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The effect of bafilomycin A1 and protease inhibitors on the degradation and recycling of a Class 5-mutant LDLR

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The low-density lipoprotein receptor (LDLR) mediates cholesterol homeostasis through endocytosis of lipoprotein particles, particularly low-density lipoproteins (LDLs). Normally, the lipoprotein particles are released in the endosomes and the receptors recycle to the cell surface. Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by mutations in the gene encoding the LDLR. These mutations are divided into five functional classes where Class 5 mutations encode receptors that suffer from ligand-induced degradation and recycling deficiency. The aim of this study was to investigate whether it is possible to prevent the fast ligand-induced degradation of Class 5-mutant LDLR and to restore its ability to recycle to the cell surface. E387K is a naturally occurring Class 5 mutation found in FH patients, and in the present study, we used Chinese hamster ovary cells transfected with an E387K-mutant LDLR. Abrogation of endosomal acidification by adding bafilomycin A1 or addition of the irreversible serine protease inhibitors, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and 3,4-dichloroisocoumarin (DCI), prevented the degradation of the E387K-mutant LDLR. However, the undegraded receptor did not recycle to the cell surface in the presence of LDL. Unexpectedly, AEBSF caused aggregation of early endosome antigen-1-positive endosomes and the intracellular trapped LDLR co-localized with these aggregated early endosomes.

Keywords familial hypercholesterolemia; LDLR; Class 5; bafilomycin A1; AEBSF

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Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by mutations in the gene encoding the low-density lipoprotein receptor (LDLR), which lead to defective clearance of lipoproteins from the circulation [1]. The disease is characterized by hypercholesterolemia, tendon xanthomas, and premature coronary heart disease. FH is one of the most common genetic disorders with a prevalence of heterozygotes of about 1/500 in most western countries [2]. At present, more than 1000 different mutations in the *LDLR* locus have been found to cause FH [3].

The LDLR is synthesized by ribosomes bound to the endoplasmic reticulum (ER) and translocated into the ER lumen, where it is partially glycosylated and appears with a molecular mass of 120 kDa [4]. Within 30–45 min, the receptor is transported to the Golgi apparatus where the oligosaccharides are modified and the receptor's apparent molecular mass is increased to 160 kDa. The 160 kDa form is transported to the cell surface where it mediates the uptake of lipoprotein particles, mainly low-density lipoprotein (LDL), by receptor-mediated endocytosis. The internalized lipoprotein particle is released in the endosomes and the receptor is recycled back to the cell surface in a process called receptor recycling [5]. A typical LDLR molecule is estimated to recycle more than 100 times before being degraded [6,7].

The extracellular part of the LDLR is composed of a ligand-binding domain that mediates the binding of LDL and an epidermal growth factor (EGF) domain, which controls the pH-dependent release of LDL in the endosomes [8]. This domain consists of two EGF-like modules (EGF-A and -B), a six-bladed β-propeller where each blade consists of a four stranded β-sheet, and a third EGF-like module (EGF-C) [9]. The EGF domain is presumed to function as an alternative ligand that displaces LDL from the ligand-binding domain upon acidification of the sorting endosome lumen [8]. If efficient ligand uncoupling is prevented, the receptor is degraded.

Mutations causing recycling deficiency are classified as Class 5 mutations. All the currently described Class 5 mutations are located in the EGF-domain and are predicted to act by preventing the acid-dependent release of ligand in the endosomes. Several Class 5 mutations, including E387K, cluster in the N-terminal W6 blade of the β-propeller [9,10].

The LDLR mutation E387K was first identified by Hobbs *et al.* [10] and classified as a Class 5 mutation causing a severe phenotype. E387 is located in the β-propeller of the EGF domain within a region fully conserved between human, mouse, rat, and *Xenopus*. The LDLR activity in fibroblasts and Epstein–Barr virus transformed lymphoblasts from homozygous FH patients with E387K-mutant LDLR was found to be $<2\%$ [10] and 5–10% of normal activity [11], respectively.

It is not known which protease(s) is (are) responsible for the degradation of normal and Class 5-mutant LDLR or in which intracellular compartment the degradation takes place. The half-life of normal LDLR in fibroblasts was determined, by pulse-chase experiments, to be 12 h [12]. However, cell-dependent variation in LDLR turnover exists. In macrophages (THP-1 and J774 cell lines), the LDLR was found to be metabolically unstable, with half-life between 2 and 4 h [13,14]. The degradation was not affected by inhibitors of lysosomal proteases in any of the cell types [14,15]. There are few reports describing the degradation of Class 5-mutant LDLRs in cells isolated from FH patients. In fibroblasts, an accelerated degradation was found for V408M-mutant LDLR ($t_{1/2} =$ 1.5 h) [16], for D412H-mutant LDLR ($t_{1/2} < 1$ h) [17], and for an LDLR with a deletion corresponding to the first two EGF-like modules ($t_{1/2} = 2$ h) [18], all suggested to be Class 5 mutations [10].

The aim of this study was to reveal if it is possible to prevent the fast ligand-induced degradation of Class 5-mutant LDLR and to restore its ability to recycle to the cell surface. This could represent a therapeutic approach for FH patients with Class 5 mutations. We have used Chinese hamster ovary (CHO) cells stably transfected with an E387K-mutant LDLR and examined the effect of an acidotropic agent and protease inhibitors on the degradation and recycling of this Class 5-mutant LDLR.

Materials and Methods

Materials

Bafilomycin A1 was purchased from Merck (Darmstadt, Germany). Tetracycline, gentamicin, blasticidin, and zeocin were purchased from Invitrogen (Carlsbad, CA, USA). The protease inhibitors (**Table 1**) and cycloheximide (CHX) were purchased from Sigma (St Louis, MO, USA). LDL was isolated from freshly prepared plasma as described by Ranheim *et al.* [19].

Plasmid constructs

The pcDNA4-LDLR construct has been described earlier [20]. The construct pcDNA4-LDLR-E387K was generated from pcDNA4-LDLR by site-directed mutagenesis using a QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's

Table 1 Protease inhibitors tested for their effects on degradation of E387K-mutant LDLR

Inhibitor	Concentration[a]	Protease
4-(2-Aminoethyl)-benzenesulfonyl fluoride	0.25 mg/ml	Serine proteases [32]
Antipain-HCl	60 μg/ml	Serine proteases such as plasmin, thrombin, and trypsin. Cysteine proteases such as calpain and papain [42]
Benzamidine-HCl	600 μg/ml	Trypsin, trypsin-like serine proteases [43]
E-64	10 μM	Cysteine proteases [43]
Leupeptin	50 μg/ml	Trypsin-like serine proteases. Cysteine proteases such as calpain, cathepsin B, H, and L, and papain [42]
Pepstatin	1 μg/ml	Aspartic proteases such as renin, chymosin, and pepsin [42]
Phosphoramidon	10 μM	Metalloendoproteinases, thermolysin, and elastases [42]
Phenylmethylsulfonyl fluoride	1 mM	Serine proteases [44]
3,4-Dichloroisocoumarin	0.1 mM	Serine proteases [45]

[a]Concentration used in the experiment described in **Fig. 4**.

protocol. The oligonucleotide 5′-CTTCACCAACCGGC ACAAGGTCAGGAAGA-3′ (mutated nucleotide in bold) was used to change codon 387 from GAG, encoding glutamic acid, to AAG, encoding lysine. To enable tetracycline-induced expression, the LDLR cDNA was located downstream of a cytomegalovirus immediate-early promoter containing two tetracycline operator 2 elements. The integrity of the entire plasmid was confirmed by DNA sequencing.

Cell culture and transfection

T-Rex CHO cells (Invitrogen) were stably transfected with pcDNA4-LDLR or pcDNA4-LDLR-E387K as described previously [21]. Transfected T-Rex CHO cells were maintained in Ham's F-12 complete medium (Euroclone, Pero, Italy) supplemented with 10% (*V*/*V*) fetal bovine serum (Sigma Aldrich), 10 μg/ml gentamicin, 10 μg/ml blasticidin, and 100 μg/ml zeocin in a humidified atmosphere (37°C, 5% CO_2). LDLR expression was induced by adding 1 μg/ml tetracycline.

A cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany), measuring lactate dehydrogenase activity in the cell medium, was used to quantify the extent of cell death caused by different cell treatments. In all experiments described in this study, the cell death was $<5\%$.

Western blot analysis

Transfected T-Rex CHO cells were grown in Ham's F-12 supplemented with 10% lipoprotein deficient serum (LPDS) and 1 μg/ml tetracycline for 24 h prior to additional treatment of the cells. Cell lysates were prepared, protein concentration determined, and western blot analysis was performed as described previously [21]. Membranes were immunostained with rabbit polyclonal anti-LDLR antibody (1:2000) (Progen Biotechnik GmbH, Heidelberg, Germany) and anti-β-tubulin antibody (1:1000) (Nordic Biosite, Täby, Sweden) for 1 h at room temperature and counterstained with a horseradish peroxidase-conjugated anti-rabbit antibody (GE Healthcare, Little Chalfont, UK). The signals were developed using SuperSignal West Dura Extended Substrate (Pierce Biotechnology, Rockford, IL, USA). ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) was used to detect the signals, and Quantity One Basic 4.4.0 software (Bio-Rad) was used to quantify band intensities. The concentrations of the antibodies were optimized to achieve low background and a linear dose-dependent increase in signal intensity.

Metabolic labeling and immunoprecipitation

The transfected T-Rex CHO cells were grown in Ham's F-12 medium supplemented with 10% LPDS and 1 μg/ml tetracycline for 24 h. The medium was changed to cysteine- and methionine-free DMEM (Invitrogen) containing 10% LPDS, 2 mM L-glutamine, and 1 μg/ml tetracycline for 30 min to deplete intracellular pools of cysteine and methionine. The cells were pulsed with 250 μCi EXPRE35S35S Protein Labeling Mix ([^{35}S]cysteine and [^{35}S]methionine; Perkin Elmer, Waltham, MA, USA) for 30 min. The cells were washed twice in PBS and chased in Ham's F-12 containing 10% LPDS, 1 μg/ml tetracycline with or without LDL (200 μg protein/ml) for 0, 2, 4, 6, and 8 h. The cells were washed twice in PBS, lysed in 500 μl lysis buffer (1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 20 mM Tris/HCl, pH 7.5) supplemented with a cocktail of protease inhibitors (Roche Diagnostics) for 30 min at 4°C and harvested by scraping. The lysates were sonicated for 10 s and centrifuged at 15,000 *g* at 4°C for 15 min to remove cell debris. Then, 60 μl Protein G Sepharose (GE Healthcare) was washed twice in PBS and incubated with 5 μg rabbit polyclonal anti-LDLR antibody at 4°C for 1 h. The conjugated Sepharose was added to the cell lysates and incubated at 4°C overnight. The Sepharose with captured immunocomplexes was washed twice in wash buffer (PBS containing 0.5% Triton X-100) and the immunocomplexes were released from the Sepharose by boiling in Laemmli sample buffer containing 5% β-mercaptoethanol. The immunoprecipitates were subjected to SDS/PAGE (4–20%). The gel was dried and exposed in a phosphorus screen, and the signals were analyzed using a PhosphorImager.

Biotinylation and immunoprecipitation

The transfected T-Rex CHO cells were grown in Ham's F-12 medium supplemented with 10% LPDS and 1 μg/ml tetracycline for 24 h before incubation with CHX (100 μg/ml), with or without LDL (200 μg protein/ml) and either 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, 0.25 mg/ml) or bafilomycin A1 (200 nM) for 4 h. The cells were washed twice in ice-cold PBS and incubated with 0.5 ml (1 mg/ml) ice-cold sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate (sulfo-NHS-LC-biotin) (Pierce Biotechnology) in PBS at 4°C for 30 min. Cells were washed twice and the reaction was quenched by incubating the cells in PBS containing 100 mM glycine at 4°C for 30 min. The incubation was followed by two washes in PBS before the cells were lysed and the LDLR immunoprecipitated as described above

using 2 μg anti-LDLR IgG-C7 antibody (Progen Biotechnik GmbH). The immunoprecipitates were subjected to SDS/PAGE and western blot analysis, as described above. Precision Protein StrepTactin-HRP Conjugate (1:20000) (Bio-Rad) was used to detect biotinylated proteins and rabbit polyclonal anti-LDLR antibody (1:2000) to detect the LDLR.

Immunocytochemistry

Cells were plated on fibronectin-coated slides (BD Biosciences) and allowed to adhere overnight before incubation with Ham's F-12 containing 10% LPDS and 1 μg/ml tetracycline for 24 h to induce LDLR expression. The cells were incubated with CHX (100 μg/ml), AEBSF (0.25 mg/ml), and LDL (200 μg protein/ml) for 4 h, washed twice in PBS, and fixed and permeabilized in 70% ethanol for 10 min at room temperature. Non-specific-binding sites were blocked by incubating the cells in blocking solution (PBS with 1% BSA and 0.1% Tween 20) overnight at 4°C. Cells were stained with mouse monoclonal anti-LDLR IgG C7 antibody (1:20) and rabbit anti-early endosome antigen-1 (EEA-1) (1:50: Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and counterstained with Alexa 488 donkey anti-mouse (1:500: Invitrogen) and Cy3 donkey anti-rabbit (1:500: Jackson ImmunoResearch Europe Ltd, Suffolk, UK) for 45 min at room temperature. The slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) and examined in a Leica TCS SP confocal microscope (Leica Microsystems Gmbh, Wetzlar, Germany) using the ×40 objective. Image processing was carried out using Adobe Photoshop version 5.5 (Adobe systems, San Jose, CA, USA).

Results

To confirm that the E387K-mutant LDLR behaves as a Class 5-mutant LDLR, the degradation rate of the receptor was analyzed in the absence and presence of LDL. T-Rex CHO cells stably transfected with wild-type LDLR or E387K-mutant LDLR were incubated in the presence of CHX (100 μg/ml), an inhibitor of protein synthesis. Following incubation, cell lysates were prepared and subjected to SDS–PAGE and western blot analysis. The turnover of the wild-type LDLR was unaffected by the presence of LDL (200 μg protein/ml) and a half-life >8 h [**Fig. 1(A)**] was observed. The E387K-mutant LDLR had a half-life of ~7 h in the absence of LDL and a half-life of ~2 h in the presence of LDL [**Fig. 1(B)**].

Treatment with CHX has limitations and might increase the stability of particular proteins due to inhibition of the continued synthesis of short-lived proteins, which might be involved in protein degradation. To eliminate an eventual effect of CHX on the turnover of LDLR, [^{35}S]methionine/cysteine pulse-chase experiments were performed. Metabolic labeling confirmed that wild-type LDLR is unaffected by the presence of LDL (200 μg protein/ml), however the stability of E387K-mutant LDLR was significantly reduced in the presence of LDL [**Fig. 1(C)**]. Turnover of proteins measured by metabolic labeling with [^{35}S]methionine/cysteine in pulse-chase experiments is not directly comparable with turnover of proteins after inhibition of protein synthesis by CHX, due to continued incorporation of cell-associated [^{35}S]methionine/cysteine after chase is initiated. However, we believe that the pulse-chase experiment described in **Fig. 1(C)** shows that CHX does not cause an artificial increase in LDLR stability. The slightly reduced stability of E387K-mutant LDLR in the absence of ligand is in agreement with previous reports and might indicate an essential role for the EGF domain in ligand-independent receptor recycling [16,18]. Thus, E387K-mutant LDLR behaved as a Class 5-mutant LDLR with a ligand-induced accelerated degradation [10].

Following internalization, the LDLR is exposed to the acidic interior of the compartments in the endosomal–lysosomal pathway or in the recycling pathway. Intra-endosomal acidification triggers ligand–receptor dissociation and is necessary for transport along the endosomal pathway [22]. Thus, intra-endosomal acidification might have an impact on the turnover of E387K-mutant LDLR. The proton pump vacuolar (H^+)-ATPase (V-ATPase) generates the acidification of most intracellular compartments [23]. To analyze the effect of alkalization on E387K-mutant LDLR, we used bafilomycin A1, a specific inhibitor of V-ATPase [24]. Transfected CHO cells were exposed to CHX (100 μg/ml) and bafilomycin A1 (200 nM) for 2 and 6 h. Cell lysates were prepared and subjected to SDS–PAGE and western blot analysis [**Fig. 2(A)**]. No significant effect of bafilomycin A1 was observed on the turnover of E387K-mutant LDLR in the absence of LDL. However, bafilomycin A1 inhibited the degradation of E387K-mutant LDLR in the presence of LDL.

Inhibition of endosomal acidification by bafilomycin A1 reduced the degradation of E387K-mutant LDLR. To analyze if the undegraded receptor is allowed to recycle during inhibition of endosomal acidification, cell surface

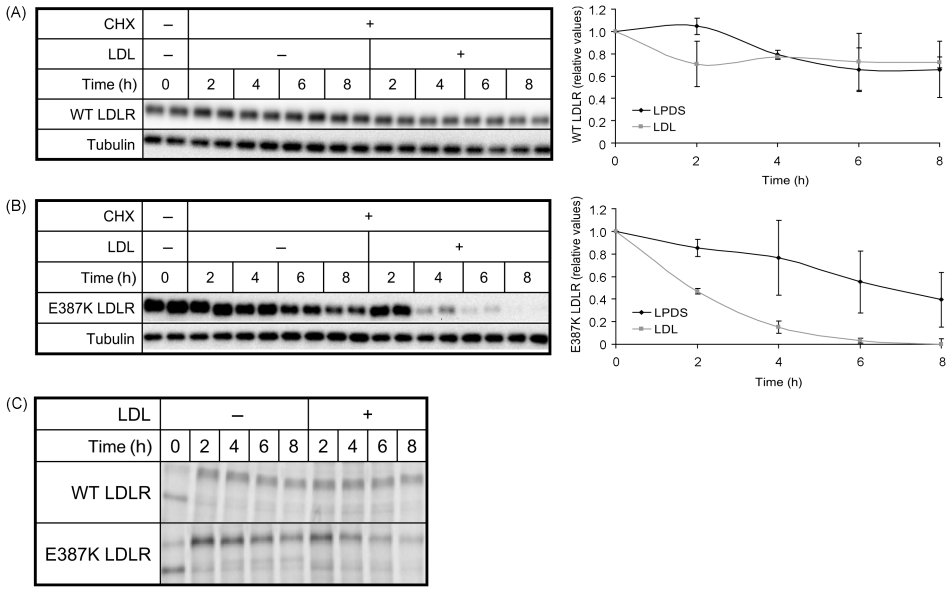

Fig. 1 Degradation rates of the receptor analyzed in the absence and presence of LDL Turnover of wild-type (A) and E387K-mutant LDLR (B) in the presence and absence of LDL. CHO cells stably transfected with wild-type (WT) and E387K-mutant LDLR were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX and LDL for the indicated times. Cell lysates were prepared and equal amounts of protein were subjected to SDS–PAGE and western blot analysis using anti-LDLR and anti-tubulin antibodies. Three independent experiments were performed from which one representative western blot is shown. The amount of LDLR was correlated to the amount of β-tubulin detected in each lane and each time point represents mean ($\pm$SD) of the three experiments. (C) Turnover of wild-type and E387K-mutant LDLR with and without LDL. CHO cells stably transfected with wild-type (WT) and E387K-mutant LDLR were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h. The cells were pulse-labeled with [^{35}S]methionine/cysteine for 30 min. Following chase at the indicated times, the LDLRs were immunoprecipitated from solubilized cells and run on SDS–PAGE. Signals were developed in a Phosphorimager. The experiment was performed three times with similar results.

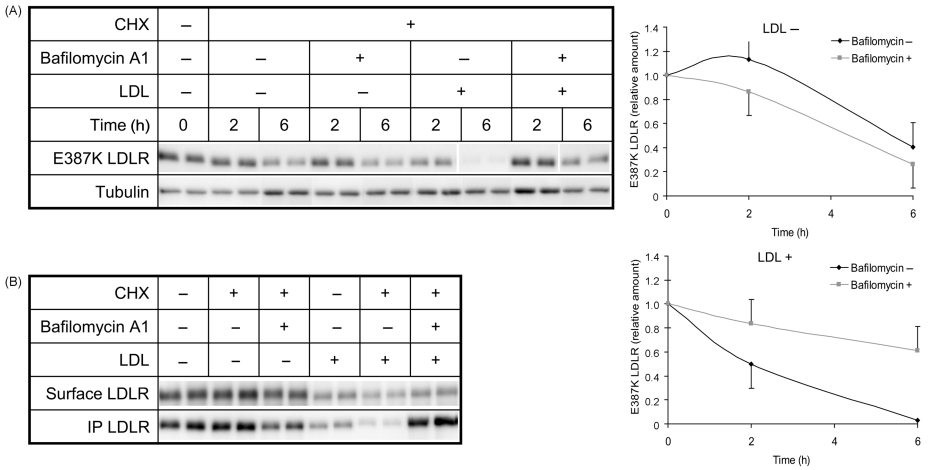

Fig. 2 The effect of bafilomycin A1 (A) On E387K-mutant LDLR turnover. Stably transfected CHO cells were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX, LDL, and bafilomycin A1 (200 nM) for the indicated times. Cell lysates were prepared and equal amounts of protein were subjected to SDS–PAGE and western blot analysis using anti-LDLR and anti-tubulin antibodies. Three independent experiments were performed from which one representative western blot is shown. The amount of LDLR was correlated to the amount of β-tubulin detected in each lane and each time point represents mean ($\pm$ SD) of the three experiments. (B) On the amount of cell surface localized E387K-mutant LDLR. Stably transfected CHO cells were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX, LDL, and bafilomycin A1 (200 nM) for 4 h. Surface proteins were biotinylated and the LDLRs were immunoprecipitated (IP) from solubilized cells and subjected to SDS–PAGE and western blot analysis using StrepTactin-HRP Conjugate and an anti-LDLR antibody. The experiment was performed three times and one representative western blot is shown.

proteins were labeled with a membrane impermeable biotin reagent (sulfo-NHS-LC-biotin) prior to immunoprecipitation of LDLR and western blot analysis of biotin-labeled LDLR and total LDLR. Cells treated with CHX do not produce new LDLR proteins, and if the internalization of the receptor is normal, the amount of cell surface exposed LDLR should decrease if recycling is inhibited. As expected, the amount of total immunoprecipitated E387K-mutant LDLR was reduced in cells incubated with LDL [**Fig. 2(B)**]. Treatment with 200 nM bafilomycin A1 for 4 h increased the amount of total immunoprecipitated E387K-mutant LDLR significantly. However, the amount of immunoprecipitated biotinylated E387K-mutant LDLR, representing the cell surface localized LDLR, was not comparably increased. This indicates that inhibition of endosomal acidification causes an intracellular accumulation of E387K-mutant LDLR in the presence of LDL. In the absence of LDL, the fraction of immunoprecipitated biotinylated E387K-mutant LDLR was not significantly reduced, indicating that recycling is not dependent on acidic pH when receptor–ligand dissociation is not an issue.

To identify the protease activity involved in the LDL-induced degradation of E387K-mutant LDLR, we tested the effect of a range of protease inhibitors (**Table 1**). These inhibitors have previously been used in cell assays and intracellular effects have been described [25–31]. Transfected CHO cells were cultured in the presence of LDL, exposed to CHX (100 μg/ml), and different protease inhibitors for 4 h. Cell lysates were prepared and subjected to SDS–PAGE and western blot analysis (**Fig. 3**). The degradation was not affected by inhibitors of cysteine proteases, aspartic proteases, metalloproteases, or by competitive inhibitors of serine proteases. However, the irreversible serine protease inhibitors, AEBSF (also known as Pefabloc) and 3,4-dichloroisocoumarin (DCI), considerably reduced the degradation of E387K-mutant LDLR. AEBSF acts by sulfonylation [32] and DCI acts by acylation [33] of a serine residue in the active site of the protease. A third irreversible serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), did not have any effect. However, PMSF has poor stability in aqueous solution and is inactivated by components in serum [30]. AEBSF was chosen for further analysis.

To analyze if the undegraded receptors following AEBSF treatment are allowed to recycle, cell surface proteins were labeled with sulfo-NHS-LC-biotin prior to immunoprecipitation of LDLR and western blot analysis of biotin-labeled LDLR and total LDLR (**Fig. 4**). As in the previous experiment, the amount of total

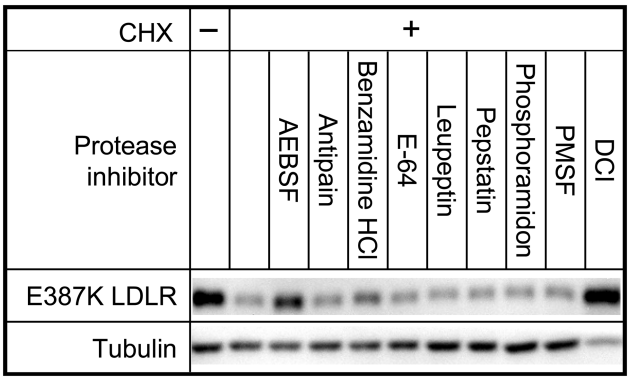

Fig. 3 The effect of protease inhibitors on the degradation of E387K-mutant LDLR in the presence of LDL CHO cells stably transfected with E387K-mutant LDLR were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX, LDL, and protease inhibitors (**Table 1**) for 4 h. Cell lysates were prepared and equal amounts of protein were subjected to SDS–PAGE and western blot analysis using anti-LDLR and anti-tubulin antibodies. Three independent experiments were performed from which one representative western blot is shown.

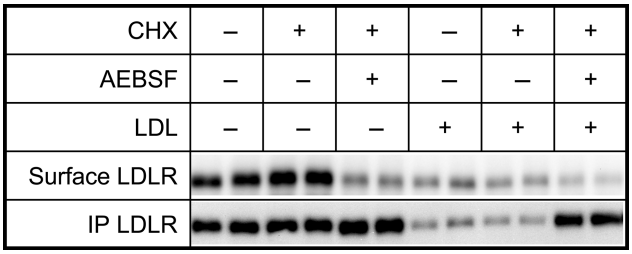

Fig. 4 The effect of AEBSF on the amount of cell surface localized E387K-mutant LDLR Stably transfected CHO cells were grown in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX, LDL, and AEBSF (0.25 mg/ml) for 4 h. Surface proteins were biotinylated and the LDLRs were immunoprecipitated (IP) from solubilized cells and subjected to SDS–PAGE and western blot analysis using StrepTactin-HRP Conjugate and an anti-LDLR antibody. Three independent experiments were performed from which one representative western blot is shown.

immunoprecipitated E387K-mutant LDLR was reduced in cells incubated with LDL. However, AEBSF caused an increase in total immunoprecipitated E387K-mutant LDLR. The amount of immunoprecipitated biotinylated E387K-mutant LDLR, representing the cell surface localized receptor, was reduced both in the presence and in the absence of LDL. Thus, AEBSF caused an intracellular accumulation of the E387K-mutant LDLR independent of the presence of LDL.

To visualize the intracellular localization of LDLR after treatment with AEBSF, we performed confocal immunofluorescence microscopy and analyzed the co-localization of both wild-type and E387K-mutant

LDLR with the early endosome antigen-1 (EEA-1) which is associated with the early endosomes [34]. The wild-type LDLR was found primarily on the cell surface both in the presence and absence of LDL [**Fig. 5(A)**]. However, AEBSF treatment induced an intracellular accumulation of the wild-type LDLR both in the presence and absence of LDL. In the presence of LDL, the E387K-mutant LDLR was nearly undetectable, however in the presence of AEBSF intracellular accumulation of E387K-mutant LDLR was shown. Thus, the confocal microscopy images confirm the results from the immunoprecipitation of biotinylated LDLR (**Fig. 4**).

As described by Wilson *et al.* [35], EEA-1 was found to be evenly distributed throughout the CHO cells [**Fig. 5(A,B)**]. However, AEBSF treatment seems to induce an aggregation of the EEA-1 labeling and a slight increase in EEA-1 was associated with the perinuclear region. This indicates that the early endosome distribution in the cells is affected by AEBSF treatment. The intracellular trapped LDLR co-localize well with these aggregated early endosomes. Thus, AEBSF seems to have an affect on the normal distribution of early endosomes, which might cause the intracellular trapping of the LDLR and prevent the transport of LDLR both to the cell surface and to the site of degradation.

Discussion

In the present study, we utilized CHO cells transfected with an E387K-mutant LDLR to analyze if it is possible to prevent the fast ligand-induced degradation of Class 5-mutant LDLR and to restore its ability to recycle to the cell surface. Initially, we confirmed that E387K-mutant LDLR suffer from an LDL-dependent increased turnover in our cell system.

If the acidification of the endosomal compartments was prevented by the V-ATPase-specific inhibitor, bafilomycin A1, the degradation of E387K-mutant LDLR was prevented. Inhibition of intra-endosomal acidification has been shown to prevent receptor–ligand dissociation and to abrogate protein trafficking between early and late endosomes [22]. V-ATPase interacts directly with the endocytic transport machinery, and inhibition of intra-endosomal acidification prevents the association of the GDP/GTP exchange factor ADP-ribosylation factor nucleotide site opener (ARNO) and Arf6 to the V-ATPase. This leads to accumulation of cargo in early endosomes [22]. The reduction in E387K-mutant LDLR degradation LDL by bafilomycin A1 indicates that the degradation of the mutant LDLR is a post early-endosome event or that the degradation is performed in the early endosomes, by a pH-sensitive protease. However, endosomal/lysosomal proteases are characterized by a broad pH optimum [36] and a complete inhibition of a possible LDLR degrading protease, caused by inhibition of intra-endosomal

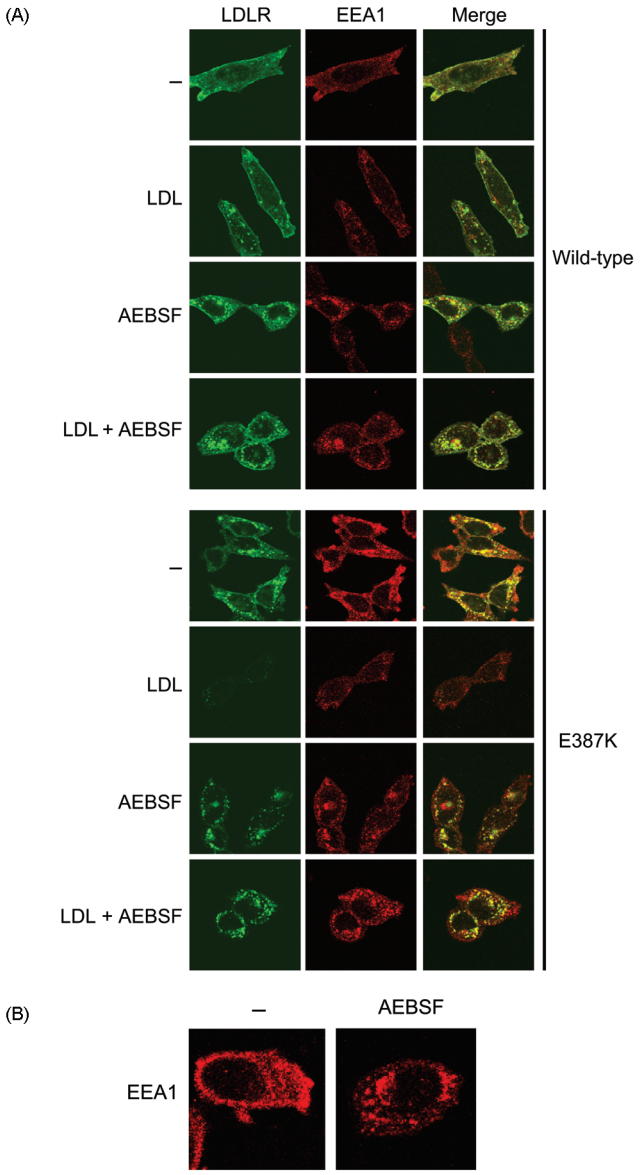

Fig. 5 Confocal laser microscopy (A) CHO cells stably transfected with wild-type and E387K-mutant LDLR were grown on fibronectin-coated slides in medium containing 10% LPDS and stimulated with tetracycline for 24 h before addition of CHX, LDL, and AEBSF for 4 h. Cells were permeabilized with 70% ethanol, stained with anti-LDLR IgG-C7 antibody and rabbit anti-EEA-1 antibody, and counterstained with Alexa 488 donkey anti-mouse (green, LDLR) and Cy 3 donkey anti-rabbit (red, EEA-1), respectively. The experiment was performed three times with similar results. (B) A close-up of CHO cells showing EEA-1 staining before and after treatment with AEBSF for 4 h.

acidification, is therefore unlikely. Although degradation is prevented by bafilomycin A1, the LDLR does not recycle in the presence of LDL, probably because ligand release is prevented. However, recycling of the receptor in the absence of LDL was found not to be acid-dependent, which is in agreement with observations by Baravalle *et al.* [37] who found that recycling of transferrin was not effected by bafilomycin A1.

Lysosomal proteases belong to the aspartic, cysteine, or serine proteinase families of hydrolytic enzymes. Although they are called lysosomal proteases, the enzymes are usually detected within all vesicles of the endocytic pathway. Previously, inhibitors of cysteine proteases (leupeptine and E64) have been found not to prevent the degradation of LDLR [14,15]. We tested a panel of protease inhibitors including aspartic, cysteine, and serine protease inhibitors. The irreversible serine protease inhibitors, AEBSF and DCI, were the only inhibitors that prevented the degradation of E387K-mutant LDLR in the presence of LDL.

We further tested if the prevention of degradation by AEBSF allowed the E387K-mutant LDLR to recycle to the cell surface. AEBSF caused an intracellular accumulation of the mutant receptor both in the presence and absence of LDL. Thus, AEBSF seems to inhibit both the degradation of E387K-mutant LDLR in the presence of LDL and the normal recycling of E387K-mutant LDLR in the absence of LDL. However, analysis of these cells by confocal microscopy showed that AEBSF had effect on the distribution of early endosome in the cells, causing an aggregation of the EEA-1-positive early endosomes. Thus, the effect of AEBSF on the E387K-mutant LDLR degradation is possibly mediated by an effect on endosomal trafficking *per se*.

AEBSF, originally developed to be an irreversible serine protease inhibitor, has also been shown to inhibit NADPH oxidase activity by chemical modification of cytochrome b_{559} and preventing the formation of an active complex [38]. Endosomal trafficking is coordinated by a series of membrane-associated protein complexes. In addition to the pH-dependent V-ATPase–ARNO–Arf6 complex, Rab proteins, and their associations with various effector partners, are central in endosomal trafficking [39,40]. The effect of AEBSF on the endosomal trafficking might represent another example of AEBSF's ability to modify proteins and prevent complex formation.

Rudenko *et al.* [8] determined the crystal structure of the LDLR at pH 5.3 and proposed a mechanism for LDL release in the endosomes whereby the β-propeller functions as an alternative substrate for the ligand-binding domain at acidic pH, displacing bound LDL. Recently, Zhao and Michaely [41] proposed an alternative allosteric model where the β-propeller at low pH interacts with the ligand-binding domain on a different site than the LDL and this interaction drives a conformational change that disrupts lipoprotein-binding, thereby facilitating release. The crystal structure of the LDLR at pH 5.3 has revealed intramolecular interactions, which are proposed to be important in the acid-dependent ligand release. The receptor adopts a closed conformation with an intramolecular interface between the top face of the β-propeller, involving the W3 and W4 blades and the ligand-binding domains 4 and 5 [8]. Interdomain movements have to take place to convert the receptor from an open ligand-binding active conformation to a closed ligand-binding inactive conformation [7]. These movements are proposed to involve the EGF-A and -B domains. The crystal structure revealed an interface between the base of β-propeller, involving the W3, W4, and W5 blades and the EGF-B domain at low pH [8]. In crystal structure determinations at neutral pH, the EGF-B was found to be disordered [9]. Hobbs *et al.* found a cluster of Class 5 mutations, among these E387K, in the N-terminal part of the β-propeller representing the W6 blade of the β-propeller. In the structural determinations [8,9], this part of the β-propeller has not been described to be involved in any intermolecular interactions so why do mutations in this part of the β-propeller have a detrimental effect on ligand release and receptor recycling?

In summary, in our model system of CHO cells, we have transfected a Class 5-mutant LDLR and shown that the LDL-induced degradation of the mutant receptor is reduced by an acidotropic agent and inhibited by irreversible serine protease inhibitors. Although the ligand-induced degradation was prevented, restoration of recycling was not accomplished. In addition, we discovered that the serine protease inhibitor AEBSF seems to disturb endosomal trafficking in cells by inducing aggregation of EEA-1-positive early endosomes.

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