Characterization of the Plasma Membrane Targeting and the Endoplasmic Reticulum-associated Degradation of Bovine Anion Exchanger 1

（牛アニオン交換輸送体 1 の細胞膜輸送と小胞体関連分解分子機構の性状解析）

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Contents

Abbreviations

Abbreviations used in this study are as follows:

AE1	anion exchanger 1 (SLC4A1, band 3)
CFTR	cystic fibrosis transmembrane conductance regulator
ER	endoplasmic reticulum
ERAD	ER-associated degradation
HS	hereditary spherocytosis
PBS	phosphate-buffered saline, pH 7.4
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis

In the present study, one-letter and three-letter abbreviations for amino acid residues are employed.

General Introduction

Membrane and secretory proteins are cotranslationally transported and folded in the endoplasmic reticulum (ER). Misfolded or unassembled proteins, including various mutant proteins, are recognized by 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) (Ellgaard and Helenius, 2003; Vember and Brodsky, 2008). 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 (Jensen *et al*., 1995; Ward *et al*., 1995; Gelman *et al*., 2002; Johnston *et al*., 1998). Like most ERAD substrates, CFTR and ΔF508-CFTR require ubiquitylation prior to targeting to the proteasome and consequent degradation by the proteasome (Ward *et al*., 1995; Gelman *et al*., 2002) (Fig. 1).

Anion exchanger 1 (AE1, SLC4A1, also called band 3) is predominantly expressed in red blood cells and in intercalated cells in renal tubules, and is one of the best studied of all membrane proteins (Alper, 1991; Reithmeier *et al*., 1996; Tanner, 1993). Various mutations of the human *AE1* gene have been reported to cause dominant hereditary spherocytosis and recessive or dominant distal renal tubular acidosis (Tse and Lux, 2001; Williamson and Toye, 2008; Yenchitosomanus *et al*., 2005). Previous studies have demonstrated possible implications in pathogenesis of the trafficking deficit/ER retardation of AE1 mutants: a nonsense mutation at Arg^{901} that truncates AE1 by 11 amino acid residues at the C-terminus (Δ11) caused its intracellular retention in non-polarized Madin-Darby canine

kidney cells (Toye *et al*., 2002) and HEK293 cells (Quilty *et al*., 2002). Although the precise mechanism for the pathogenesis of dominant distal renal tubular acidosis caused by Δ11 mutation is still unclear, the 11 C-terminal residues are likely to involve some determinant for membrane trafficking of AE1 to the selective compartment of the plasma membrane (Devonald *et al*., 2003; Toye *et al*., 2004).

On the other hand, previous studies on patients with various mutations responsible for dominant hereditary spherocytosis associated with partial deficiency of AE1 failed to demonstrate the presence of the mutant AE1 protein in their red cell membranes (Tse and Lux, 2001). Although this was partly explained by the absence of the mutant transcripts, different explanations such as defective trafficking and folding (Dhermy *et al*., 1999; Quilty and Reithmeier, 2002) and proteolytic degradation (Inaba *et al*., 1996; Ito *et al*., 2006) have been suggested. However, no mutations in the C-terminal tail of AE1 associated with abnormal red cell shapes including HS have been reported to date and therefore the possible significance in membrane targeting and/or recognition by the ERAD machineries of the C-terminal region other than the last 11 amino acids has not been defined.

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, is degraded via ERAD in transfected K562 and HEK293 cells (Adachi *et al*., 2009; Ito *et al*., 2006; Ito *et al*., 2007) (Fig. 1). However, the mechanisms underlying degradation of R664X AE1 appeared to differ significantly from those of CFTR. The most notable difference was the absence of AE1 ubiquitylation (Ito *et al*., 2006). Although there is accumulating evidence that a number of cytosolic proteins are privileged ubiquitin-independent proteasomal

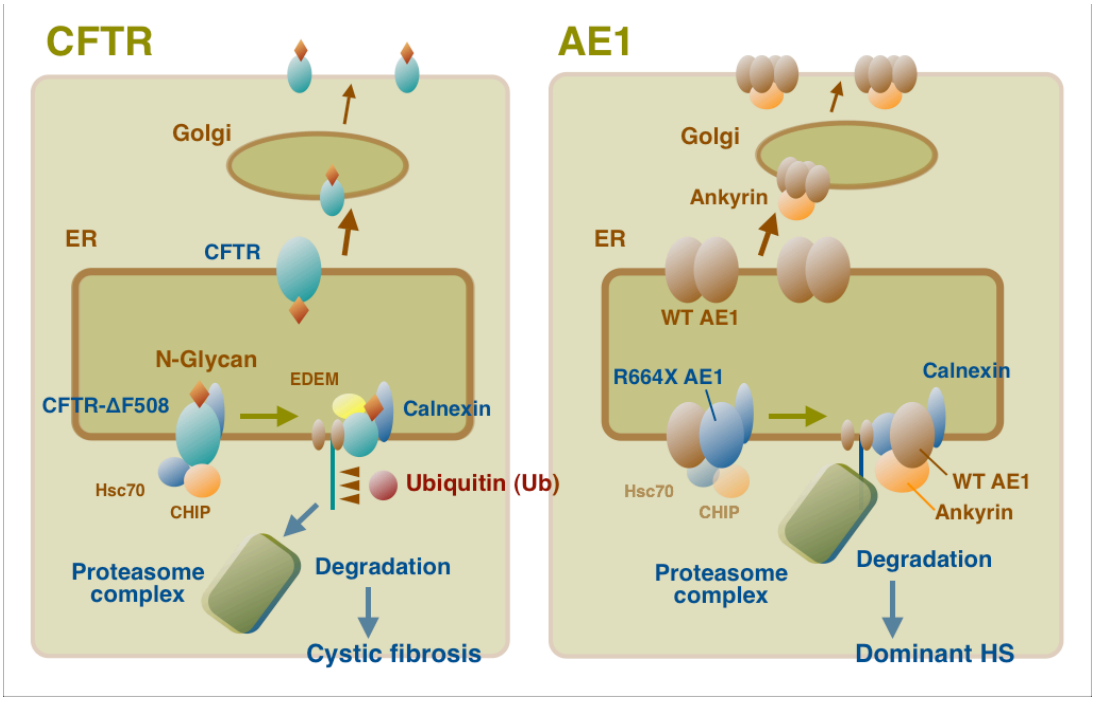

Figure 1. Different characteristics of the ERAD for the CFTR and the AE1 (Ito *et al.*, 2006).

substrates (Sheaff *et al*., 2000; Hwang *et al*., 2005; Jariel-Encontre *et al*., 2008), to the best of our knowledge no substantial data have been reported for ubiquitylation-independent ERAD of polytopic membrane proteins.

Other noticeable difference between AE1 and CFTR degradation are seen in their retrotranslocation and the lack of *N*-glycan-dependent recognition by the ER chaperones (Ito *et al*., 2006) (Fig. 1), the latter of which is due to the absence of *N*-glycan in bovine AE1 (Ito *et al*., 2006; Ito *et al*., 2007). The idea for difference in retrotranslocation is based on the distinct responses of AE1 and CFTR when the proteasome function are inhibited in the transfected cells (Ito *et al*., 2006). Proteasome inhibition does not reduce extraction of CFTR from the ER; rather, increasing levels of misfolded CFTR in the cytosol lead to aggresome formation (Johnston *et al*., 1998; Kopito, 2000). The aggresome is a microscopically visible,

pericentriolar structure and is now known to be involved in the aggresome-autophagy pathway (Kopito, 2000; Garcia-Mata *et al*., 2002; Xie and Klionsky, 2007). 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 (Ito *et al*., 2006). Thus, the proteasomal degradation of R664X AE1 appears to be spatially linked to its retrotranslocation from the ER. However, the mechanisms for retrotranslocation, extraction from the ER, of these polytopic proteins are poorly understood.

Based on these previous findings, the present study was conducted to further characterize the transport from the ER to the plasma membrane and the ERAD of bovine AE1. In Chapter 1, the roles of the C-terminal cytoplasmic tail of AE1 in the ER exit and the plasma membrane expression were investigated in HEK293 cells using a series of truncation and substitution mutants. In Chapter 2, aggresome formation was further studied for R664X AE1 and ΔF508-CFTR in transfected cells. Some parts of the present study have been published as follows:

1. Adachi, H., Ito, D., Kurooka, T., Otsuka, Y., Arashiki, N., Sato, K., and Inaba, M. (2009) Structural implications of the EL(K/Q)(L/C)LD(A/G)DD sequence in the C-terminal cytoplasmic tail for proper targeting of anion exchanger 1 to the plasma membrane. *Jpn. J. Vet. Res.* **57**, 135-146.
2. Adachi, H., Kurooka, T., Otsu, W., and Inaba, M. (2010) The forced aggresome formation of a bovine anion exchanger 1 (AE1) mutant through association with DF508-cystic fibrosis transmembrane conductance-regulator upon proteasome inhibition in HEK293 cells. *Jpn. J. Vet. Res.* **58**, in press

Chapter 1

Structural implications of the EL(K/Q)(L/C)LD(A/G)DD sequence in the C-terminal cytoplasmic tail for proper targeting of AE1 to the plasma membrane

Introduction

Anion exchanger 1 (AE1, SLC4A1, also called band 3) is the most abundant transmembrane protein in red blood cells and one of the best studied of all membrane proteins (Alper, 1991; Reithmeier *et al*., 1996; Tanner, 1993). AE1 is composed of two functionally distinct domains. The 47-kDa N-terminal cytoplasmic domain plays a critical role in the formation and stabilization of red cell membranes by anchoring the membrane skeletal proteins spectrin and actin to lipid bilayers through association with ankyrin (Low *et al*., 1991; Zhang *et al*., 2000). The 52-kDa C-terminal transmembrane domain consists of 12-14 transmembrane spans and has a function as an anion exchanger that mediates rapid Cl^-/HCO_3^- exchange across the cell membrane (Tanner, 1997; Vince and Reithmeier, 1996). The C-terminal region at the end of the transmembrane domain has a cytoplasmic location and contains the last 40 amino acid residues of AE1 (Zhu *et al*., 2003). Various studies have reported to date that the C-terminal cytoplasmic tail has physiological and pathological roles in functional expression of AE1 in the plasma membrane.

Various mutations of the human *AE1* gene have been reported to cause dominant hereditary spherocytosis and recessive or dominant distal renal tubular acidosis (Tse and Lux, 2001; Williamson and Toye, 2008; Yenchitosomanus *et al*., 2005). Previous studies on the C-terminus Δ11 mutation showed that this mutation of AE1 caused its intracellular retention in non-polarized Madin-Darby canine kidney cells (Toye et al., 2002) and HEK293 cells (Quilty et al., 2002). Although the precise mechanism for the pathogenesis of dominant distal renal tubular acidosis caused by Δ11 mutation is still unclear, the 11 C-terminal residues are

likely to involve some determinant for membrane trafficking of AE1 to the selective compartment of the plasma membrane (Devonald *et al*., 2003; Toye *et al*., 2004). However, it is likely that the 11 C-terminal residues are not principally required for membrane targeting in erythroid cells, since red cells with AE1 Δ11 mutation have only mildly reduced content and substantial anion transport activity of AE1 (Toye *et al*., 2002) and avian erythroid AE1 does not have this sequence (GenBank accession number NM_025522).

On the other hand, previous studies on patients with various mutations responsible for dominant hereditary spherocytosis associated with partial deficiency of AE1 failed to demonstrate the presence of the mutant AE1 protein in their red cell membranes (Tse and Lux, 2001). Although this was partly explained by the absence of the mutant transcripts, different explanations such as defective trafficking and folding (Dhermy *et al*., 1999; Quilty and Reithmeier, 2002) and proteolytic degradation (Inaba *et al*., 1996; Ito *et al*., 2006) are plausible. However, no mutations in the C-terminal tail of AE1 associated with abnormal red cell shapes including HS have been reported to date and therefore the possible significance of the C-terminal region other than the last 11 amino acids has not been defined. Moreover, the acidic sequence LDADD in the C-terminal tail was reported to interact with carbonic anhydrase II (CAII), allowing smooth HCO_3^- metabolism across the cell membrane (Vince *et al*., 2000; Vince and Reithmeier, 1998; Vince and Reithmeier, 2000), and appears to involve novel intra-protomeric regulation of Cl^-/HCO_3^- exchange within dimeric AE1 (Dahl *et al*., 2003). However, it remains unclear if this association with CAII has an important role in proper targeting of AE1 to the plasma membrane. In addition, as suggested by a previous study (Mori *et al*., 1995), AE1 and some other polytopic membrane proteins share the

sequence containing the core CAII binding site. Although this region was suggested to participate in the proper membrane insertion and membrane targeting of AE1, no substantial roles have been elucidated as yet.

The purpose of the present study was to examine the effects of structural alterations in the C-terminal tail of AE1 on its membrane expression and to define the sequence essential for proper and efficient trafficking of AE1 to the plasma membrane. We first created several C-terminal truncation mutants of bovine AE1 to survey the sequence required for membrane targeting in HEK293 cells by immunofluorescence microscopy and detection of AE1 at the cell surface by biotinylation. Subsequent analyses were concentrated on the intracellular localization and turnover of a series of AE1 mutants with Ala substitutions at the residues in the sequence of EL(K/Q)(L/C)LD(A/G)DD, highly conserved among various mammals, to determine amino acid residues essential for efficient trafficking and stability of AE1.

Materials and Methods

Antibodies

Murine monoclonal antibody cdb3-64 to bovine erythroid AE1 was described previously (Ito *et al*., 2006). Other antibodies used were anti-calnexin (Stressgen, Victoria, BC, Canada), anti-GM130 (BD Biosciences Clontech, Palo Alto, CA, USA), and anti-Lamp2 (Santa Cruz Biotechnology, Santa Cruz, CA).

Construction of plasmids

Plasmids pcAE1WT and pEGFP-AE1WT in the present study were originally reported as pcbebWT and pEGFP-bebWT, respectively, in our previous report and contained the cDNAs for the entire coding region of wild-type (WT) bovine erythroid AE1 and the WT AE1 N-terminally tagged with enhanced green fluorescence protein (EGFP), respectively (Ito *et al*., 2006). The cDNAs for bovine AE1 with truncation mutations ΔCt28 (K903X) and ΔCt18 (N913X) lacking the 28 and 18 C-terminal amino acids, respectively (Fig. 2), were obtained by PCR amplification using the primer BEBrev1 (5'-GCGGCCGCTCAGATCACTGCA-3') (Ito *et al*., 2006) and appropriate reverse primers introducing a stop codon at the corresponding amino acids residue with the *Sal* I site at the 5'-ends. The cDNAs obtained were cloned into pCRII vectors (Invitorogen, San Diego, CA, USA) and then subcloned into pcDNA3.1(-) (Invitrogen) and pEGFP-C3 (BD Biosciences Clontech) vectors. To obtain C-terminal tail mutants E901A, L902A, L905A, D906A, D908A, D909A, D906E, and D908A/D909A, partial AE1 cDNA fragments were generated by PCR amplification using

appropriate primers, to alter a codon for the indicated amino acid residue to an Ala codon, and substituted for the corresponding region of the WT cDNA in the plasmids using the endogenous *Hind* III sites in the WT cDNA and the vectors. Several C-terminal tail mutants of murine AE1 were also prepared using plasmid pBL containing the murine AE1 cDNA; pBL was kindly provided by S. L. Alper (Harvard Medical School). Nucleotide sequences of these clones were confirmed using a CEQ 8800 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 Chemical Co., St. Louis, MO, USA) 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 for a well in 6-well plate) using TransIt-LT1 reagent (Mirus, Madison, WI, USA) and used for 48 hours after transfection.

Cell surface biotinylation

Cell surface biotinylation was carried out essentially as described previously (Ito *et al*., 2006). Transfected cells were washed with ice-cold borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, pH 9.0) and treated twice with 1 ml of 0.8 mM EZ-link NHS-SS-biotin (Pierce Chemical Co., IL, USA) in borate buffer for 30 minutes at 4˚C. The cells were then rinsed in 192 mM glycine, 25 mM Tris/Cl (pH 8.3) solution to quench any

unreacted reagent. Subsequently, cells were lysed with RIPA buffer (1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/Cl, pH 7.6) containing protease inhibitors, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM 4-(2-aninoethyl)-benzenesulfonyl fluoride (all from Sigma), for 30 minutes on ice. After removal of insoluble materials by centrifugation at 18,000 *g* for 15 minutes at 4˚C, an aliquot of the lysate was saved for immunoblotting. NutrAvidin beads (Pierce) were added to the lysate for 1 hour at 4˚C to bind the biotinylated proteins. The supernatant was removed and an aliquot was saved for immunoblotting. The NutrAvidin beads were washed four times with RIPA buffer. Captured proteins were released from the beads in the sample buffer for SDS-PAGE containing 10% 2-mercaptethanol for 1 hour at ambient temperature. Insoluble materials after solubilization and centrifugation were also dissolved in the sample buffer for SDS-PAGE followed by passage through QIAshredder columns (QIAGEN, Tokyo, Japan). Proteins were separated by SDS-PAGE and were analyzed for AE1 contents by immunoblotting. Signals for bovine AE1 were detected with cdb3-64 antibody using the ECL plus Western blotting detection system (Amersham, Buckinghamshire, UK).

Pulse-chase assay

HEK293 cells transiently transfected with WT and C-terminal mutants were pulse-labeled for 20 minutes with 100 μCi/ml of [^{35}S]methionine (EXPRE^{35}S^{35}S, NEN Life Science Products) in methionine-free Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% dialyzed fetal bovine serum, and chased for the indicated periods with or without 10 μM

lactacystin (Peptide Institute, Inc., Osaka, Japan). Cells were washed in phosphate-buffered saline (PBS) and then lysed with solubilizing buffer, containing 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 25 mM Tris/Cl, pH 7.6 and protease inhibitors described above, for 30 minutes on ice. After removal of cell debris by centrifugation at 18,000 *g* for 15 minutes at 4°C, the resultant supernatant was incubated with cdb3-64 antibody overnight at 4°C with gentle agitation. To this was added Protein G-Sepharose 4FF (Amersham). After incubation for 2 hours, beads were thoroughly washed 4 times in solubilizing buffer, twice in the same buffer containing 500 mM NaCl, and finally once with 0.1% Triton X-100 in 25 mM Tris/Cl, pH 7.5. Immunoprecipitates thus obtained were analyzed by SDS-PAGE and autoradiography.

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 minutes at -20°C, washed in PBS, and blocked with 1% bovine serum albumin in PBS for 30 minutes at ambient temperature. Subsequently, cells were incubated with the anti-calnexin, anti-GM130, or anti-Lamp2 antibodies for 1 hour, washed with PBS and then incubated with a 1:500 dilution of AlexaFluor 568-labeled anti-murine or rabbit IgG 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 Nikon ECLIPSE microscope equipped with a deconvolution apparatus (Nikon, Tokyo, Japan). When the cells transfected with AE1 WT or its mutants without EGFP tags, the cells were first reacted with the anti-bovine AE1 antibody cdb3-64 or anti-mouse AE1 antibody described above.

Results

The plasma membrane expression of the C-terminal truncation mutants of bovine AE1 in HEK293 cells

We first generated EGFP-tagged C-terminal mutants of bovine erythroid AE1 missing 18 (ΔCt18) or 28 (ΔCt28) C-terminal amino acid residues. Figure 2A shows the predicted topology model for the last two transmembrane spans and the C-terminal cytosolic region of bovine AE1 based on Cys-scanning mutagenesis analysis of human AE1 (Zhu *et al*., 2003) and the positions of the stop codons introduced to generate ΔCt18 and ΔCt28 mutants.

HEK293 cells transfected with EGFP-AE1 WT and ΔCt18 showed prominent fluorescent signals at the cell periphery, indicating that these proteins were expressed at the plasma membrane (Fig. 3). Fluorescent signals in the cytoplasm with reticular patterns comparable to those of the ER marker calnexin were not obvious for EGFP-AE1 WT and ΔCt18 mutants. In contrast, the localization of EGFP-AE1 ΔCt28 was limited intracellularly, consistent with that of the ER (Fig. 3A). Some of the signals of EGFP-AE1 ΔCt28 with juxtanuclear localization were merged with those of the Golgi-resident protein GM130 but not with those of the lyzosome marker Lamp2, demonstrating that EGFP-AE1 ΔCt28 principally localized to the ER and partly in the Golgi apparatus, but not in lysosomes. These results suggested that 18 C-terminal amino acids were not indispensable for membrane trafficking of bovine AE1 and that the region surrounding ΔCt28 mutation had some crucial role in plasma membrane targeting of AE1. We also confirmed by immunofluorescent detection with the cdb3-64 antibody that the proteins without EGFP tags had the same localizations, *i.e.,* AE1

WT and ΔCt18 profoundly localized to the plasma membrane, whereas AE1 ΔCt28 exhibited intracellular localization (data not shown).

To confirm the data from (immuno)fluorescence microscopy analyses, expression of bovine AE1 mutants at the plasma membrane was examined by cell-surface biotinylation (Fig. 3B). In immunoblotting, signals with the appropriate size of 105 kDa were observed for WT AE1 in the fraction of biotinylated proteins bound to Streptavidin beads, as well as in other fractions (Fig. 3B, *WT, Bound*). The signal was not detected in the eluate from the biotin-labeled fraction prepared from the cells expressing the WT that were mock-treated without biotin compounds (Fig. 3B, *WT(-), Bound*) and immunoblotting for actin in the same fraction gave no signals (data not shown), confirming the specific detection of biotinylated AE1 in our procedure. Considering that the biotinylated protein fraction contained a sample that was overloaded 12-fold compared with other fractions, by densitometric scanning of the blot we estimated that about 5.5% (n = 2, mean value) of total WT AE1 present in the transfected cells, the sum of the total and detergent-insoluble fractions, was expressed at the cell surface. The C-terminal truncation mutants ΔCt18 gave immunoblot patterns similar to that for the WT and the proteins present at the cell surface were estimated to be about 4.5% (n = 2, mean value) of the total contents for ΔCt18 mutant, indicating their presence at the cell surface. In contrast, no signal was detected for ΔCt28 mutant in the bound fraction containing biotinylated proteins (Fig. 3B) in good agreement with the lack of fluorescent signals in the plasma membrane (Fig. 3A).

Effects of mutations of the conserved amino acid residues in the C-terminal tail on the stability and membrane trafficking of AE1

The amino acid sequence surrounding the ΔCt28 mutation site contained six amino acid residues, Glu^{901}, Leu^{902}, Leu^{905}, Asp^{906}, Asp^{908}, and Asp^{909}, in the sequence E901L(K/Q)(L/C)LD(A/G)DD909, which is highly conserved among various mammals and includes binding site LDADD for CAII (Vince *et al*., 2000; Vince and Reithmeier, 1998; Vince and Reithmeier, 2000) (Fig. 2B). We generated a series of AE1 mutants in which these amino acids were replaced by Ala, or Glu (Fig. 4A), and analyzed the effect of substitutions on the membrane expression of AE1 in HEK293 cells.

When EGFP-tagged AE1 E901A, L902A, L905A, and L906A were transfected into HEK293 cells, fluorescent signals of these proteins showed intracellular localization without apparent signals at the plasma membrane (Fig. 4B). The signals were comparable with those for the ΔCt28 mutant described above and were principally merged with those of calnexin (data not shown). By contrast, EGFP-AE1 D908A, D909A, and D908A/D909A presented abundant signals at the cell periphery, indicating localization at the plasma membrane as observed for the WT. In addition, substitution of Glu for Asp^{906} (D906E) appeared to have little effect on the membrane localization, suggesting that the presence of a negatively charged residue at this position was essential. Similar results were obtained for AE1 mutants without EGFP tags by immunofluorescence microscopy in transiently transfected HEK293 cells (data not shown).

In cell-surface biotinylation analysis, AE1 D908A and D909A were found in the biotin-bound fractions, confirming the plasma membrane expression of these mutants,

although the signal intensities relative to the total amount were 2.0-2.4% (n=2, mean value), much less than those observed for the WT (Fig. 4B). In contrast, none of the AE1 mutants, E901A, L902A, L905A, and D906A, showed immunoreactive bands in the fractions containing biotinylated proteins at a demonstrable level (Fig. 4B). These data demonstrated that AE1 E901A, L902A, L905A, and D906A were not expressed at the cell surface but retained inside the cells, whereas other mutants were transported to the plasma membrane. These results were principally the same as those obtained in microscopic analyses, and indicated that the conserved amino acids Glu^{901}, Leu^{902}, Leu^{905}, and Asp^{906}, but not Asp^{908} and Asp^{909} in the sequence E901L(K/Q)(L/C)LD(A/G)DD909 were important in expression of AE1 in the plasma membrane.

We also analyzed localization of the WT and several mutants of murine AE1, since two amino acids, Lys^{903} and Leu^{904}, intervening between Leu^{902} and Leu^{905} in bovine AE1 were different from those of Gln and Cys found in AE1 homologues of other mammalian species (Fig. 2B) and might cause different localization of the protein. As shown in Fig. 4C, while murine AE1 WT presented plasma membrane localization, all of the EGFP-tagged murine AE1 mutants, E900A, L901A, L904A, and L905A, demonstrated intracellular distribution in transfected HEK293 cells, consistent with those observed for corresponding bovine mutants E901A-D906A. These data indicated that the species difference of amino acid residues at the positions corresponding to Lys^{903} and Leu^{904} in bovine AE1 did not affect the membrane expression of AE1.

The results obtained above suggested that the conserved region, particularly the sequence EL(K/Q)(L/C)LD, would have some roles in the membrane trafficking or stability

of AE1. Therefore, we examined if the mutations at the conserved residues affected the stability of proteins. Transfected HEK293 cells were pulse-labeled with [^{35}S]methionine for 20 minutes and chased for 0, 1, 4, or 8 hours in the presence or absence of 10 μM lactacystin followed by immunoprecipitation of AE1 proteins (Fig. 5A). Figure 5B summarizes the densitometric scanning of the signals of autoradiography for retained proteins at the indicated chase periods. AE1 D908A, which had cell surface expression, showed reduction pattern similar to that for the WT with a half-life ($t_{1/2}$) of about 6 hours, while ΔCt28 and E901A mutants exhibited slightly increased turnovers compared to the WT ($t_{1/2}$ = 4.5 ~ 5 hours). The turnover rates of WT and mutants in the presence of lactacystin were markedly increased compared with those seen in the absence of inhibitor and ΔCt28 and E901A mutants had accelerated turnovers ($t_{1/2} \approx$ 8 hours) compared to those for WT and D908A AE1 ($t_{1/2} \geq$ 8 hours).

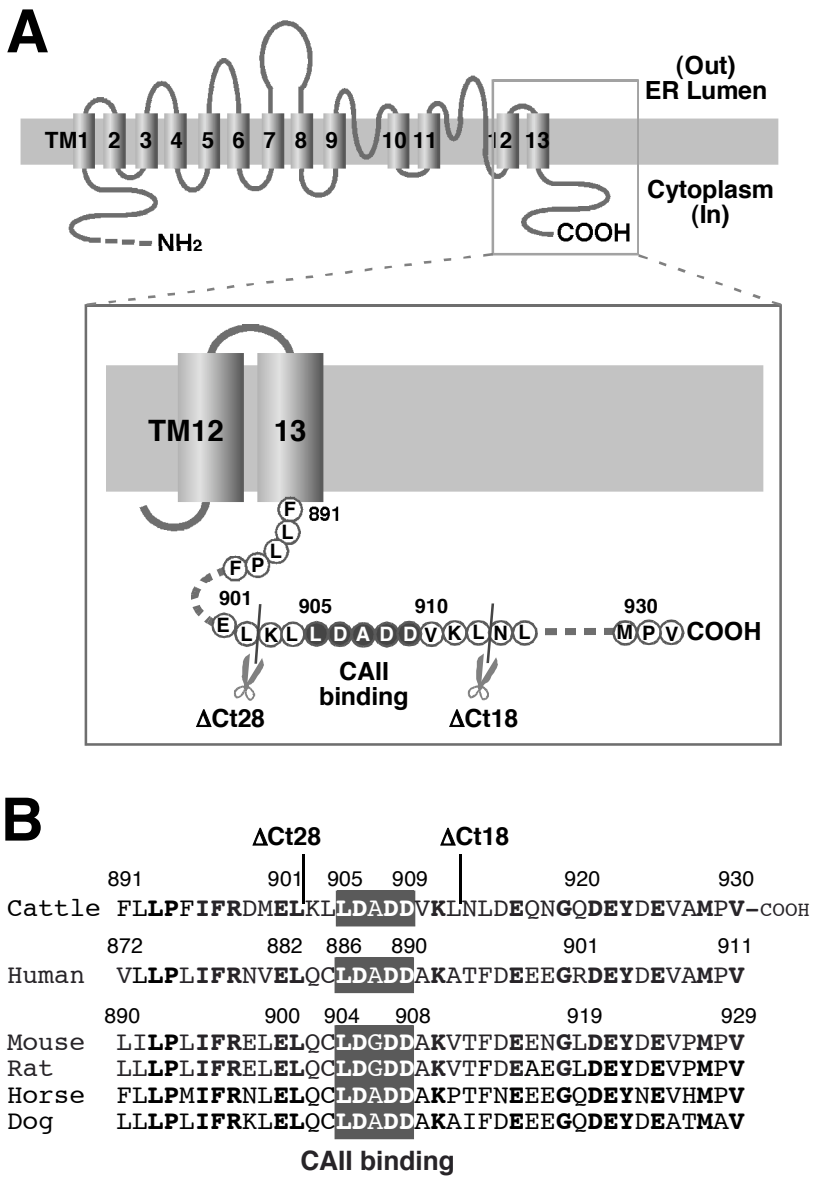

Figure 2. Schematic model of bovine AE1 and comparison of the sequences of the cytoplasmic tails of AE1 from various mammals.

A. Schematic model of the transmembrane domain of bovine AE1 with the positions of introduced truncation mutations used in this study (*ΔCt28* and *ΔCt18*). Membrane topology of AE1 is based on the study by Zhu *et al.* (2003). B. Comparison of amino acid sequences of the C-terminal cytosolic tails of AE1 from various mammals. Amino acid residue numbers of bovine, human, and murine AE1 and the positions of truncation mutations are indicated for comparison. Conserved amino acid residues among mammals are indicated in bold face. Amino acid residues in the CAII binding sequence LD(A/G)DD are highlighted. Bovine, equine, and canine sequences were determined in our laboratory (GenBank accession numbers NM_181036, AB242565, and AB242566, respectively).

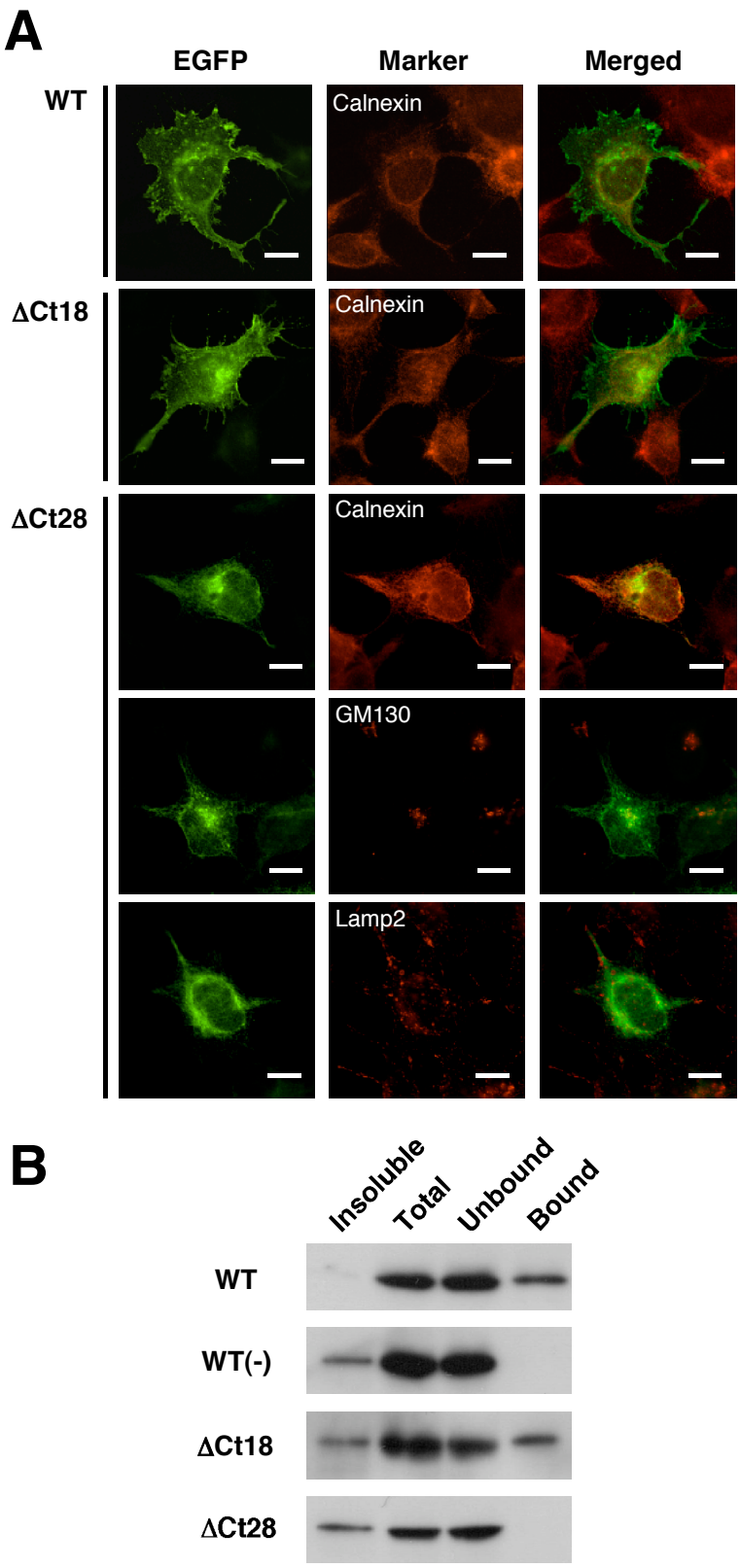

Figure 3. Expression of the C-terminal truncation mutants of bovine AE1 in transfected HEK293 cells.

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Figure 3. Expression of the C-terminal truncation mutants of bovine AE1 in transfected HEK293 cells.

A. HEK293 cells transfected with EGFP-tagged bovine AE1 WT, ΔCt18, and ΔCt28, were probed with anti-calnexin, anti-GM130, and anti-Lamp2 antibodies, followed by detection with a secondary antibody labeled with AlexaFluor 568 and examined by fluorescence microscopy. Bars = 10 μm. B. Cell surface biotinylation analysis for AE1 WT, ΔCt18, and ΔCt28. The immunoblots show the total amounts of AE1 in the soluble fractions (*Total*), AE1 not bound to NutrAvidin beads (*Unbound*), and biotin-labeled AE1 bound to the NutrAvidin beads (*Bound*). The amounts of AE1 in the detergent-insoluble fractions of the transfected cells are also indicated (*Insoluble*). The *WT(-)* panel shows the blot obtained for the cells transfected with AE1 WT and processed by exactly the same procedure except for no addition of biotinylating reagent. The fraction bound to the Streptavidin beads was 12-fold overloaded with respect to other fractions to obtain comparable band densities within the linear range of the chemiluminescence.

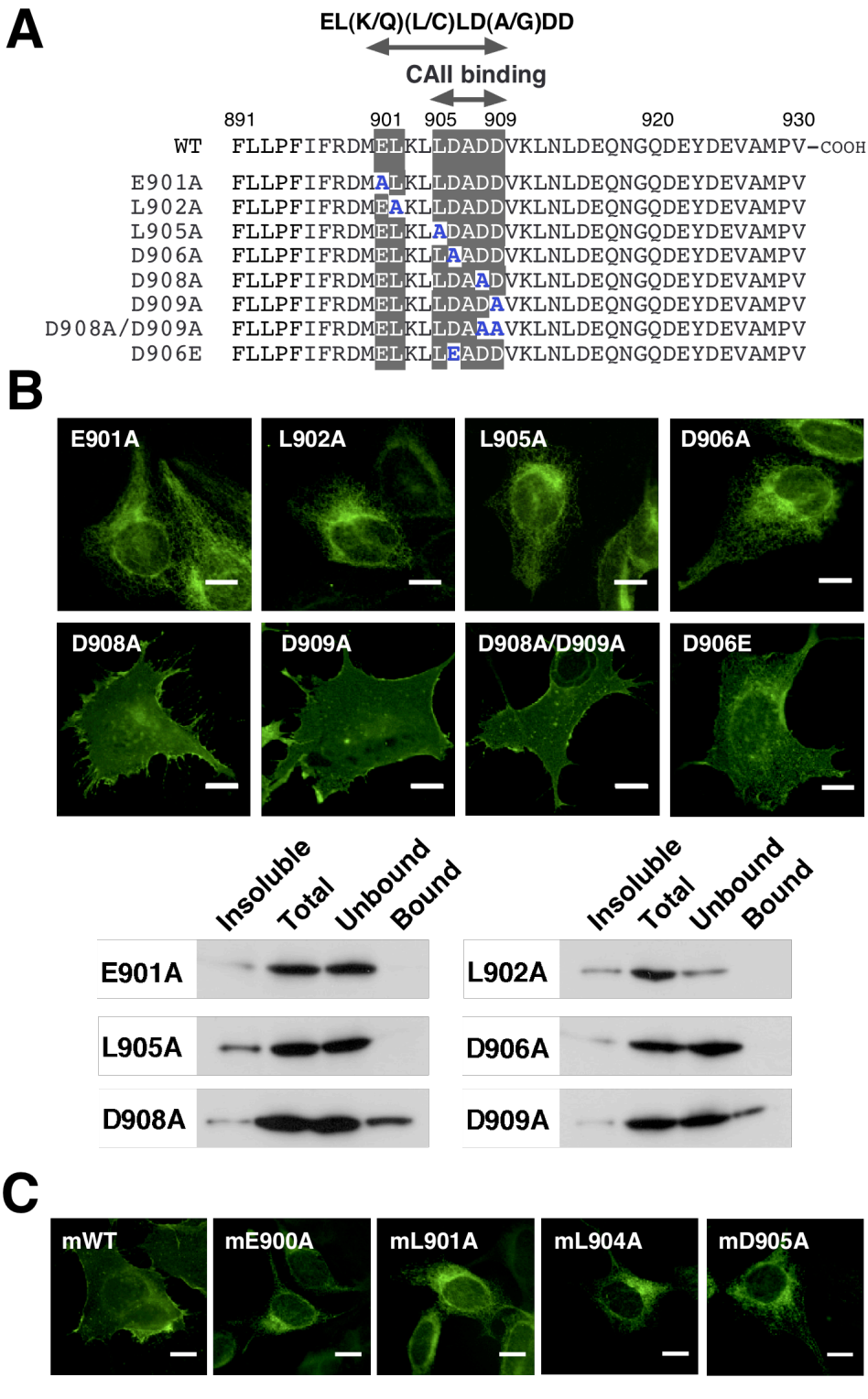

Figure 4. Effects of Ala conversion mutations in the C-terminal tail on the membrane expression of AE1 in transfected HEK293 cells.

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Figure 4. Effects of Ala conversion mutations in the C-terminal tail on the membrane expression of AE1 in transfected HEK293 cells.

A. Positions of the introduced Ala substitutions in the sequence of EL(K/Q)(L/C)LD(A/G)DD in various bovine AE1 mutants are indicated. B. Distributions of EGFP-tagged AE1 E901A, L902A, L905A, D906A, D908A, D909A, D908A/D909A, and D906E mutants in transfected HEK293 cells. Bars = 10 μm. Cell surface expressions of AE1 E901A, L902A, L905A, D906A, D908A, and D909A mutants were also determined by biotinylation and are shown as described in the legend for Fig. 2. C. EGFP-tagged mouse AE1 WT and Ala conversion mutants E900A, L901A, L904A, and D905A, corresponding to bovine mutants E901A, L902A, L905A, and D906A, respectively, were expressed in HEK293 cells and examined for their subcellular localization by fluorescence microscopy. Bars = 10 μm.

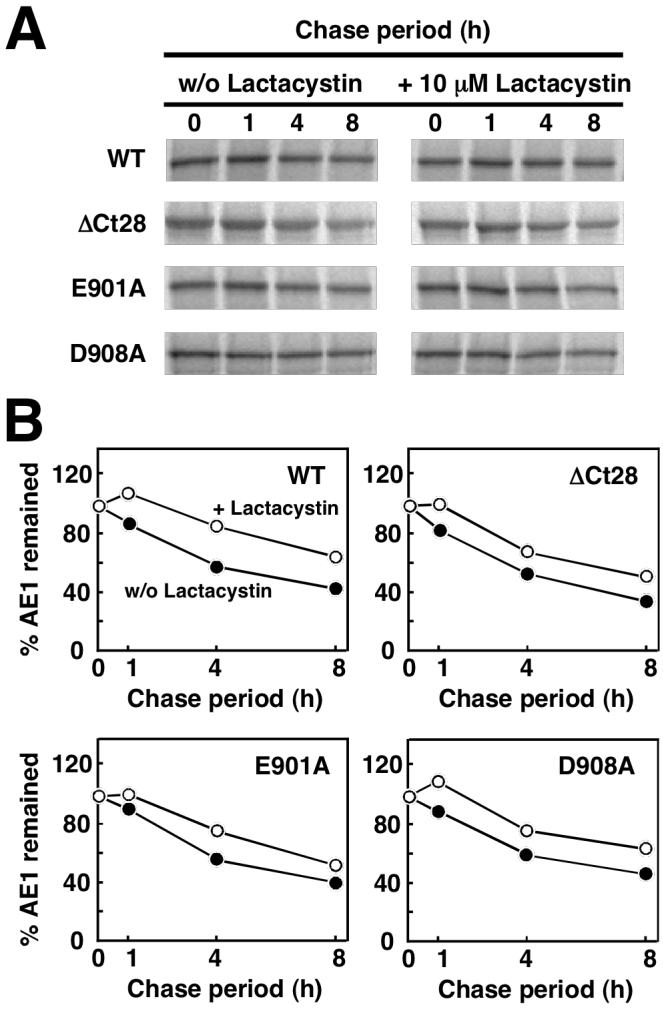

Figure 5. Turnovers of the WT and various C-terminal mutants of AE1 in transfected HEK293 cells.

A. Transfected cells were labeled with 100 μCi/ml of [^{35}S]methionine for 20 minutes and then chased for 0, 1, 4, and 8 hours in the presence or absence (*w/o*) of 10 μM lactacystin. Labeled AE1 proteins (WT and ΔCt28, E901A, and D908A mutants) were then immunoprecipitated with the anti-bovine AE1 antibody cdb3-64, collected with Protein-G Sepharose, separated by SDS-PAGE, and visualized by autoradiography. B. The intensities of AE1 polypeptides retained after the indicated chase periods were quantitated by densitometric scanning of autoradiography and expressed as percentages of the initial values without chase. The data represent the mean of two independent experiments.

Discussion

The present study demonstrates that 4 amino acids Glu^{901}, Leu^{902}, Leu^{905}, and Asp^{906} within the sequence E901LKLLDADD909 in the C-terminal tail are important for the expression of bovine AE1 in the plasma membrane. Although bovine AE1 differs from other known AE1 in amino acid residues within this sequence at the 3rd and 4th positions (Fig. 2B), loss of the plasma membrane expression of murine AE1 mutants corresponding to bovine AE1 E901A, L902A, L905A, and D906A in HEK293 cells (Fig. 4C) supports the hypothesis that, in general, Glu^{1}, Leu^{2}, Leu^{5}, and Asp^{6} in the sequence EL(K/Q)(L/C)LD(A/G)DD conserved among various mammals are critical for proper membrane expression of the AE1 protein.

Previous studies by Zhu *et al*. (Zhu *et al*., 2003) demonstrated that 40 C-terminal amino acid residues of human AE1 had cytoplasmic localization by analyzing accessibilities to biotin-derivatives and anion transport activities in HEK293 cells of a series of Cys substitution mutants for Phe^{806} through Gln^{884}. Their data also showed that the mutations at Glu^{882} and Leu^{883}, corresponding to Glu^{901} and Leu^{902} in bovine AE1, generated functionally inactive mutants with no substantial anion exchange activity in transfected HEK293 cells, whereas mutants with Cys substitution at the surrounding residues Asn^{880}, Val^{881}, and Gln^{884} exhibited anion transport activities comparable to that of the wild type (Zhu *et al*., 2003). The authors hypothesized that these mutations affected the binding of CAII to the region L886DADD890 adjacent to the mutation sites, which had been shown to be required for full AE1 activity (Vince *et al*., 2000; Vince and Reithmeier, 1998; Vince and Reithmeier, 2000),

or that the mutations at these sites impaired processing of AE1 to the cell surface.

Although the mutations in the core CAII binding site of LDADD to ADADD or LDAAA abolished the CAII binding (Vince and Reithmeier, 2000), our present study demonstrated that the corresponding constructs L905A and D908A/D909A showed distributions in the transfected cells totally distinct from each other, *i.e.*, AE1 L905A was internally retained, whereas D908A/D909A was expressed at the cell surface (Fig. 4B). These data are consistent with the previous observation in *Xenopus* oocytes by Dahl *et al*. (Dahl *et al*., 2003). They showed that ADADD and LDAAA mutants of AE1 retained Cl^-/Cl^- exchange activity at a level similar to that of the wild-type AE1, whereas these mutants exhibited no Cl^-/HCO_3^- exchange, indicating that the mutants ADADD and LDAAA were present at the cell surface but their Cl^-/HCO_3^- exchange activities were abolished due to the absence of CAII binding to the protein. In addition, bovine AE1 D906A showed intracellular retention (Fig. 4B), whereas the corresponding mutation of LDADD to LAADD caused no change in binding with CAII (Vince and Reithmeier, 2000).

These findings indicate that impaired membrane expression of AE1 with selected missense mutations in the EL(K/Q)(L/C)LD(A/G)DD sequence and the lack of CAII binding to the protein do not correlate with each other. Therefore, the residues Glu^{901}, Leu^{902}, Leu^{905}, and Asp^{906} with the conserved sequence E901L(K/Q)(L/C)LD906 are likely to be involved in the folding or membrane trafficking of AE1 and substitution of each of these residues by Ala is enough to impair the processing of AE1 to the plasma membrane. First, all of the AE1 mutants, E901A, L902A, L905A, and D906A, as well as the C-terminal truncation mutant AE1 ΔCt28, showed intracellular retention with primary localization to the ER (Figs. 3 and 4)

and we demonstrated that some of them (ΔCt28 and E901A) had slightly shortened half-lives compared to WT AE1 (Fig. 5). Moreover, degradation of ΔCt28, E901A, and D908A mutants as well as WT AE1 involves the proteasomal pathway in the ER (Fig. 5) as we reported previously for WT and R664X AE1 (Ito *et al*., 2006). These findings indicate that the mutants are principaly retained in the ER and degraded by the proteasomal ER-associated degradation presumably due to their structural abnormalities (Ellgaard and Helenius, 2003). Such ER-retarded structural abnormality is probably implicated in recognition with ERAD machineries to be degraded via proteasomal pathway. Second, Cordat *et al*. (Cordat *et al*., 2003) demonstrated that truncation mutants of human AE1 missing 20 and 35 C-terminal amino acid residues, but not the last 15 residues, were misfolded and showed reduced expression when transfected into HEK293 cells. Taken together with that ΔCt18 AE1 showed effective expression in the plasma membrane (Fig. 3), our findings are in good agreement with their observation and suggest that the region required for proper folding may extend several residues to the C-terminal side of the EL(K/Q)(L/C)LD sequence.

The structural study by Mori *et al*. (Mori *et al*., 1995) reported that the region containing the sequence EL(K/Q)(L/C)LD became susceptible to trypsin only after alkaline treatment of the membrane, suggesting that this region was shielded. They suggested that the sequence (V/L)EXXXLD(A/G)DD, found in the C-terminal tail in both AE1 and aquaporin-1, might contribute to some role of the C-terminal region in polytopic membrane proteins in proper membrane insertion and consequent folding of this region into the molecule *in situ*, or targeting to the plasma membrane (Mori *et al*., 1995). Our data partly supported this assumption and defined the amino acid residues essential for cell surface expression of AE1.

Amino acid sequences similar to EL(K/Q)(L/C)LD are actually found in the corresponding regions of two other members of the human *SLC4A1* gene family, AE2 and AE3, with the sequences EMKCLD and ELQALD, respectively, indicating that the 1st, 5th, and the last amino acids are identically conserved among anion exchangers AE1-AE3 and the 2nd positions are hydrophobic. This assumption also fits the corresponding sequences of AE1 from non-mammalian vertebrates including chicken and zebrafish AE1 (GenBank accession numbers NM_025522 and NM_198338, respectively). Taking into account that the AE1 D906E mutant showed plasma membrane targeting as shown in Fig. 4, the last amino acid residue should be acidic. Two-dimensional crystallographic analysis (Wang *et al*., 1993) of the membrane domain of AE1 revealed a mobile subunit in the protein, which may be the last two transmembrane spans (Groves and Tanner, 1999). Thus, it is likely that the folding confered by the EL(K/Q)(L/C)LD sequence occurs in these last two membrane spans or in the membrane spans with the preceding candidate re-entrant loop (Zhu *et al*., 2003).

Finally, our finding on the plasma membrane expression of bovine AE1 ΔCt18 mutant (Fig. 3) is totally different from the previous observations for intracellular retention of Δ11 mutant of human AE1 responsible for dominant renal tubular acidosis (Devonald *et al*., 2003; Quilty *et al*., 2002; Toye *et al*., 2004; Toye *et al*., 2002). In parallel experiments, we actually observed that bovine ΔCt11 but not human ΔCt11 AE1 was transported to the plasma membrane (W. Otsu, T. Kurooka, D. Ito, H. Adachi, Y. Otsuka, K. Sato, and M. Inaba, unpublished observation). There would be some difference between human and bovine AE1 in regulation of their trafficking to the plasma membrane, though the precise mechanism remains unknown.

In conclusion, the present study demonstrates that well-conserved amino acid residues Glu^{1}, Leu^{2}, Leu^{5}, and Asp^{6} in the conserved sequence of EL(K/Q)(L/C)LD(A/G)DD found in the C-terminal cytoplasmic tail have essential structural consequences in expression of AE1 in the plasma membrane. Demonstration of the binding site for intra- or intermolecular association of this region is of interest, since it is involved in membrane expression of AE1 as shown in the present study, and it also appears to be related to anion transport by AE1 through interprotomeric interaction of CAII bound to the C-terminal tail with an adjacent molecule in the AE1 homodimer (Dahl *et al*., 2003).

Summary for Chapter 1

While the C-terminal cytoplasmic tail of anion exchanger 1 (AE1, band 3) has been reported to possess important physiological roles, including one for proper membrane trafficking, its precise characteristics remain unclear. To clarify the overall structural consequences of the conserved sequence EL(K/Q)(L/C)LD(A/G)DD, containing the core binding sequence LDADD for carbonic anhydrase II, in the C-terminal region, we analyzed the membrane expression and turnover of bovine AE1 with a series of truncation and substitution mutations in HEK293 cells. Immunofluorescence microscopy and cell-surface biotinylation demonstrated that truncation mutants missing 18 C-terminal residues targeted the plasma membrane, but the one lacking the conserved region, by truncation of 28 amino acid residues, was retained inside the cells. Substitutions of Ala for Glu^{901}, Leu^{902}, Leu^{905}, and Asp^{906} in the sequence $E^{901}L(K/Q)(L/C)LDADD^{909}$ of bovine AE1 or those in the corresponding murine sequence also caused intracellular retention, though these mutants had half-lives comparable to that for wild-type AE1. These data demonstrate that the conserved amino acid residues Glu^{1}, Leu^{2}, Leu^{5}, and Asp^{6} in the EL(K/Q)(L/C)LD(A/G)DD region have essential structural consequences in stable expression of AE1 at the plasma membrane regardless of the ability in binding to carbonic anhydrase II of this region.

Chapter 2

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

Introduction

Misfolded or unassembled proteins in the ER are recognized by ERAD machineries of the ER quality control mechanisms and are retrotranslocated from the ER into the cytosol for degradation by the proteasome system, a process known as ERAD (Ellgaard and Helenius, 2003; Vember and Brodsky, 2008). CFTR and its most common mutant, ΔF508-CFTR are typical transmembrane proteins in mammalian cells whose ERAD have been well characterized (Jensen *et al*., 1995; Ward *et al*., 1995; Gelman *et al*., 2002; Johnston *et al*., 1998). Like most ERAD substrates, CFTR and ΔF508-CFTR require ubiquitylation prior to binding to the proteasome and consequent degradation by the proteasome (Ward *et al*., 1995; Gelman *et al*., 2002). Our previous study showed that bovine AE1 with a nonsense mutation of R664X (band $3^{Bov.Yamagata}$), which is responsible for hemolytic anemia associated with HS in cattle, is degraded via ERAD in transfected K562 and HEK293 cells (Adachi *et al*., 2009; Ito *et al*., 2006; Ito *et al*., 2007). The studies in the previous Chapter demonstrated that various mutants of bovine AE1, as well as the wild-type AE1, can be ERAD substrates (Fig. 5). However, our previous study showed that the mechanisms underlying degradation of R664X AE1 differed significantly from those of CFTR (Fig. 1). The most notable difference was the absence of AE1 ubiquitylation (Ito *et al*., 2006). Although there is accumulating evidence that a number of cytosolic proteins are privileged ubiquitin-independent proteasomal substrates (Sheaff *et al*., 2000; Hwang *et al*., 2005; Jariel-Encontre *et al*., 2008), 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 (Johnston *et al*., 1998; Kopito, 2000). The aggresome is a microscopically visible, pericentriolar structure and is now known to be involved in the aggresome-autophagy pathway (Garcia-Mata *et al*., 2002; Kopito, 2000; Xie and Klionsky, 2007). 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 (Ito *et al*., 2006). 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 (Appenzeller and Hauri, 2006) prior to retrotranslocation and degradation. Similar findings have been reported for several other transmembrane proteins (Mayer *et al*., 1998; Plemper *et al*., 1998), 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 (Inaba *et al*., 1996; Ito *et al*., 2006). 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 (Ito *et al*., 2006). The plasmid clone pEGFP-DF508-CFTR (Loffing-Cueni *et al*., 2001) 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 (Michaely and Bennett, 1995; Stefanovic *et al*., 2007). 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 hours of incubation; when present, the cells were incubated for 4 hours with nocodazole, after which lactacystin was added, followed by further incubation for 8 hours.

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 minutes on ice. After removal of cell debris by centrifugation at 18,000 *g* for 15 minutes at 4˚C, proteins in the supernatant were separated by 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 hours, 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 minutes at -20˚C, washed in PBS, and blocked with 1% bovine serum albumin in PBS for 30 minutes at ambient temperature. Subsequently, cells were incubated with the appropriate antibodies for 1 hour, washed with PBS and then incubated with a 1:500 dilution of anti-murine or rabbit IgG labeled with 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 (Ito *et al*., 2006) (Fig. 6A). When the cells were incubated with the proteasome inhibitor 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 (Kopito, 2000). 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. 6B), in agreement with a previous report (Johnston *et al*., 1998). Similar characteristic aggresome formation was observed for EGFP-AnkN90 consisting of the N-terminal AE1-binding domain of ankyrin (Michaely and Bennett, 1995; Stefanovic *et al*., 2007) (Fig. 6C). 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 (Ito *et al*., 2006) 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. 7A). 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. 7A). These small aggregates, which had a distribution consistent with the ER marker calnexin, were not observed in the cells expressing R664X AE1 alone (Fig. 6A), and appeared to represent the aggregates found in nocodazole- and lactacystin-treated cells expressing EGFP-ΔF508-CFTR (Fig. 6B). 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 (Michaely and Bennett, 1995; Stefanovic *et al*., 2007). However, their signals were basically consistent with those of the ER and were not detected in the areas of pericentriolar aggresomes (Fig. 7B). 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. 8). 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 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.

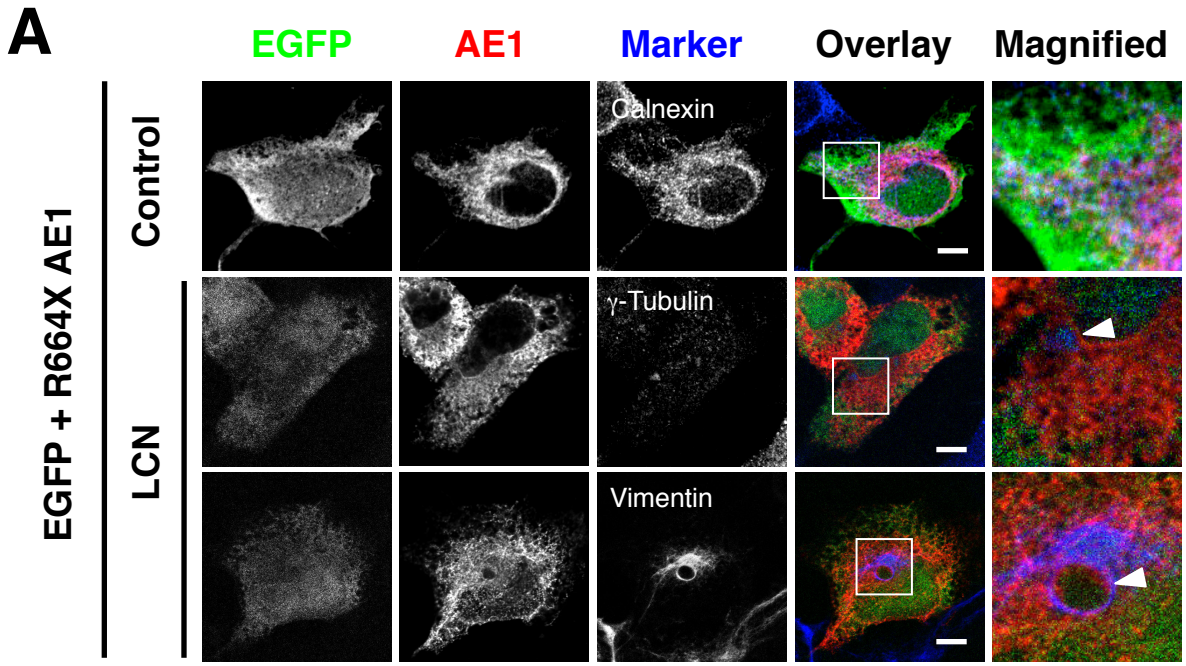

Figure 6. Difference in aggresome formation between R664X AE1 and ΔF508-CFTR.

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.

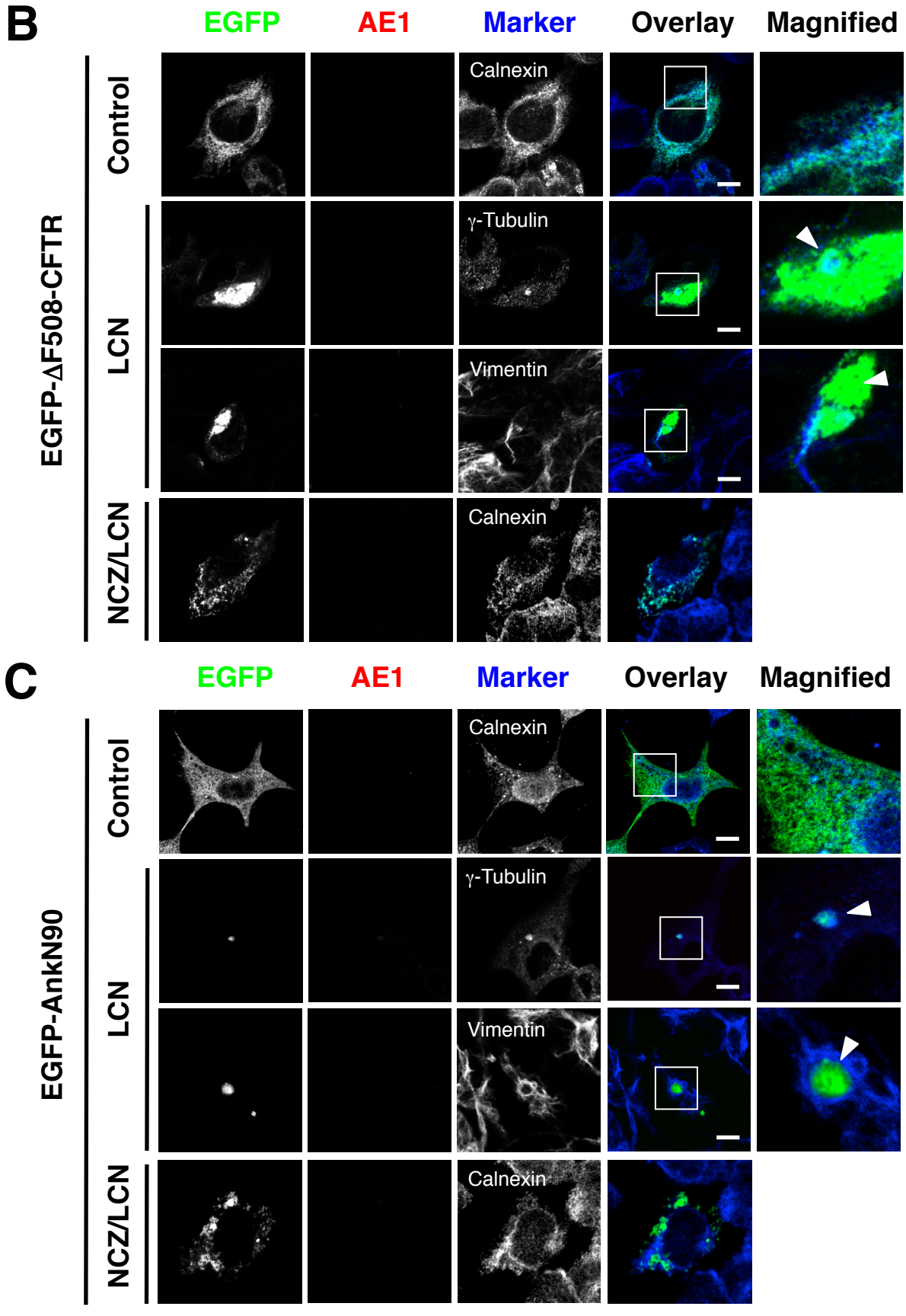

Figure 6. (Continued)

A
EGFP-ΔF508-CFTR + R664X AE1
Control
LCN
NCZ/LCN
EGFP
AE1
Marker
EGFP/AE1
Overlay
Magnified
Calnexin
γ-Tubulin
Vimentin
Calnexin

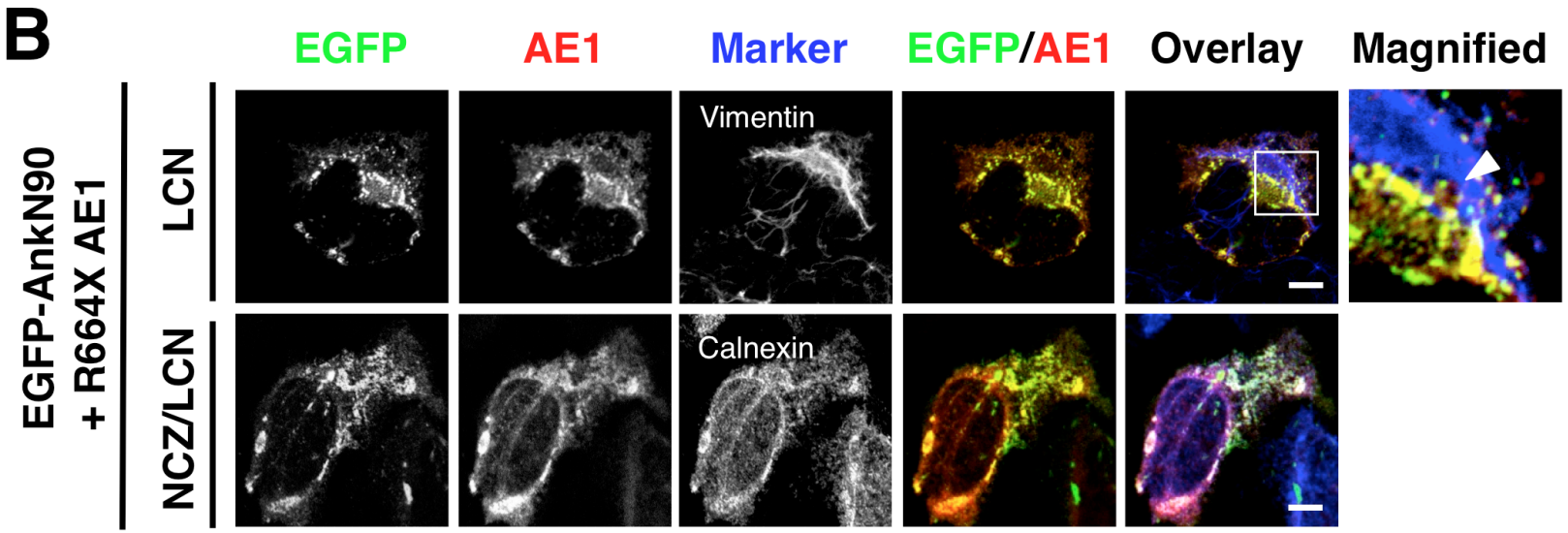

Figure 7. Intracellular localization and forced aggresome formation of R664X AE1 in the presence of ΔF508-CFTR.

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.

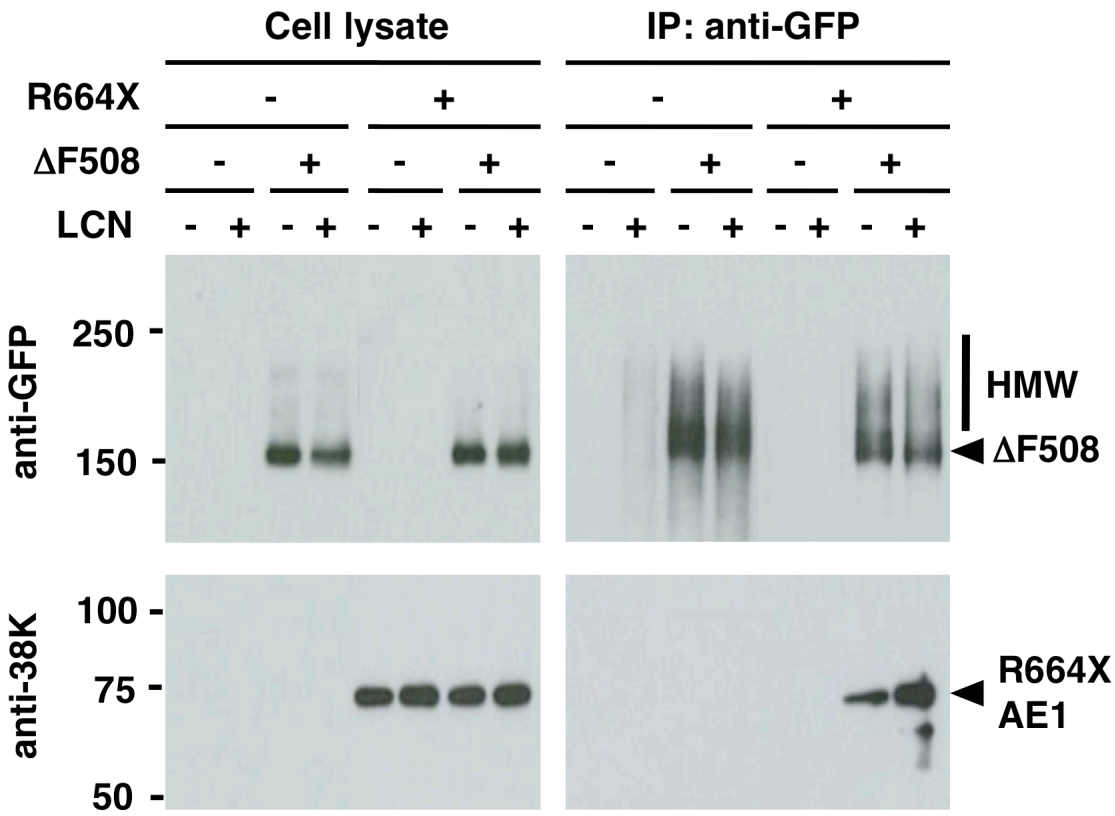

Figure 8. Co-immunoprecipitation of R664X AE1 in the immunoprecipitates of ΔF508-CFTR from the cells expressing these proteins.

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.

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 (Vembar and Brodsky, 2008). Those include recognition of the ERAD machinery Pind *et al*., 1994; Farinha and Amara, 2005), retrotranslocation from the ER to the cytosol (Jensen *et al*., 1995; Ward *et al*., 1995), and degradation by the ubiquitin-proteasome system (Ward *et al*., 1995; Gelman *et al*., 2002; Johnston *et al*., 1998). On the other hand, various mutations of the *AE1* (*SLC4A1*) gene have been reported to cause dominant HS or dominant/recessive renal tubular acidosis in humans and/or cattle (Perrotta *et al*., 2008; Yenchitosomanus *et al*., 2005; Inaba *et al*., 1996), and several AE1 mutants have been shown to be degraded via the proteasome pathway (Kittanakom *et al*., 2004; Ito *et al*., 2006).

Our previous study revealed significant differences in ERAD characteristics between R664X AE1 and ΔF508-CFTR (Ito *et al*., 2006). 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. 7). 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 (Mayer *et al*., 1998; Plemper *et al*., 1998; Nakatsukasa and

Brodsky, 2008). A study by Nakatsukasa *et al*. (Nakatsukasa *et al*., 2008) 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 (Ito *et al*., 2006), may involve mechanisms totally different from that hypothesized for various polytopic membrane proteins in the current model (Vembar and Brodsky, 2008). Cotranslocational ER protein degradation, during protein synthesis, via a proteasomal pathway through functions of the translocon Sec61 (Oyadomari *et al*., 2006) 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 ER, resulting in aggresome formation in co-localization with ΔF508-CFTR (Fig. 8). Considering that EGFP-AnkN90, which principally has cytoplasmic distribution and can associate with AE1, did not affect the ER retention of R664X AE1 (Fig. 7B), 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. 8), 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 (Nakatsukasa *et al*., 2008; Nakatsukasa and Brodsky, 2008). 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 (Kopito, 2000; Garcia-Mata *et al*., 2002; Xie and Klionsky, 2007). 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 (Tanner, 1993; Tanner, 2002), whereas CFTR is present in epithelial cells in diverse tissues (Bertland and Frizzell, 2003). 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 (Guggino and Stanton, 2006) or through a direct protein-protein association hypothesized for CFTR-ENaC interaction (Berdiev et al., 2009).

Moreover, AE1 is known to associate with glycophorin A (Auffray *et al*., 2001), one of the major sialoglycoproteins, and also forms a complex with several other transmembrane proteins in the red cell membrane (Mohandas and Gallagher, 2008; Anong *et al*., 2009). 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.

Summary for Chapter 2

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.

General Conclusion

It remains unclear how ERAD substrates are selected from proteins that are properly folded or that are on the correct folding pathway. Particularly, ERAD of polytopic membrane proteins remains mysterious (Vembar and Brodsky, 2008). Based on previous findings on the ER quality control of CFTR and ΔF508-CFTR, it is known that ERAD process of integral membrane proteins consists of several steps including recognition in the ER, retrotranslocation, and degradation by the cytoplasmic proteasomes.

The present study demonstrated that the intracellular distribution of R664X AE1 in transfected HEK293 cells is totally different from that of ΔF508-CFTR, when proteasomes are inhibited, although both proteins are found predominantly in the ER of the transfected cells under steady state condition. The ΔF508-CFTR forms aggresomes at the pericentriolar locus by the function of microtubules, whereas R664X AE1 does not. This finding was strengthened by the observation that R664X AE1 was localized in aggresomes in association with co-transfected ΔF508-CFTR due to a characteristic association of R664X AE1 with ΔF508-CFT. These results indicate that R664X AE1 can form aggresomes once dislocated into the cytosol, suggesting, in turn, that ERAD of AE1 would occur on the ER membrane without apparent ubiquitylation and retrotranslocation of the polypeptides into the cytoplasmic space.

The present study also showed that the conserved amino acid sequence, the EL(K/Q)(L/C)LD(A/G)DD sequence, within the C-terminal tail is structurally important. The data demonstrate that the conserved amino acid residues ELXXLD (X is any amino acid

residue) in this region have essential structural consequences in stable expression of AE1 at the plasma membrane regardless of the ability in binding to carbonic anhydrase II of this region. AE1 mutants, in which the conserved sequence was disrupted by substitution mutations, showed retardation in the ER followed by proteasomal degradation. The molecular structure within the cytoplasmic region of the polytopic proteins would thus be included in recognition by ERAD molecules in addition to the putative function for exit from the ER.

Taken together, these findings suggest that the N-terminal and C-terminal cytoplasmic regions of the AE1 protein may possess some signals for intracellular trafficking and/or degradation via the ERAD pathway.

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Abstract in Japanese（要　旨）

Characterization of the Plasma Membrane Targeting and the Endoplasmic Reticulum-associated Degradation of Bovine Anion Exchanger 1

（牛アニオン交換輸送体 1 の細胞膜輸送と小胞体関連分解分子機構の性状解析）

小胞体(ER)で合成された膜内在性タンパク質は、多くの分泌タンパク質と同様、ER における品質管理機構の作用で選別を受けながら細胞内の目的部位に輸送される。構造に異常のあるポリペプチドは、細胞質でユビキチン–プロテアソームシステムにより分解される(ER 関連分解、ERAD)。哺乳動物細胞における ERAD の分子機構は、従来、主に嚢胞性線維症の原因タンパク質である cystic fibrosis transmembrane conductance regulator (CFTR)と、そのΔF508 変異体(ΔF508-CFTR)を中心に進められてきた。本研究は、CFTR とは仕組みが異なることが示唆されるアニオン交換輸送体 AE1 (band 3)の ERAD についてその性状を明らかにすることを目的とした。

ERAD は、ER における認識、ER から細胞質への逆行輸送、ならびにユビキチン–プロテアソーム系によるポリペプチドの分解からなる一連のプロセスである。第 1 章では、ERAD 関連分子による認識に関わる AE1 の分子内領域を特定する一助として、C 末端細胞質内領域に焦点を充てた検討を行った。ヒト AE1 の C 末端 11 アミノ酸残基欠損変異体は、細胞膜輸送異常を来し、家族性尿細管性アシドーシスの原因となることが知られる。まず、この C 末端細胞質内ドメイン(Phe^{891}-...-Val^{903}-COO^-)の 11、18、28 各アミノ酸残基を欠失させた牛 AE1 変異体(それぞれΔCt11、ΔCt18、ΔCt28)の HEK293 細胞における細胞内分布を調べたところ、ΔCt11 とΔCt18 は野生型(WT)と同程度に細胞膜に分布したのに対し、ΔCt28 は ER に滞留した。ΔCt28 が Lys^{903} 以降の配列を欠く変異体であることから、種間で配列が保たれ、また炭酸脱水酵素 II 結合部位(L905DADD)を含む近傍領域 Glu^{901}-...-Asp^{909} のアミノ酸置換変異体を作製して ER 滞留の有無とターンオーバーを解析した。その結果、Glu^{901}、Leu^{902}、Leu^{905}、ならびに Asp^{906} の Ala 置換変異体が細胞膜に移行せず ER に留まった。これら ER 滞留変異体のターンオーバーは WT のそれと著しくは異ならなかったが、いずれもプロテアソーム阻害

剤 lactacystin 存在下での延長が認められた。この領域の配列が数カ所異なるマウス AE1 の各アミノ酸置換変異体でも同様の結果が得られた。これらの結果から、AE1 の ER 滞留、あるいは ER 以降の細胞内輸送に関わる認識に C 末端細胞質内ドメインの EL(K/Q)(L/C)LD(A/G)DD 配列の構造が関与することが明らかになった。

第２章では、従来の研究から示唆される、AE1 の ERAD がユビキチン非依存性であること、またプロテアソーム阻害時に細胞質内凝集体アグリソーム(aggresome)を形成しないことについて遺伝性球状赤血球症の原因となる牛 AE1 のナンセンス変異体 R664X AE1 を用いた検討を行った。ΔF508-CFTR の ER 滞留が見られる HEK293 細胞のプロテアソーム系を lactacystin で阻害すると、ΔF508-CFTR は微小管依存性に中心小体周囲で大きなアグリソームを形成するが、R664X AE1 の局在は変化せず ER に留まり、アグリソームには全く分布が認められなかった。ところが、両者を同時に発現させてプロテアソームを阻害すると、R664X AE1 はΔF508-CFTR とともに中心小体周囲で中間径フィラメントに囲われたアグリソームに局在した。この細胞では、R664X AE1 がΔF508-CFTR と特異的に結合していることが免疫沈降法で明らかになった。これらの結果は、AE1 の ERAD の一連の過程がΔF508-CFTR のそれと異なり ER 膜上で生じること、両者の相互作用によりΔF508-CFTR の ERAD 関連分子群の作用が AE1 の ER から細胞質への移行を生じることが明らかになった。

以上のように、本研究は、牛 AE1 の ERAD と細胞膜輸送に関する細胞質内ドメインの特定領域の関与とその ERAD が ER 膜上において生じることとを明らかにした。これらの知見は哺乳動物細胞の ER における膜内在性タンパク質の品質管理機構における未知分子機構の存在を示唆するものである。