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FULL PAPER

The forced aggresome formation of a bovine anion exchanger 1 (AE1) mutant through association with ΔF508-cystic fibrosis transmembrane conductance regulator upon proteasome inhibition in HEK293 cells

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Abstract

The endoplasmic reticulum (ER)-associated degradation of various polytopic proteins, involving the most common mutant of cystic fibrosis transmembrane-conductance regulator (CFTR), ΔF508-CFTR, involves retrotranslocation of the polypeptide into the cytosol, leading to aggresome formation when the proteasome activity is attenuated. By contrast, an R664X nonsense mutant of the bovine anion exchanger 1 (AE1) is retained in the ER and does not form aggresomes upon proteasome inhibition in transfected HEK293 cells. Here, we report that R664X AE1 formed a large cytoplasmic aggregate when cells co-transfected with enhanced green fluorescence protein (EGFP)-ΔF508-CFTR were exposed to the proteasome inhibitor lactacystin. R664X AE1 and EGFP-ΔF508-CFTR showed co-localization in the aggregates and signals of which coincided with γ-tubulin and were caged by vimentin at the pericentriolar locus, demonstrating aggresome formation. On the other hand, EGFP-AnkN90, consisting of the N-terminal AE1 binding domain of ankyrin, a cytoplasmic protein, also exhibited co-localization with R664X AE1, but was found throughout the ER. Moreover, R664X-mutant protein was specifically immunoprecipitated with EGFP-ΔF508-CFTR from the cells co-expressing these proteins. These findings indicate that R664X AE1 is forcibly extracted from the ER to reside in aggresomes through association with ΔF508-CFTR.

Key words: AE1, aggresome, CFTR, ER, proteasomal ERAD

Introduction

Membrane and secretory proteins are cotranslationally transported and folded in the endoplasmic reticulum (ER). Misfolded or unassembled proteins are recognized by

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ER quality control mechanisms and are retrotranslocated from the ER into the cytosol for degradation by the proteasome system, a process known as ER-associated degradation (ERAD)[7,35]. ERAD of transmembrane proteins in mammalian cells has been characterized primarily in studies of cystic fibrosis transmembrane conductance regulator (CFTR) and its most common mutant, ΔF508-CFTR[10,17,18,36]. Like most ERAD substrates, CFTR and ΔF508-CFTR require ubiquitylation prior to targeting to the proteasome and consequent degradation by the proteasome[10,36]. Our previous study showed that bovine anion exchanger 1 (AE1) with a nonsense mutation of R664X, which is the causative mutant for hereditary spherocytosis (HS) in cattle[13], is degraded via ERAD in transfected K562 and HEK293 cells[1,14,15]. However, the mechanisms underlying degradation of R664X AE1 differed significantly from those of CFTR. The most notable difference was the absence of AE1 ubiquitylation[14]. Although there is accumulating evidence that a number of cytosolic proteins are privileged ubiquitin-independent proteasomal substrates[12,16,31], to the best of our knowledge no substantial data have been reported for ubiquitylation-independent ERAD of polytopic membrane proteins.

Another difference between AE1 and CFTR degradation is seen in their retrotranslocation. Proteasome inhibition does not reduce extraction of CFTR from the ER; rather, increasing levels of misfolded CFTR in the cytosol lead to aggresome formation[18,20]. The aggresome is a microscopically visible, pericentriolar structure and is now known to be involved in the aggresome-autophagy pathway[9,20,37]. Therefore, aggregated CFTR is terminally degraded by autophagy, a lysosome-dependent process. By contrast, in the case of AE1, proteasome inhibitors do not cause aggresome formation but instead increase retention of R664X AE1, primarily in the ER[14]. Interestingly, enhanced ER retention of R664X AE1 is evident in specified perinuclear regions within the cell, suggesting sequestration of R664X AE1 to a compartment of the ER such as the ER-exit site[3] prior to retrotranslocation and degradation. Similar findings have been reported for several other transmembrane proteins[22,30], suggesting that retrotranslocation of these proteins is coupled to proteasome function. However, the mechanisms for retrotranslocation, extraction from the ER, of these polytopic proteins are poorly understood.

In the present study, to further characterize the difference in ERAD between R664X AE1 and CFTR, we investigated the fate of R664X AE1 in HEK293 cells co-transfected with these proteins. Unexpectedly, we found interaction between these two transmembrane proteins and a change in the terminal intracellular localization of R664X AE1 protein when the proteasome pathway was inhibited.

Materials and Methods

Antibodies: Murine monoclonal antibody cdb3-64 and the rabbit polyclonal antibody (anti-38K) to the N-terminal cytoplasmic domain of bovine erythroid AE1 were described previously[13,14]. Other antibodies used were anti-calnexin (Stressgen, Victoria, BC, Canada), anti-γ-tubulin, anti-vimentin (Sigma, St. Louis, MO, USA), and anti-green fluorescent protein (GFP) (Medical and Biological Laboratories, Nagoya, Japan).

Construction of plasmids: Plasmid pcAE1RX in the present study was originally reported as pcbebRX in our previous report and contained the cDNAs for the entire coding region of R664X mutant bovine erythroid AE1[14]. The plasmid clone pEGFP-ΔF508-CFTR[21] was a generous gift from Dr. Bruce A. Stanton (Dartmouth Medical School, Hanover, NH, USA).

A partial cDNA fragment encoding the 90-kDa N-terminal AE1 (band 3)-binding domain of bovine erythroid ankyrin, termed AnkN90

(GenBank accession number AF222766), was ligated to *Hind* III and *Eco* RI sites of vector pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA, USA). In the resultant recombinant protein, EGFP-AnkN90, AnkN90 lacked 26 N-terminal amino acid residues but contained core regions required for association with band 3[23,32]. Nucleotide sequences of these clones were confirmed using an 8800 CEQ DNA sequencer (Beckman Coulter, Fullerton, CA, USA).

Cell culture and transfection: HEK293 cells were purchased from Health Science Research Resources Bank (Osaka, Japan) and grown in minimum essential medium Eagle (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The cells were transiently transfected with the various plasmid constructs (1 μg of DNA per well in a 6-well plate) using TransIt-LT1 reagent (Mirus, Madison, WI, USA) and used for 48 hr after transfection. When the cells were treated for proteasome inhibition and microtubule disruption, they were incubated in the presence or absence of 10 μM lactacystin (Peptide Institute, Inc., Osaka, Japan) and/or 5 μg/ml nocodazole (Sigma) in the last 12 hr of incubation; when present, the cells were incubated for 4 hr with nocodazole, after which lactacystin was added, followed by further incubation for 8 hr.

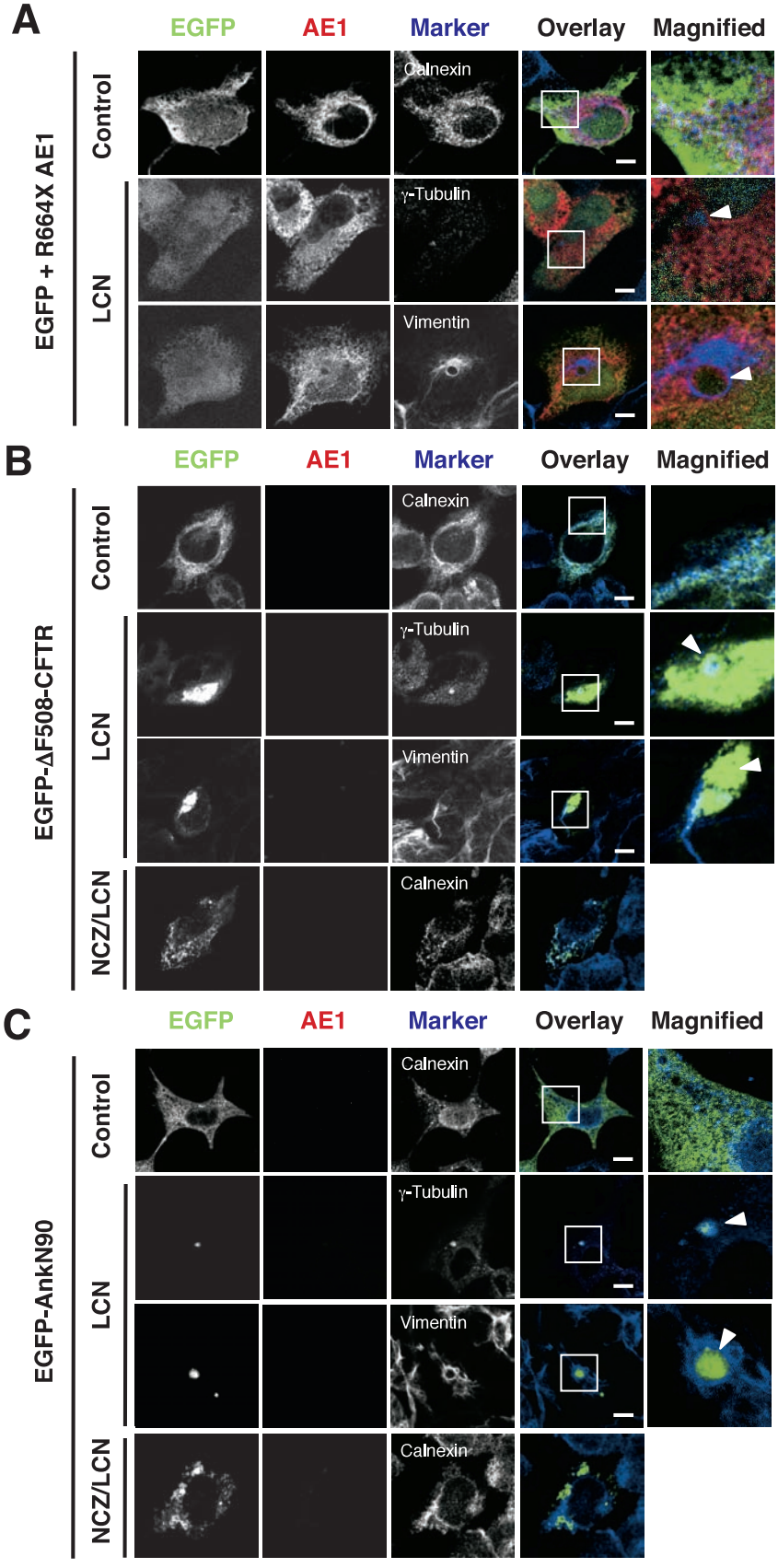
Analysis of proteins: After incubation, cells were washed in phosphate-buffered saline (PBS) and lysed in IP buffer containing 20 mM Tris/Cl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (all from Sigma) for 30 min on ice. After removal of cell debris by centrifugation at 18,000 *g* for 15 min at 4°C, proteins in the supernatant were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Signals were detected using the ECL plus Western blotting detection system (Amersham, Buckinghamshire, UK). Protein concentration was determined by the method of Bradford using a Bio-Rad protein assay kit.

For immunoprecipitation, the supernatant of the cell lysate was incubated with appropriate antibodies overnight at 4°C with gentle agitation. Protein G-Sepharose beads were added and, after incubation for 2 hr, the beads were thoroughly washed 4 times in IP buffer containing 0.1% SDS, twice in IP buffer containing 500 mM NaCl, and once with 0.1% Triton X-100 in 20 mM Tris/Cl (pH 7.5). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence microscopy: Cells were grown on collagen-coated coverslips (Iwaki Glass Co., Tokyo, Japan). After washing in PBS, they were fixed with methanol for 7 min at −20°C, washed in PBS, and blocked with 1% bovine serum albumin in PBS for 30 min at ambient temperature. Subsequently, cells were incubated with the appropriate antibodies for 1 hr, washed with PBS and then incubated with a 1 : 500 dilution of anti-murine or rabbit IgG labeled with AlexaFluor 568, AlexaFluor 568, and/or AlexaFluor 405, all obtained from Molecular Probes (Eugene, OR, USA), at ambient temperature. After washing with PBS, cells were mounted in ProLong antifade reagent (Molecular Probes) and examined under a Zeiss confocal laser LSM5 PASCAL microscope.

Results

The R664X AE1 exhibited ER retardation as reported previously when expressed in HEK293 cells, with its immunofluorescent signals consistent with those of the ER marker calnexin[14] (Fig. 1A). When the cells were incubated with the proteasome inhibitor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lactacystin, a large focus, which was surrounded by intermediate filaments, was found at a pericentriolar locus indicated by the signals of γ-tubulin, indicating formation of the aggresome[20]. However, R664X AE1 showed no significant change in intracellular localization and was not detected in the area of aggresomes. In contrast, EGFP-ΔF508-CFTR that was retained in the ER showed profound deposition of aggresomes in the cells treated with lactacystin, and disruption of microtubules with nocodazole totally disrupted aggresome formation (Fig. 1B), in agreement with a previous report[18]. Similar characteristic aggresome formation was observed for EGFP-AnkN90 consisting of the N-terminal AE1-binding domain of ankyrin[23,32] (Fig. 1C). These results confirmed our previous observation that R664X AE1, as well as its wild type, was retained in the ER without formation of aggresomes upon proteasome inhibition[14] and suggest that this protein is not extracted into the cytosol in its ERAD process.

To further characterize different responses to attenuated proteasome functions, we examined intracellular localization of R664X AE1 in the cells co-transfected with EGFP-ΔF508-CFTR. In HEK293 cells expressing both of these proteins, most immunofluorescence signals of R664X AE1 coincided with EGFP signals in the ER, indicating co-localization of the AE1 mutant and EGFP-ΔF508-CFTR in the ER (Fig. 2A). Unexpectedly, proteasome inhibition caused aggregation of R664X AE1; signals of R664X AE1 were consistent with those of the CFTR mutant, located in the pericentriolar region, and were caged by vimentin signals. Moreover, nocodazole inhibited this aggresome formation and generated dispersed aggregates with signals of both proteins (Fig. 2A). These small aggregates, which had a distribution consistent with the ER marker calnexin, were not observed in the cells expressing R664X AE1 alone (Fig. 1A), and appeared to represent the aggregates found in nocodazole- and lactacystin-treated cells expressing EGFP-ΔF508-CFTR (Fig. 1B). On the other hand, when HEK293 cells doubly transfected with R664X AE1 and EGFP-AnkN90 were exposed to lactacystin (and nocodazole), coincident localization of these proteins was evident, demonstrating their interactions, presumably through oligomer formation[23,32]. However, their signals were basically consistent with those of the ER and were not detected in the areas of pericentriolar aggresomes (Fig. 2B). These findings suggested that there was an interaction between R664X AE1 and ΔF508-CFTR and that this interaction forced the AE1 mutant to be extracted from the ER to form aggresomes.

To verify this hypothesis, we examined interaction of these proteins by immunoprecipitation using the anti-GFP antibody. At first, detergent-solubilized supernatants from transfected cells contained 75-kDa R664X AE1, 170-kDa EGFP-ΔF508-CFTR, or both of them, as shown in the immunoblots (Fig. 3). Cells treated with lactacystin exhibited relatively decreased levels of EGFP-ΔF508-CFTR, and this was likely due to aggresome formation of this protein since the detergent-insoluble precipitates from the cells incubated in the presence of the proteasome inhibitor contained EGFP-ΔF508-CFTR at a level much more abundant than that in control

Fig. 1. Difference in aggresome formation between R664X AE1 and ΔF508-CFTR. HEK293 cells were transfected with R664X AE1 and EGFP (*A*), EGFP-ΔF508-CFTR (*B*), and EGFP-AnkN90 (*C*), and incubated in the presence (*LCN* and *NCZ/LCN*) or absence (*Control*) of nocodazole (*NCZ*) and/or lactacystin (*LCN*). The cells were stained for AE1 with the anti-AE1 monoclonal antibody cdb3-64 (*AE1*). Cells were also stained for the ER with anti-calnexin (*Calnexin*), centrosomes with anti-γ-tubulin (*γ-Tubulin*), and intermediate filaments with anti-vimentin (*Vimentin*) antibodies. *Overlay* shows the image in which all EGFP, AE1, and marker signals are merged. The indicated areas in the overlay images are magnified (*Magnified*) and aggresomes at the pericentriolar locus are indicated by arrowheads. Bars, 10 μm.

cells (data not shown). Immunoblot analysis demonstrated the presence of both R664X AE1 and EGFP-ΔF508-CFTR in the immunoprecipitates from the cells expressing these proteins, whereas only the CFTR mutant was found in the immunoprecipitates from the cells transfected with EGFP-ΔF508-CFTR alone. Moreover, no signals were detected for these proteins in immunoprecipitates of the cells expressing R664X AE1 alone. These data indicated that the immunoprecipitation worked immunospecifically and demonstrated that R664X AE1 associated with EGFP-ΔF508-CFTR in the transfected cells. It is also notable that higher molecular weight species of EGFP-ΔF508-CFTR, indicative of polyubiquitylation, were evident, whereas no such signals were found for the mutant AE1.

Discussion

The current model for ERAD of polytopic membrane proteins in mammalian cells is largely based on investigations on CFTR and its most common mutant, ΔF508-CFTR[35]. Those include recognition of the ERAD machinery[8,29], retrotranslocation from the ER to the cytosol[17,36], and degradation by the ubiquitin-proteasome system[10,18,36]. On the other hand, various

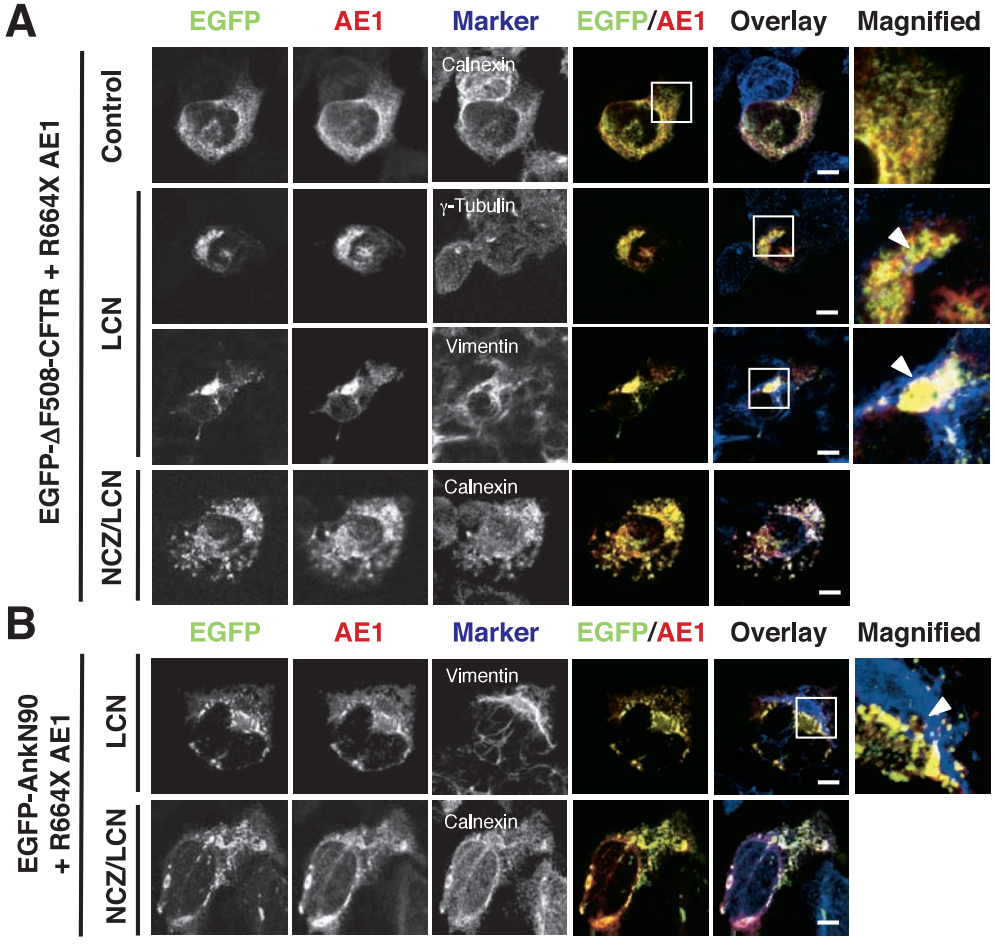

Fig. 2. Intracellular localization and forced aggresome formation of R664X AE1 in the presence of ΔF508-CFTR. HEK293 cells were co-transfected with R664X AE1 and EGFP-ΔF508-CFTR (*A*) or EGFP-AnkN90 (*B*), followed by incubation and staining as described in the legend for Fig. 1. Merged images for EGFP and AE1 signals (*EGFP/AE1*) and all of EGFP, AE1, and markers (*Overlay*) are shown. Boxed areas in merged images are magnified (*Magnified*) and arrowheads indicate aggresomes. Bars, 10 μm.

mutations of the *AE1* (*SLC4A1*) gene have been reported to cause dominant HS or dominant/recessive renal tubular acidosis in humans and/or cattle[13,28,38], and several AE1 mutants have been shown to be degraded via the proteasome pathway[14,19].

Our previous study revealed significant differences in ERAD characteristics between R664X AE1 and ΔF508-CFTR[14]. The present study further confirmed particulars of ERAD of R664X AE1, including an apparent lack of ubiquitylation and profound ER retention instead of cytoplasmic aggresome formation upon proteasome inhibition; ΔF508-CFTR and AnkN90 were concentrated in aggresomes at a pericentriolar locus, whereas R664X AE1 exhibited increased ER retention with the total absence at the focus caged by vimentin, relevant to the aggresome, formed in the same cell (Fig. 2). These findings suggest that proteasomal degradation of R664X AE1 and its retrotranslocation are tightly coupled and occur at the ER membrane, as has been reported for several other polytopic membrane proteins[22,25,30]. A study by Nakatsukasa *et al.*[26] has demonstrated that ubiquitylated polytopic membrane proteins in yeast strains can be extracted from the ER in a solubilized form, and that, in general, aggresomes may form from the retrotranslocation of a polytopic protein in the ER membrane. Hence, ERAD of R664X AE1, and possibly that of the wild-type AE1[14], may involve mechanisms totally different from that hypothesized for various polytopic membrane proteins in the current model[35]. Cotranslocational ER protein degradation, during protein synthesis, via a proteasomal pathway through functions of the translocon Sec61[27] might be involved in this quality control process.

The most notable finding of the present study therefore is that the particular ER retardation of R664X AE1 was altered and the mutant AE1 was forcibly dislocated from the

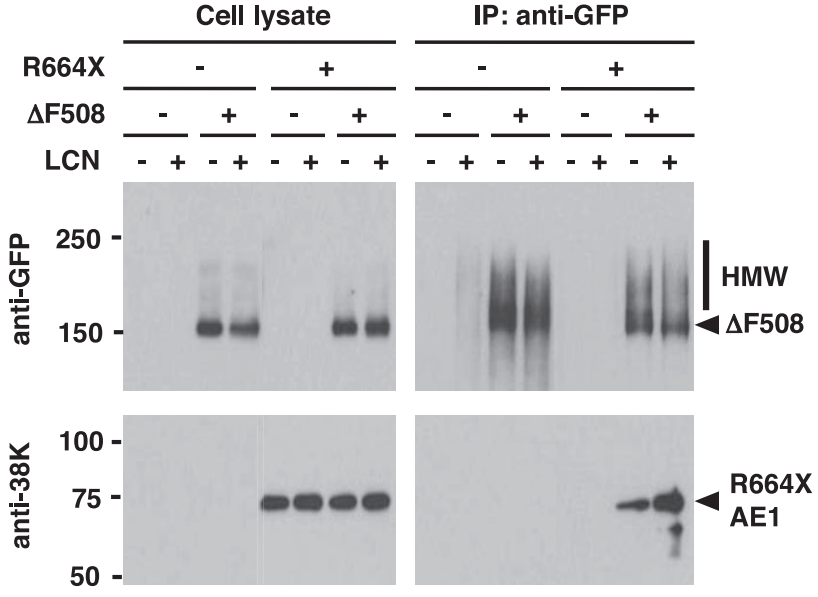

Fig. 3. Co-immunoprecipitation of R664X AE1 in the immunoprecipitates of ΔF508-CFTR from the cells expressing these proteins. HEK293 cells were transfected (+) with R664X AE1, EGFP-ΔF508-CFTR, or both of them, and incubated in the presence (+) or absence (−) of 10 μM lactacystin. The cells were also transfected with a corresponding amount of an empty vector (−). After incubation, detergent-solubilized supernatants (*Cell lysate*) were immunoprecipitated with the anti-GFP antibody, and immunoprecipitates (*IP: anti-GFP*) were analyzed by SDS-PAGE and immunoblotting for EGFP-ΔF508-CFTR (*ΔF508*) and R664X AE1 (*R664X AE1*) using anti-GFP (*anti-GFP*) and anti-38K (*anti-38K*) antibodies, respectively. Higher molecular weight species (*HMW*) and migrating positions of size markers in kDa are also shown.

ER, resulting in aggresome formation in co-localization with ΔF508-CFTR (Fig. 3). Considering that EGFP-AnkN90, which principally has cytoplasmic distribution and can associate with AE1, did not affect the ER retention of R664X AE1 (Fig. 2B), forced aggresome formation likely involves interaction with ΔF508-CFTR, and subsequent extraction from the ER coincides with the translocation of ΔF508-CFTR. Nevertheless, the mechanisms whereby R664X AE1 is extracted from the ER membrane, and by which the association with the CFTR mutant governs the fate of R664X AE1, still remain mysterious and are of great interest since R664X AE1 alone does not appear to undergo retrotranslocation into the cytoplasm, as discussed above. Ubiquitylation, which is prerequisite to retrotranslocation, was not apparent in R664X AE1, even under coexistence with ΔF508-CFTR (Fig. 3), and therefore altered ubiquitylation status in the dislocation process can be ruled out. It is possible that interaction with ΔF508-CFTR, the ERAD machinery for ΔF508-CFTR, and/or other proteins (discussed below) might affect the hydrophobic status of the AE1 mutant, as reported for extraction of Ste6p*, a 12 transmembrane protein in yeast, from the ER to the cytosol in a soluble form[25,26]. Meanwhile, our finding demonstrates that R664X AE1 is terminally degraded by autophagy, a lysosome-dependent system, instead of the native degradation via the ERAD pathway[9,20,37]. More generally, our data indicate that proteolytic degradation of one polytopic protein can change that of another protein.

Another notable finding is the specific interaction of R664X AE1 with ΔF508-CFTR in co-expressed cells, although it is unclear whether the interaction is direct or indirect. On the other hand, association between wild-type AE1 and ΔF508-CFTR, as judged by immunoprecipitation, was rather less abundant (data not shown), indicating that ER retardation is important for this interaction. AE1 is an anion exchanger that is expressed in red cells and intercalated cells in the renal collecting ducts[33,34], whereas CFTR is present in epithelial cells in diverse tissues[6]. Therefore, the physiological and/or pathological significance of their association remains unclear at present. However, the interaction of these proteins itself is not so surprising since CFTR is known to regulate several transport proteins, including the epithelial sodium channel (ENaC), anion exchanger, and aquaporins, by forming a macromolecular signaling complex mediated by PDZ domain scaffolding proteins[11] or through a direct protein-protein association hypothesized for CFTR-ENaC interaction[5]. Moreover, AE1 is known to associate with glycophorin A[4], one of the major sialoglycoproteins, and also forms a complex with several other transmembrane proteins in the red cell membrane[2,24]. Thus, under the conditions with attenuated ERAD activity, changes may occur in the terminal degradative pathway of these transmembrane proteins and their interactors.

In conclusion, our present study demonstrates novel ERAD without cytosolic retorotranslocation in quality control of the polytopic membrane protein AE1 and its change to the aggresome-autophagy pathway governed by ERAD of ΔF508-CFTR through their specific interaction. Our findings provide insights into the attenuated ERAD of polytopic membrane proteins under some disease conditions.

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