

Degradation of the LDL receptor class 2 mutants is mediated by a proteasome-dependent pathway

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Abstract Familial hypercholesterolemia is a genetic disorder that results from various gene mutations, primarily within the LDL receptor (LDLR). Approximately 50% of the LDLR mutations are defined as class 2 mutations, with the mutant proteins partially or entirely retained in the endoplasmic reticulum. To determine the degradation pathway of the LDLR class 2 mutants, we examined the effects of inhibition of several potential pathways on the levels of the wild-type LDLR and its four representative class 2 mutants (S156L, C176Y, E207K, and C646Y) stably expressed in Chinese hamster ovary (CHO) cells. We found that proteasome inhibitors MG132 and lactacystin blocked the degradation of the LDLR mutants, but not that of the wild-type LDLR. Treatment of CHO cells with these proteasome inhibitors led to a significant accumulation of the mutants at steady state. Furthermore, cell surface levels of the LDLR mutants were significantly increased upon inhibition of the proteasome degradation pathway. In contrast to the proteasome inhibitors, inhibitors of trypsin-like proteases, chymotrypsin-like proteases, and lysosomal pathway inhibitors did not affect the levels of the LDLR mutants. Taken together, these data demonstrate that the proteasome is the principal degradation pathway for LDLR class 2 mutants.—Li, Y., W. Lu, A. L. Schwartz, and G. Bu. **Degradation of the LDL receptor class 2 mutants is mediated by a proteasome-dependent pathway.** *J. Lipid Res.* 2004. 45: 1084–1091.

Supplementary key words familial hypercholesterolemia • mutation • degradation

Familial hypercholesterolemia (FH) is a common genetic disorder with an estimated frequency of about 1 in 500. The primary defects in FH patients are mutations in the gene encoding the LDL receptor (LDLR) (1, 2). 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. Loss of LDLR function leads to decreased LDL catabolism and elevated levels of plasma

LDL and cholesterol. At present, more than 600 different mutations in the *LDLR* gene have been identified. Among them, ~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).

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 (3–5). In previous studies, we identified receptor-associated protein (RAP) as a specialized chaperone for members of the LDLR family; RAP functions in the folding and trafficking of these receptors along the early secretory pathway (6). In the case of the LDLR, we have shown that RAP is associated with the LDLR in vivo and plays an important role in the folding and maturation of the LDLR and several of its class 2 mutants (7).

Prolonged retention of misfolded and incompletely folded proteins in the ER leads to their degradation. Recent evidence indicates that misfolded, damaged, and unassembled proteins are retrotranslocated from the ER lumen to the cytoplasm and thereafter degraded via the proteasomal degradation pathway (8–10). Indeed, increasing numbers of misfolded or unassembled yeast and eukaryotic integral membrane and secreted proteins have been shown to be substrates for the proteasomal degradation (8).

Although the biogenesis and endocytic trafficking of the LDLR are well understood, little is known about the mechanism(s) governing the turnover of its mutants. To examine whether the degradation of LDLR class 2 mutants is regulated via the proteasomal degradation pathway, we analyzed the effects of proteasome inhibitors on the cellular turnover of LDLR mutants. Our data demonstrate that proteasomal degradation is the principal pathway for LDLR mutant degradation.

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; LDLR, LDL receptor; LRP, LDL receptor-related protein; RAP, receptor-associated protein; SREBP, sterol-regulatory element binding protein.

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EXPERIMENTAL PROCEDURES

Materials

MG132 (Z-Leu-leu-leu-H aldehyde) was from Peptide Institute (Minosh-shi, Osaka, Japan). Lactacystin, leupeptin, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester loxistatin, and pepstatin A were obtained from Calbiochem-Novabiochem (La Jolla, CA). Endoglycosidase H (endo H) was from Boehringer Mannheim (Germany). Chloroquine, NH_4Cl , *N*-*p*-tosyl-L-lysine chloromethyl ketone, and *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone were from Sigma (St. Louis, MO). Mouse anti-hemagglutinin (anti-HA) antibody was from Babco (12CA5; Richmond, CA). FITC-conjugated goat anti-mouse antibody was from BD Biosciences-PharMingen (San Diego, CA). Peroxidase-labeled anti-mouse antibody and ECL system were from Amersham Life Science. All tissue culture media, serum, and plasticware were from Life Technologies. Immobilon-P transfer membrane was from Millipore. Protein A-agarose beads were from Repligen (Cambridge, MA). Rainbow molecular weight markers were from Bio-Rad. Proteinase inhibitor cocktail Complete™ was from Boehringer Mannheim. [^{35}S]cysteine was obtained from ICN Pharmaceuticals.

Constructs, cell culture, and transfection

The construction of full-length human wild-type LDLR and its mutants S156L, C176Y, E207K, and C646Y with an HA epitope near the N terminus in pcDNA3 vector has been described previously (7). LDL receptor-related protein (LRP)-null Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum. Stable transfection into CHO cells was achieved by transfection of 30 μg plasmid DNA in 10 cm dishes. Stable transfectants were selected using 700 $\mu\text{g}/\text{ml}$ G418 and maintained with 400 $\mu\text{g}/\text{ml}$ G418.

Metabolic pulse-chase labeling and immunoprecipitation

Metabolic labeling with [^{35}S]cysteine was performed essentially as described previously (7). For pulse-chase experiments, cells were generally pulse labeled for 30 min with 200 $\mu\text{Ci}/\text{ml}$ [^{35}S]cysteine in cystine-free medium and chased with serum-containing medium for 0 to 22 h. Cells were lysed with 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Immunoprecipitation was carried out essentially as described previously (7).

Western blot analysis

CHO cells stably transfected with wild-type LDLR or its mutants were seeded at 5×10^5 cells per well in 6-well plates and cultured overnight before experiments. Cells were incubated in the presence or absence of various proteasome inhibitors or lysosome inhibitors at 37°C as indicated in each experiment. Cell monolayers were then washed twice in prechilled phosphate-buffered saline and lysed with 0.5 ml of lysis buffer. Equal quantities of protein were subjected to SDS-PAGE (6%) under reducing or nonreducing conditions. After transfer to Immobilon-P transfer 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.

Endo H digestion

CHO cells stably transfected with wild-type LDLR or its mutants were seeded at 5×10^5 cells per well in 6-well plates and cultured overnight before experiments. Cells were lysed with 0.5 ml of lysis buffer, and lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitates were eluted from protein A

beads by boiling in 100 nM sodium acetate, pH 5.5, containing 0.4% SDS. Each elution was divided into two equal parts. The control portion was combined with an equal volume of 100 nM sodium acetate, pH 5.5, without endo H. The experimental portion was added to an equal volume of the same buffer containing 1 mU of endo H. The digestion was carried out at 37°C for 16 h before the samples were analyzed via Western blot.

Flow cytometric analysis of cell surface LDLR

Cell surface LDLR measurement was carried out using the method described previously (11). Briefly, CHO cells stably transfected with wild-type LDLR or its mutants were seeded at 1.0×10^6 cells per T25 flask and cultured overnight before experiments. Cells were washed and incubated with or without proteasome inhibitors for 8 h. Cells were then detached by incubation with nonenzymatic cell dissociation solution (Sigma). Successive incubations with affinity-purified anti-HA IgG (25 $\mu\text{g}/\text{ml}$) and goat anti-mouse Ig-FITC were carried out at 4°C for 45 min each. Background fluorescence intensity was assessed in the absence of primary antibody and subtracted from all samples. Mean fluorescence values were obtained from at least triplicate analysis on a FACScalibur (BD Biosciences-PharMingen), and data were analyzed with Cell Quest software.

RESULTS

Stable expression of wild-type LDLR and its mutants in CHO cells

To investigate the degradation pathway for LDLR and its mutants, we stably transfected wild-type LDLR and four of its class 2 mutants, S156L, C176Y, E207K, and C646Y, into CHO cells. The four LDLR class 2 mutants we selected are naturally occurring mutations found in FH patients (1, 2). The S156L mutation occurs within ligand binding repeat 4 of the LDLR, C176Y and E207K within ligand binding repeat 5, and C646Y within epidermal growth factor precursor repeat 3. Following stable transfection, the steady-state levels of the receptor in transfected cells were analyzed by Western blotting with anti-HA antibody (Fig. 1A). (All LDLR constructs were tagged with an HA epitope near their carboxyl termini.) As seen in the figure, the wild-type LDLR and its mutants exhibit distinct expression patterns. The lower bands migrating at ~ 120 kDa represent the full-length ER precursor form that lacks complex sugar modification, whereas the upper bands migrating at ~ 160 kDa represent the mature form of the receptor that has been correctly folded and glycosylated within the *trans*-Golgi network. For the wild-type LDLR, nearly all the receptor at steady state is in the mature form, reflecting efficient ER folding and the relatively long half-life of the mature receptor. For mutants S156L and C176Y, both the ER precursor forms and the mature forms are seen. However, for mutants E207K and C646Y, only the ER precursor forms are detected (Fig. 1A). To confirm the nature of the ER precursor and mature forms, we examined the susceptibility of the wild-type LDLR and its mutants to endo H. Figure 1B shows that all the ER precursor forms of the receptors were sensitive to endo H (faster migration upon endo H treatment), while the mature forms were resistant to endo H (data not

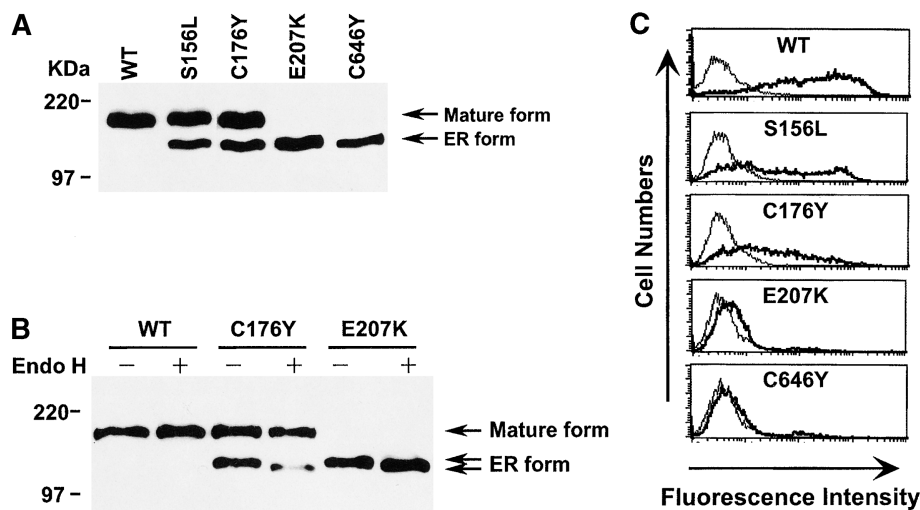


Fig. 1. LDL receptor (LDLR) mutants exhibit partial or complete endoplasmic reticulum (ER) retention. **A:** Western blotting analysis of the wild-type LDLR and its mutants stably transfected in Chinese hamster ovary (CHO) cells. Aliquots of cell lysates from CHO cells stably transfected with wild-type LDLR or its mutants were separated via 6% SDS-PAGE under reducing conditions and Western blotted with anti-hemagglutinin (anti-HA) antibody. The ER and mature forms of the LDLR and its mutants are indicated. **B:** Endoglycosidase H (endo H) susceptibility of the wild-type LDLR and its mutants. Wild-type LDLR or its mutants C176Y and E207K stably transfected in CHO cells was immunoprecipitated from cell lysates using anti-HA antibody. The immunoprecipitates were subjected to digestion without or with endo H, followed by Western blotting with anti-HA antibody. Note, the ER forms of mutants C176Y and E207K migrated faster following endo H digestion. **C:** Flow cytometric analysis of CHO cells stably transfected with the wild-type LDLR and its mutants. The negative controls without the primary antibody are indicated by light lines, whereas the signals from receptor staining are shown in dark lines.

shown for the mutants S156L and C646Y). The endo H-sensitive LDLR mutants represent those proteins within the ER, whereas the endo H-resistant LDLR and its mutants must have exited the ER and trafficked beyond the *medial*-Golgi compartments (12). Examination of cell surface receptor expression by flow cytometric analysis revealed very low levels of cell surface antibody staining for mutants E207K and C646Y, moderate levels of cell surface antibody staining for mutants S156L and C176Y, and a high level of cell surface antibody staining for the wild-type LDLR (Fig. 1C). These results are consistent with partial (mutants S156L and C176Y) and complete (mutants E207K and C646Y) ER retention of misfolded LDLR mutants.

Trafficking and turnover of the wild-type LDLR and its class 2 mutants

To investigate the trafficking and turnover of the wild-type LDLR and its mutants, we performed metabolic pulse-chase analyses. CHO cells stably transfected with wild-type LDLR and its mutants were metabolically pulse labeled with [³⁵S]cysteine for 30 min and chased for 0, 0.5, 1, 2, 5, 10, or 22 h. After each chase period, cells were lysed and quantitatively immunoprecipitated with anti-HA antibody and analyzed via SDS-PAGE under reducing conditions. As seen in Fig. 2, wild-type LDLR exhibited efficient maturation, with the majority of the receptor converted to the mature form after a 1–2 h chase. Mutants S156L and C176Y exhibited impaired receptor maturation, and mutants E207K and C646Y exhibited no recep-

tor maturation. For the S156L and C176Y mutants, after 1 h of chase, only 11–12% of the receptors were converted to the mature form. No conversion was seen for the E207K or C646Y mutants (Fig. 2). In contrast, ~75% of the wild-type LDLR had converted to the mature form after 1 h of chase (Fig. 2). The absence of the mature form

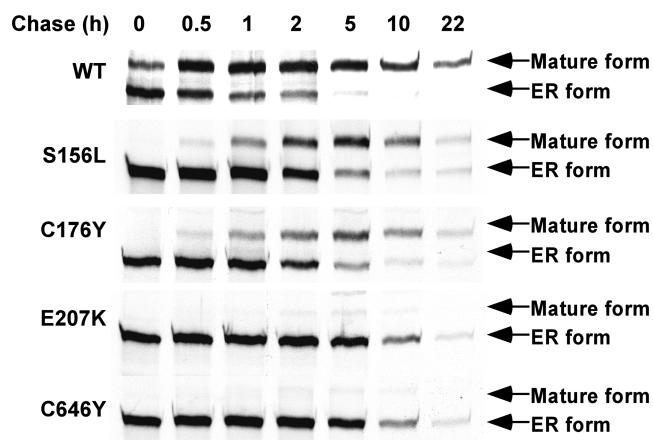


Fig. 2. Cellular trafficking and turnover of wild-type LDLR and its mutants. CHO cells stably transfected with wild-type LDLR or its mutants were metabolically pulse-labeled with [³⁵S]cysteine for 30 min and chased for the indicated times. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by 6% SDS-PAGE under reducing conditions. The ER and mature forms of the LDLR and its mutants are indicated. These data are representative of two such experiments performed.

for the E207K and C646Y mutants suggests that the effects of these mutations on receptor folding and trafficking are more severe than those of mutants S156L and C176Y. From these pulse chase analyses, although less conversion to the mature form was seen for each of the mutants when compared with the wild-type LDLR, it was apparent that their ER forms disappeared readily during the chase period. As a result, the overall half-lives of the wild-type LDLR and its mutants were shown to be similar (~ 6 h for the wild-type LDLR and mutants E207K and C646Y, and ~ 4.5 h for mutants S156L and C176Y).

LDLR class 2 mutants, but not the wild-type LDLR, are targets for the proteasomal degradation pathway

We next investigated the degradation pathway for the wild-type LDLR and its four class 2 mutants. We examined the effects of two highly selective proteasome inhibitors, MG132 and lactacystin, on receptor levels in these stably transfected CHO cell lines. Cells were incubated with proteasome inhibitors or DMSO (as vehicle control) for 8 h, and the levels of wild-type LDLR and its mutants were analyzed by Western blotting with anti-HA antibody under both reducing and nonreducing conditions. As seen in Fig. 3, MG132 and lactacystin blocked the degradation of the LDLR mutants, but not that of the wild-type LDLR. Treatment of CHO cells with these proteasome inhibitors led to a significant accumulation of the mutants at steady state (Fig. 3). For mutant S156L, proteasome inhibitor treatment resulted in a significant accumulation of its ER form, and a slight increase of its mature form. For mutant C176Y, proteasome inhibitor treatment resulted in a significant accumulation of both the ER form and the mature form. For mutants E207K and C646Y, proteasome inhibitor treatment resulted in significant accumulation of

their ER forms. Furthermore, when analyzed under non-reducing conditions, significant accumulation of ER “aggregates” was seen with mutants E207K and C646Y. These “aggregates” migrated at the top of the stacking and separating gels, and likely represent intermolecular disulfide bond-linked receptor molecules, because they were reduced to monomeric receptor species under reducing conditions.

To confirm the role of the proteasomal pathway in the degradation of the wild-type LDLR and its mutants, we studied the effect of proteasomal inhibitor MG132 on the receptor degradation in metabolic pulse-chase analysis. As seen in Fig. 4, MG132 blocked the degradation of the LDLR mutant E207K, but not that of the wild-type LDLR.

To further characterize human LDLR class 2 mutant degradation via the proteasomal pathway, we examined the kinetics of LDLR mutant accumulation upon proteasome inhibitor treatment. As seen in Fig. 5, incubation of CHO cells stably transfected with LDLR mutants C176Y and E207K with 20 μ M MG132 for various times (4–8 h) resulted in a time-dependent accumulation of these LDLR mutants. Increased levels were detected as early as 4 h after MG132 addition, and continued over the time course tested.

Inhibitors of nonproteasome pathways do not block human LDLR mutant degradation

The overwhelming majority of intracellular protein degradation in mammalian cells is via the proteasomes and the lysosomes. The latter are acidic vesicular compartments in which numerous proteases and glycosidases are localized (13). To investigate a potential role of other cellular proteolytic pathways in LDLR class 2 mutant degradation, the effect of various protease inhibitors was exam-

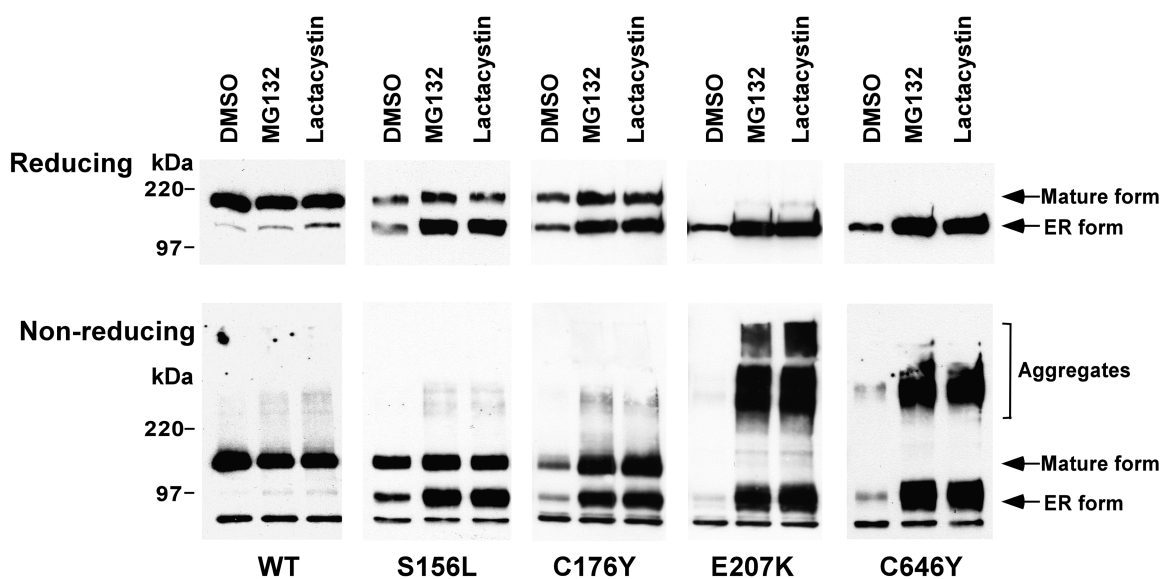


Fig. 3. The proteasome inhibitors MG132 and lactacystin block human LDLR class 2 mutant degradation. CHO cells stably transfected with wild-type LDLR or its mutants were treated with either vehicle DMSO alone, MG132 (20 μ M), or lactacystin (10 μ M) for 8 h. Identical aliquots of cell lysates were separated via 6% SDS-PAGE under reducing or nonreducing conditions and Western blotted with anti-HA antibody. The ER and mature forms of the LDLR and its mutants are indicated. These data are representative of several such experiments performed.

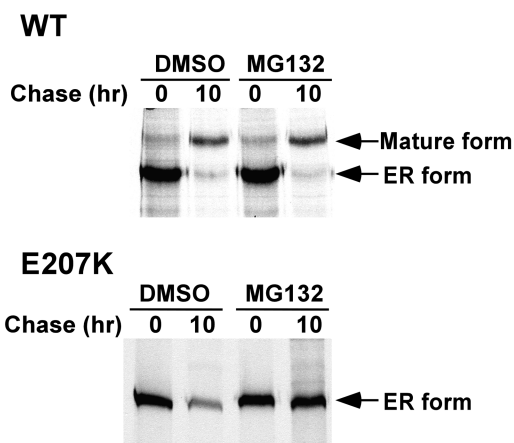


Fig. 4. MG132 blocks the ER degradation of the LDLR mutant E207K in a pulse-chase analysis. CHO cells stably transfected with the wild-type LDLR and its mutant E207K were pulse labeled with [³⁵S]cysteine for 30 min and chased for indicated times in the absence or presence of MG132 (5 μ M). Cell lysates were then immunoprecipitated with anti-HA antibody.

ined. Stably transfected CHO cells were incubated in the absence or presence of various inhibitors for 8 h, and the levels of the LDLR mutants were detected by Western blotting with anti-HA antibody. Incubation of cells with the broad-spectrum lysosomal acidification inhibitors chloroquine (150 μ M) and NH₄Cl (10 mM) did not alter the levels of the LDLR mutants (Fig. 6), whereas incubation with MG132 resulted in a significant accumulation of the LDLR class 2 mutants C176Y and E207K. Similarly, incubation of cells with a mixture of lysosomal protease inhibitors, including (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester loxistatin (EST, 100 μ M), leupeptin (50 μ M), and the aspartate protease inhibitor pepstatin-A (50 μ M) did not alter mutant receptor levels

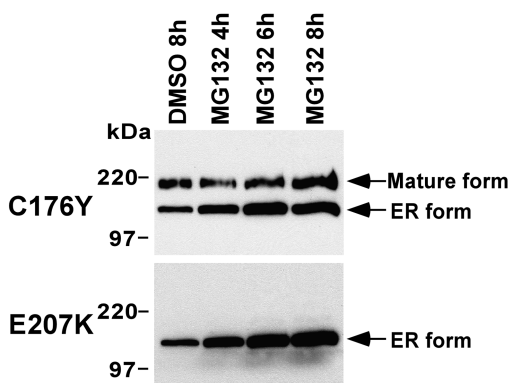


Fig. 5. Time course for human LDLR class 2 mutant accumulation upon MG132 treatment. CHO cells stably transfected with LDLR mutants C176Y or E207K were treated with either vehicle DMSO alone or MG132 (20 μ M) for 4, 6, or 8 h. Identical aliquots of cell lysates were separated via 6% SDS-PAGE under reducing conditions and Western blotted with anti-HA antibody. The ER and mature forms of the LDLR mutants are indicated. These data are representative of three such experiments performed.

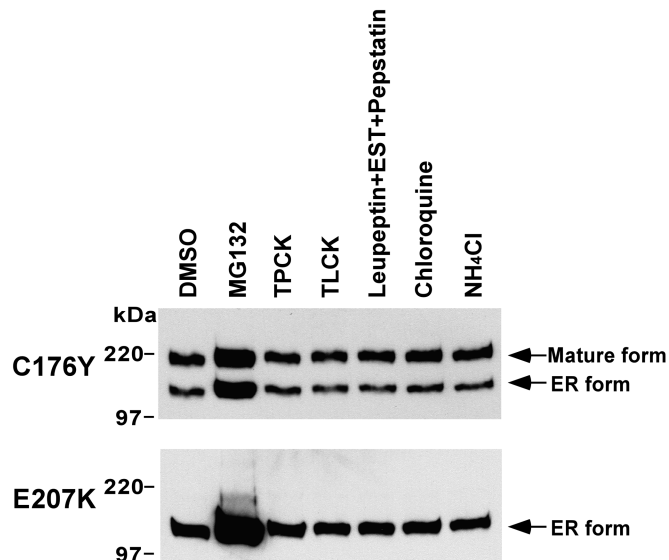


Fig. 6. Inhibitors specific for trypsin-like proteases, chymotrypsin-like proteases, or lysosomal pathway enzymes do not block LDLR class 2 mutant degradation. CHO cells stably transfected with LDLR mutants C176Y or E207K were incubated for 8 h in the presence of vehicle DMSO only, 20 μ M MG132, 100 μ M *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 100 μ M *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), the lysosome protease inhibitors leupeptin (50 μ M), pepstatin-A (50 μ M), and (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester loxistatin (EST, 100 μ M), 150 μ M chloroquine, or 10 mM NH₄Cl. Identical aliquots of cell lysates were separated via 6% SDS-PAGE under reducing conditions and Western blotted with anti-HA antibody. The ER and mature forms of the LDLR mutants are indicated. These data are representative of two such experiments performed.

(Fig. 6). In addition, incubation with the trypsin-like protease inhibitor (*N*-*p*-tosyl-L-lysine chloromethyl ketone, 100 μ M), or the chymotrypsin-like protease inhibitor (*N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, 100 μ M) also failed to increase the levels of mutants C176Y and E207K (Fig. 6). Thus, these studies suggest that the proteasomal pathway plays the primary role in human LDLR class 2 mutant degradation.

Treatment of cells with proteasome inhibitors increases cell surface levels of LDLR mutants

To determine whether class 2 mutants are able to reach the cell surface, we compared the cell surface levels of the LDLR mutants via flow cytometric analysis in the absence or presence of proteasome inhibitors. As seen in Fig. 7, for each of the four LDLR class 2 mutants, we consistently observed increases of cell surface LDLR levels upon treatment with MG132 or lactacystin. Specifically, CHO cells expressing LDLR mutants S156L and C176Y exhibited moderate levels of LDLR cell surface staining in the presence of DMSO alone. Treatment of these cells with proteasome inhibitors for 8 h resulted in \sim 1.5- and 3.1-fold increase in cell surface levels of the S156L mutant and the C176Y mutant, respectively (Fig. 7B). Unlike mutants S156L and C176Y, mutants E207K and C646Y display no mature forms (Fig. 1A), and exhibited very low levels of

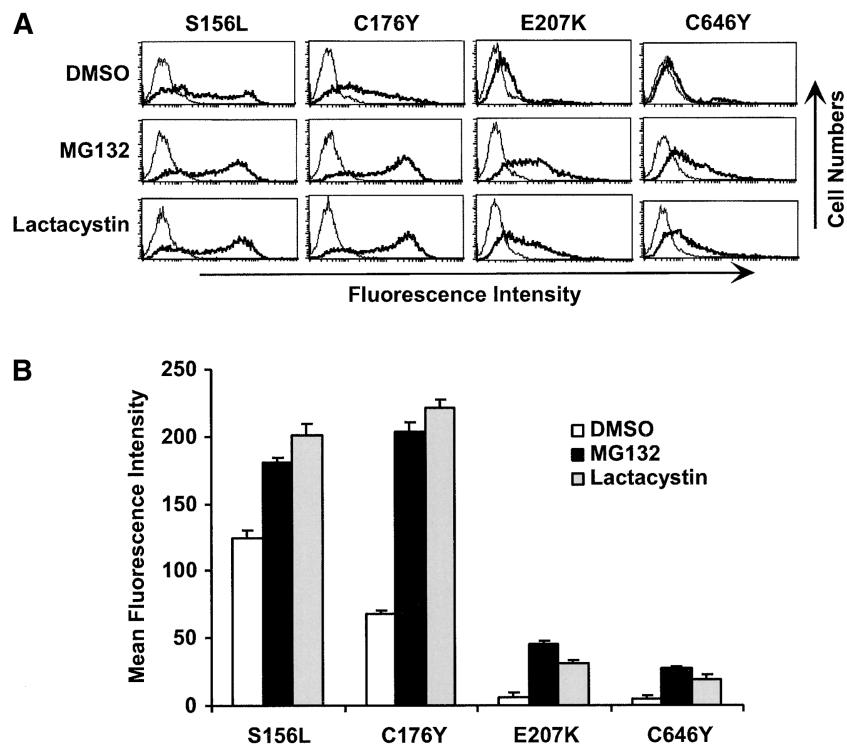


Fig. 7. The proteasome inhibitors increase cell surface levels of LDLR mutants. A: Histograms of cytofluorimetric analysis of LDLR mutants in CHO cells. CHO cells stably transfected with LDLR mutants S156L, C176Y, E207K, or C646Y were treated with either vehicle DMSO alone, MG132 (20 μ M), or lactacystin (10 μ M) for 8 h. Cells were then dissociated, labeled with anti-HA antibody, and detected with goat anti-mouse IgFITC. Background fluorescence intensity was assessed in the absence of primary monoclonal antibody (thin lines). B: Cell surface levels of LDLR mutants were expressed as mean fluorescence intensities. Each value represents the difference between total and background fluorescence intensities and is the average of triple determinations. Error bars indicate SD. This experiment is representative of several such experiments performed.

cell surface staining when cells expressing these mutants were incubated with DMSO alone. However, treatment of cells expressing the E207K and C646Y mutants with proteasome inhibitors also resulted in significant increases in levels of these two mutants at the cell surface (Fig. 7A). For the E207K mutant, the level of cell surface receptor was increased by 7.0- and 4.8-fold when the cells were treated with MG132 and lactacystin, respectively. Similarly, the level of cell surface C646Y mutant was increased by 6.0- and 4.2-fold upon treatment with MG132 and lactacystin, respectively (Fig. 7B). Taken together, these results confirm the principal role for the proteasome in LDLR class 2 mutant degradation and demonstrate that these mutants are capable of trafficking to the cell surface upon inhibition of the ER degradation pathway.

DISCUSSION

A variety of quality control mechanisms operate in the ER and in the late secretory pathway to ensure the fidelity and regulation of protein expression at the posttranslational level. If proper maturation fails, the aberrant products are degraded (3, 5, 8–10). In the current study, we demonstrate that the proteasomal degradation pathway

plays a principal role in the LDLR class 2 mutant degradation.

For some proteins, even minor changes in the protein's primary structure can cause their retention within the ER, as exemplified in many human disorders such as cystic fibrosis, α 1-antitrypsin deficiency, and FH (14, 15). One of the best examples of proteasomal degradation of an ER protein is that of the cystic fibrosis transmembrane conductance regulator (CFTR), an integral membrane protein that facilitates chloride transport across the apical membrane of epithelial cells. The most prevalent mutation in patients with cystic fibrosis is the CFTR Δ F508 allele. This mutation interferes with the correct folding of the polytopic protein in the ER. Although functional if expressed at the cell surface, essentially all of the Δ F508 protein is retained in the ER and is thereafter rapidly degraded via the proteasomal degradation pathway (16, 17).

Approximately 50% of the characterized mutations in the *LDLR* gene of FH patients lead to partial or complete retention of mutant proteins in the ER (i.e., class 2 mutations) (1, 2). Among the four naturally occurring LDLR class 2 mutants examined in this study, we found that two are partially retained in the ER, while the other two are completely retained in the ER. Four lines of evidence from this study allow us to draw the conclusion that the

proteasomal system is the principal pathway for degradation of misfolded LDLR class 2 mutants. First, proteasome inhibitors, but not inhibitors of lysosomal or other proteolytic pathways, significantly increased steady-state levels of LDLR class 2 mutants. Second, the degradation of LDLR class 2 mutants, but not of the wild-type LDLR, was inhibited by the proteasome inhibitors. Third, the proteasome inhibitors blocked LDLR mutant degradation in a time-dependent manner. Fourth, treatment of cells with proteasome inhibitors significantly increased the cell surface levels of the LDLR mutants.

The proteasomal degradation system also plays an important role in mediating both receptor endocytosis and sorting to the degradation pathway for several cell surface receptors (18–21). The LRP is a large endocytic receptor that belongs to the emerging LDLR family. One unique feature of LRP is its rapid endocytosis (11, 22) when compared with other members of the LDLR family. In a previous study, we analyzed the effects of proteasome inhibitors on the endocytic trafficking and cellular turnover of LRP, and found that the delivery of LRP to the degradation pathway is blocked within a compartment from which recycling of the receptor still occurs (23). In the present study, we did not detect any increase of wild-type LDLR stably expressed in CHO cells upon treatment with the proteasome inhibitors MG132 and lactacystin. This result indicates that sorting of the wild-type LDLR to the degradation pathway is not significantly regulated by the proteasomal degradation pathway. However, the expression of endogenous LDLR is indirectly downregulated by this system. Sterol-regulatory element binding proteins (SREBPs) are involved in the transcriptional regulation of genes encoding the LDLR as well as key enzymes of cholesterol and triglyceride biosynthesis (24). In the nucleus, SREBPs are modified by polyubiquitin chains and rapidly degraded by the proteasome (24). In the presence of proteasome inhibitors, the stabilized nuclear SREBPs are capable of enhancing the expression of their responsive genes, such as *LDLR* (25, 26).

In the present study, we have used several alternative approaches to detect a potential ubiquitination of LDLR mutants, but have not obtained conclusive results (data not shown). At least two possibilities may account for this. First, an ancillary protein (e.g., a chaperone), which itself may be ubiquitinated and thus regulated by the proteasomal degradation pathway, may function as a regulatory protein for LDLR mutant turnover. Second, there may be proteasomal degradation pathway(s) that is independent of protein ubiquitination. Future studies are needed to address these possibilities.

The mechanism by which misfolded proteins are recognized by the proteasomal degradation pathway is still under intense investigation. Prevailing evidence suggests that many of the same molecular chaperones involved in protein folding in the ER are also involved in the removal of substrates via the proteasomal degradation (3). In this regard, Jorgensen et al. (27) have shown that the general ER chaperone BiP binds LDLR and is involved in the ER retention of misfolded LDLR mutants, suggesting that BiP

is a potential key regulator in the ER quality control process of the newly synthesized LDLR. In a previous study, we demonstrated that a specialized ER chaperone for members of the LDLR family, RAP, promotes folding and maturation of wild-type LDLR and its class 2 mutants (7). Whether this specialized chaperone is also involved in ER retention and/or the proteasomal degradation of the LDLR mutants requires further investigation.

In summary, using CHO cells stably expressing LDLR class 2 mutants, we determined that proteasomal degradation is the principal pathway for LDLR mutant degradation. Our results demonstrate that misfolded receptors, such as the LDLR, that contain extensive disulfide bonds can be transported to the proteasome-mediated degradation pathway. Future studies with fibroblasts derived from FH patients with LDLR class 2 mutations should allow us to fully understand the molecular and cellular mechanisms underlying the retention and degradation of these mutants in the ER. **■**

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