our results suggest that the defects caused by certain class 2 mutations can be at least partially overcome by the regulation

of RAP expression. Thus, future studies on RAP regulation may provide clues for the development of therapeutic strategies for some FH patients.

Approximately 50% of the characterized mutations in the LDLR gene lead to mutant proteins partially or totally retained in the ER (*1*, *2*). Among the six naturally occurring LDLR class 2 mutants examined, we found that RAP promotes the folding and processing of four of these mutants. In addition, the amount of receptor "aggregates" of these mutants was significantly decreased in the presence of RAP coexpression. Thus, these results confirm an important role for RAP in the LDLR folding. However, we cannot exclude the possibility that the "mature" glycosylated forms of the LDLR mutants may still have intramolecular scrambled disulfide bonds within the module in which the mutation lies. It has been demonstrated that point mutations of key residues within the LDLR ligand-binding repeats have essentially the same effects on ligand binding as deletion of the module in which the mutations were introduced (*38*, *39*). In the current study, we demonstrated that cellular ^{125}I-LDL degradation via the LDLR and its mutant was significantly increased in the presence of RAP coexpression. There are two possible interpretations of RAP effect on ^{125}I-LDL degradation by the D283N mutant receptor shown in Figure 7. The first possibility is that, although coexpression of RAP prevents aggregation of the LDLR mutant by suppressing formation of intermolecular disulfide bonds, the module in which D283N lies may still retain some scrambled disulfide bonds, and as a results the D283N mutant still binds LDL poorly. Alternatively, coexpression of RAP may permit formation of native disulfide bonds within the repeat containing D283N, but the mutant receptor is intrinsically crippled in LDL binding even when the module is folded properly.

Among the class 2 mutants examined herein were D200G and E207K. The crystal structure of LDLR repeat 5 indicates that both the D200 and E207 residues are involved in Ca^{2+} binding (*28*). The D200G mutation represents the loss of an acid residue for the formation of the Ca^{2+} cage, whereas the E207K mutation generates an opposite charge when compared to the native structure. Thus, the D200G mutation may result in reduced affinity of repeat 5 for the Ca^{2+} ion and a corresponding defect in receptor folding. On the other hand, the E207K mutation may result in total disruption of the Ca^{2+} cage, and hence inability for the receptor to fold properly, even in the presence of RAP coexpression. Future studies directed at examination of the folding of other LDLR mutants may provide further insights into the role of both cis-elements (i.e., LDLR residues) as well as trans-elements (i.e., molecular chaperones) in LDLR folding and regulation.

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