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FULL PAPER

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

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Abstract

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 E901L(K/Q)(L/C)LDADD909 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.

Key words: AE1, band 3, carboxyl terminal tail, membrane trafficking, 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[1,14,15]. 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[10,27]. The

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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[16,20]. 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[28]. 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[19,25,26]. Previous studies on a nonsense mutation at Arg^{901} that truncates AE1 by 11 amino acid residues at the C-terminus (Δ11) showed that this mutation of AE1 caused its intracellular retention in non-polarized Madin-Darby canine kidney cells[17] and HEK293 cells[13]. 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[4,18]. 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[17] 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[19]. Although this was partly explained by the absence of the mutant transcripts, different explanations such as defective trafficking and folding[5,12] and proteolytic degradation[8,9] 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 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[21-23], and appears to involve novel intra-protomeric regulation of Cl^-/HCO_3^- exchange within dimeric AE1[3]. 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[11], 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[9]. 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[9]. The cDNAs for bovine AE1 with truncation mutations ΔCt28 (K903X) and ΔCt18 (N913X) lacking the 28 and 18 C-terminal amino acids, respectively, (Fig. 1) were obtained by PCR amplification using the primer BEBrev1 (5'-GC GGCCGCTCAGATCACTGCA-3')[9] 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

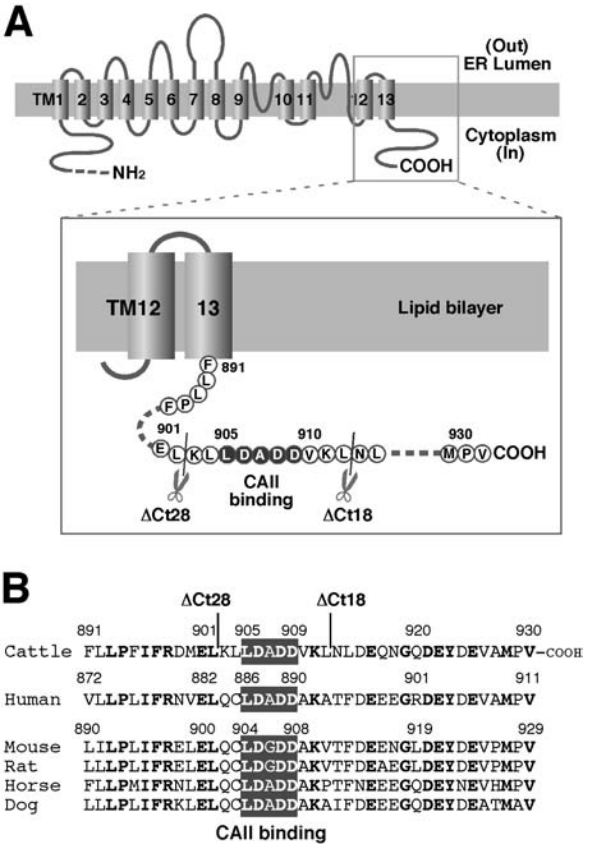


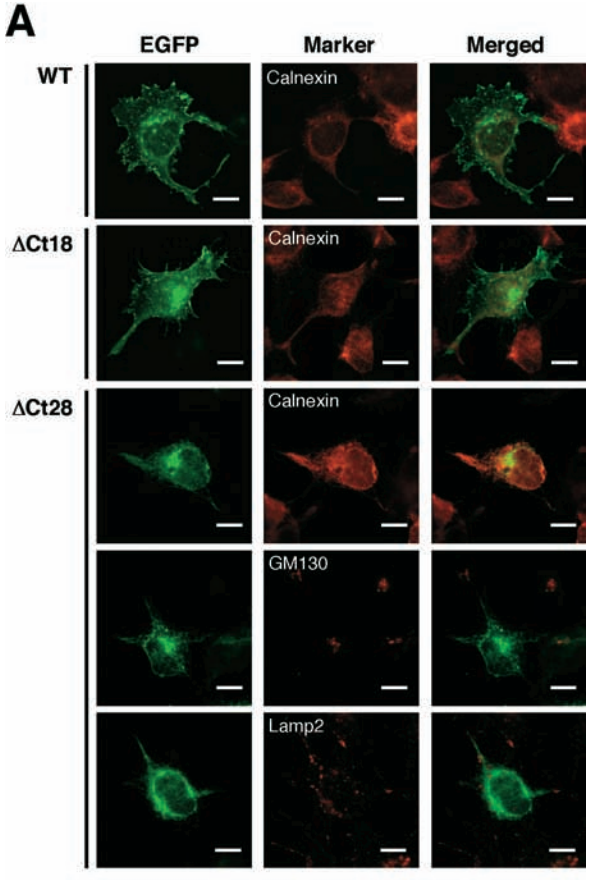
Fig. 1. Schematic model of bovine AE1 and comparison of the sequences of the cytoplasmic tails of AE1 from various mammals. A. Schematic model of the transmembrane domain of bovine AE1 with the positions of introduced truncation mutations used in this study (*ΔCt28* and *ΔCt18*). Membrane topology of AE1 is based on the study by Zhu *et al.*[28]. 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).

48 hr after transfection.

Cell surface biotinylation: Cell surface biotinylation was carried out essentially as described previously[9]. 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 min 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 (AEBSF) (all from Sigma), for 30 min on ice. After removal of insoluble materials by centrifugation at 18,000 *g* for 15 min at 4°C, an aliquot of the lysate was saved for immunoblotting. NutrAvidin beads (Pierce) were added to the lysate for 1 hr 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 hr 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 K.K., 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 min 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 min on ice. After removal of cell debris by centrifugation at 18,000 *g* for 15 min 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 hr, 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 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 anti-calnexin, anti-GM130, or anti-Lamp2 antibodies for 1 hr, 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.



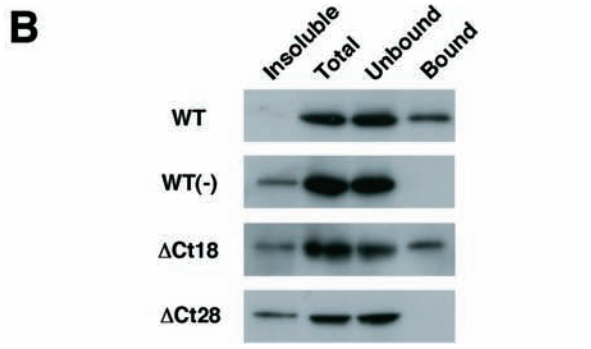


Fig. 2. Expression of the C-terminal truncation mutants of bovine AE1 in transfected HEK293 cells. A. HEK293 cells transfected with EGFP-tagged bovine AE1 WT, ΔCt18, and ΔCt28, were probed with anti-calnexin, anti-GM130, and anti-Lamp2 antibodies, followed by detection with a secondary antibody labeled 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.

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. Fig. 1A 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[28] 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. 2). Fluorescent signals in the cytoplasm with reticular patterns comparable to those of the endoplasmic reticulum (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. 2A). 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. 2B). 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. 2B, *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. 2B, *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. 2B) in good agreement with the lack of fluorescent signals in the plasma membrane (Fig. 2A).

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[21-23] (Fig. 1B). We generated a series of AE1 mutants in which these amino acids were replaced by Ala, or Glu (Fig. 3A), 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. 3B). 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. 3B). 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. 3B). These data

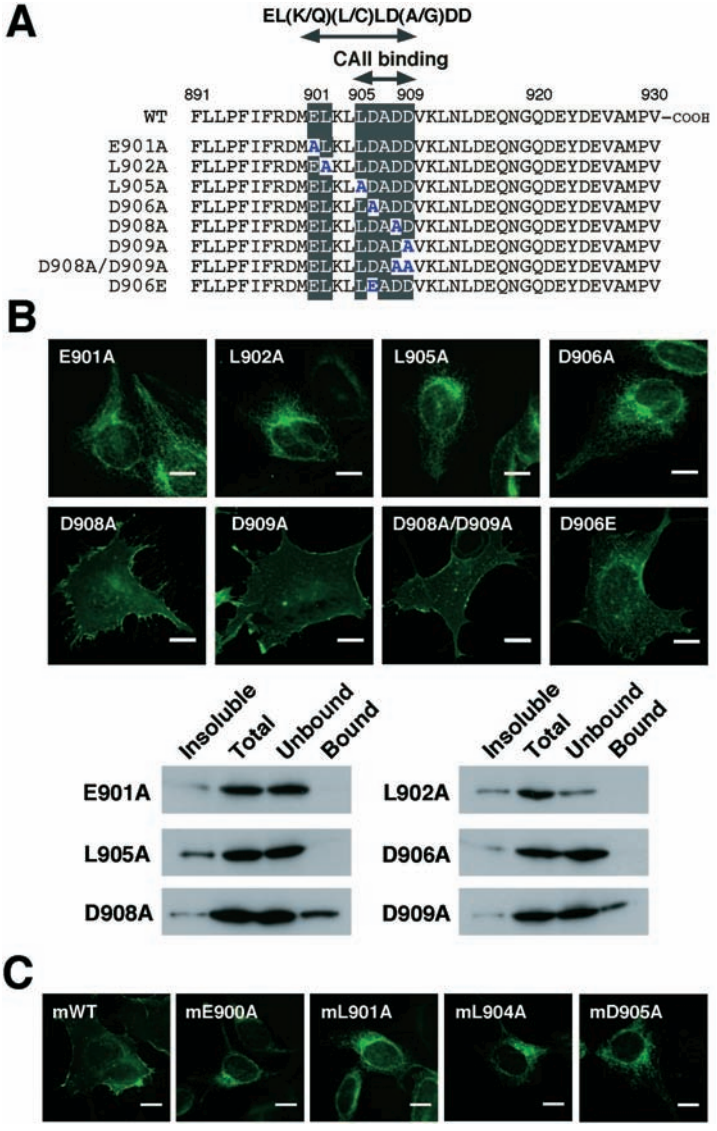


Fig. 3. Effects of Ala conversion mutations in the C-terminal tail on the membrane expression of AE1 in transfected HEK293 cells. A. Positions of the introduced Ala substitutions in the sequence of EL(K/Q)(L/C)LD(A/G)DD in various bovine AE1 mutants are indicated. B. Distributions of EGFP-tagged AE1 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.

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. 1B) and might cause different localization of the protein. As shown in Fig. 3C, 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 min and chased for 0, 1, 4, or 8 hr in the presence or absence of 10 μM lactacystin followed by immunoprecipitation of AE1 proteins (Fig. 4A). Fig. 4B 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 hr, while ΔCt28 and E901A mutants exhibited slightly increased turnovers compared to the WT ($t_{1/2} = 4.5 < 5$ hr). 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 ($t_{1/2} \approx 8$ hr) compared to those for WT and D908A AE1 ($t_{1/2} \geq 8$ hr).

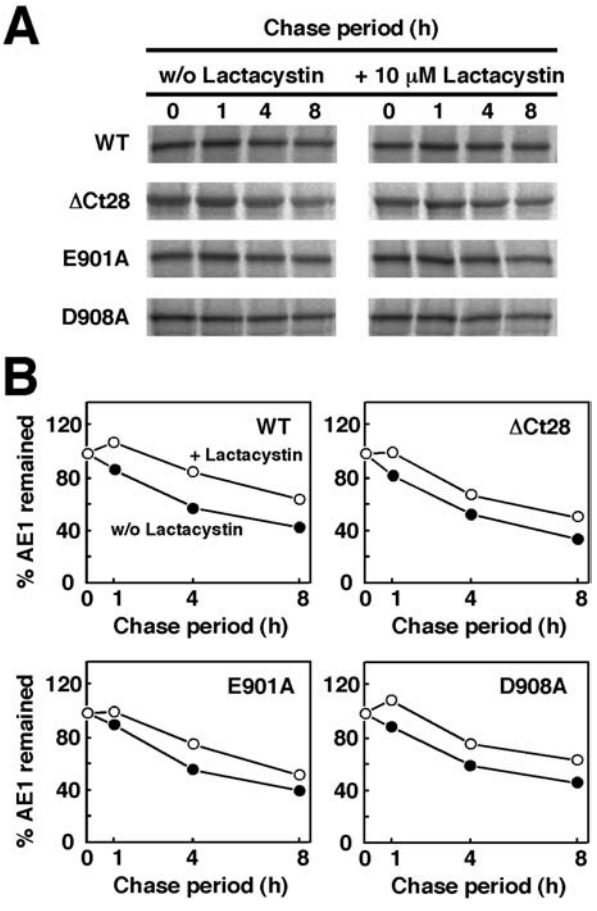


Fig. 4. Turnovers of the WT and various C-terminal mutants of AE1 in transfected HEK293 cells. A. Transfected cells were labeled with 100 μCi/ml of [^{35}S]methionine for 20 min and then chased for 0, 1, 4, and 8 h in the presence or absence (*w/o*) of 10 mM lactacystin. Labeled AE1 proteins (WT and 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. 1B), loss of the plasma membrane expression of murine AE1 mutants corresponding to bovine AE1 E901A, L902A, L905A, and D906A in HEK293 cells (Fig. 3C) 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.*[28] 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[28]. 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[21–23], 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[22], 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. 3B). These data are consistent with the previous observation in *Xenopus* oocytes by Dahl *et al.*[3]. 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. 3B), whereas the corresponding mutation of LDADD to LAADD caused no change in binding with CAII[22].

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. 2 and 3) and we demonstrated that some of them (ΔCt28 and E901A) had slightly shortened half-lives compared to WT AE1 (Fig. 4). Moreover, degradation of ΔCt28, E901A, and D908A mutants as well as WT AE1 involves the proteasomal pathway in the ER (Fig. 4) as we reported previously for WT and R664X AE1[9]. 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[6]. Second, Cordat *et al.*[2] 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. 2), 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.*[11] 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[11]. 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. 3, the last amino acid residue should be acidic. Two-dimensional crystallographic analysis[24] of the membrane domain of AE1 revealed a mobile subunit in the protein, which may be the last two transmembrane spans[7]. 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[28]. The precise relationship between membrane expression of AE1 and the structural consequences of the sequence EL(K/Q)(L/C)LD remains to be clarified.

Finally, our finding on the plasma membrane expression of bovine AE1 ΔCt18 mutant (Fig. 2) is totally different from the previous observations for intracellular retention of Δ11 mutant of human AE1 responsible for dominant renal tubular acidosis[4,13,17,18]. 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[3].

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