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NOTE

Lumenal localization in the endoplasmic reticulum of the C-terminal tail of an AE1 mutant responsible for hereditary spherocytosis in cattle

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Abstract

An R664X nonsense mutant AE1 is responsible for dominant hereditary spherocytosis in cattle and is degraded by the proteasomal endoplasmic reticulum-associated degradation. The present study demonstrated that R664X AE1 translated *in vitro* had the trypsin-sensitve site identical to that of the wild-type AE1. The P661S/R664X mutant containing a possible *N*-glycosylation site at Asn^{660} showed an increase in size by 3 kDa both in the cell-free translation system and in transfected HEK293 cells. Moreover, steady state levels of R664X and P661S/R664X in HEK293 cells were markedly increased in the presence of a proteasome inhibitior. These findings indicate that the truncated C-terminal region of R664X AE1 has lumenal localization in the endoplasmic reticulum and is not accessible to proteasomal machineries in the cytosol.

Key Words : AE1, endoplasmic reticulum, proteasome

Anion exchanger1 (AE1, band 3) is the most abundant transmembrane protein in mammalian red blood cells, with apparent molecular masses of 95-105 kDa, and accounts for about 25% of the total membrane proteins (for review, see references 1, 12, and 15). The N-terminal cytoplasmic domain of AE1 participates in maintaining mechanical properties of red cell membranes by attaching the membrane skeleton to the lipid bilayer through association with ankyrin and the C-terminal half of AE1 consists of the transmembrane domain that mediates rapid Cl^-/HCO_3^- exchange across the plasma membrane.

Various mutations of the human *AE 1*

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(*SLC4A1*) gene have been reported to cause dominant hereditary spherocytosis, a common congenital hemolytic anemia characterized by spherocytic and osmotically fragile red cells, associated with partial deficiency of AE1 by 20-40% in the red cell membrane from heterozygous patients[15,16]. Dominant hereditary spherocytosis in Japanese black cattle (band $3^{Bov.Yamagata}$) is also caused by a nonsense mutation (R664X; Arg^{664}→Stop) of bovine *AE1* and is associated with total and partial deficiency of red cell AE1 in homozygous and heterozygous animals, respectively, leading to marked instability of red cell membranes[4,5].

Our recent study has demonstrated that the R664X mutant has dominant negative effect on the functional expression of wild-type AE1 in the plasma membrane and is degraded by the endoplamic reticulum (ER)-associated degradation (ERAD) through the proteasomal system in a unique manner independent of ubiquitination and *N*-glycosylation[6]. However, the precise mechanisms for recognition and degradation of the mutant protein still remain unknown.

The amino acid residue Arg^{664}, at which C-terminal truncation due to R664X mutation occurs, resides in the middle region of the 4th extracellular loop of the wild-type AE1 flanked by the 7th and 8th transmembrane segments (TMs) according to the predicted membrane topology of the human AE1[2,12,17] (Fig. 1A). Interestingly, the region preceding the R664X mutation site contains the sequence Lys^{649}-Leu-Ser-Val-Pro-Glu-Gly-Leu-Ser-Val-Ser-Asn-Pro-Thr-Glu^{663}-COOH compatible with the criteria of the PEST sequence, being hydrophilic stretches of amino acids greater than 12 residues in length, enriched in Pro (P), Glu (E), Ser (S), and Thr (T), and uninterrupted by positively charged residues. The PEST sequence has been shown to target proteins for degradation by the proteasome[11]. If the ERAD of R664X mutant AE1 involves recognition of the putative PEST sequence, this region should be localized at the cytoplasmic side of the ER due to the impaired membrane insertion of preceding TMs possibly occurring in the mutant (Fig. 1B). To test this hypothesis, in the present study we examined the membrane topology of the mutant AE1 and the localization of its C-terminal tail region.

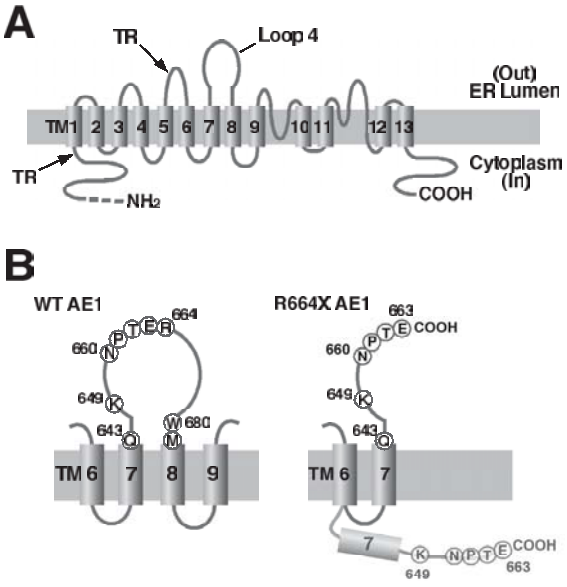

Fig. 1 Schematic illustration for membrane topology of AE1. A, a model for membrane topology of transmembrane domain of bovine AE1 predicted based on the model for human AE1[2,17] is presented with the orientation of lumenal/extracellular (*Out, ER lumen*) and cytoplasmic (*In, Cytoplasm*) localizations. Two among several distinct trypsin-sensitive sites (*TR*) in bovine AE1 are indicated: one in the cytoplasmic domain at the position adjacent to the first transmembrane span (*TM*) and the other one in the transmembrane domain at one of the extracellular loops. B, predicted lumenal localization of the 4th loop flanked by preceding TM7 and following TM8 in wild-type AE1 (*WT AE1*) and lumenal or cytoplasmic localization of the C-terminal tail of R664X mutant AE1 with correct (*upper*) or impaired (*lower*) membrane insertion of TM7.

Bovine AE1 is divided into two membrane-

bound fragments when intact red cells are treated with trypsin, while trypsin treatment of red cell inside-out vesicles generates two soluble fragments from the cytoplasmic domain (39-kDa and the preceding N-terminal 11-kDa fragments) due to an additional cleavage within the N-terminal region[8,9] in addition to the transmembrane domain. *In vitro* translation of cDNA clones in the presence of canine pancreatic microsomes produced the wild-type and R664X mutant AE1 polypeptides with their predicted sizes of 105 kDa and 75 kDa (Fig. 2A). Trypsin treatment of intact microsomal membranes containing the wild-type AE1 generated two major fragments of 52-kDa and 40-kDa polypeptides. The latter fragment was also observed in tryptic digests of R664X protein and was lost in trypsin-treated microsomes after incubation in an alkaline solution followed by centrifugation to thoroughly remove soluble and/or peripheral proteins. This indicates that the 40-kDa fragment is derived from the cytoplasmic domain, most likely equivalent to the C-terminal 39-kDa segment of the cytoplasmic domain described in previous studies on bovine red cell AE1[8,9]. On the other hand, a 28-kDa polypeptide was obtained instead of the 52-kDa fragment in tryptic digestion of microsomes containing the mutant protein. Both 52-kDa and 28-kDa polypeptides were retained in microsomal membranes after alkaline treatment (Fig. 2A), suggesting that these fragments were the transmembrane domain of wild-type AE1 and a truncated transmembrane domain from R664X AE1, respectively. These observations demonstrate that the R664X mutant AE1 has the trypsin-sensitive site at the position identical to that in the wild type at the cytoplasmic surface with localization of the other site in the ER lumen as observed for the wild type.

As shown in our previous study[6], bovine AE1 lacks a potential site for *N*-linked glycosylation, Asn-X-Ser/Thr (X=any amino acid residue except Pro)[3], exhibiting Asn^{660}-Pro-Thr rather than the corresponding Asn^{642}-Ser-Ser of human AE1, to which the *N*-glycan chain is attached[12]. To examine whether the Lys^{649}-Glu^{663} region is localized in the lumen side or the cytoplasmic surface of the ER, we created several AE1 constructs bearing P661S mutation in which Ser was substituted for Pro^{661} to generate a possible *N*-glycosylation site at the residue Asn^{660}, and analyzed their expression in both *in vitro* and HEK293 cells.

When translated *in vitro* in the presence of pancreatic microsomes (+*CPM*), all of these mutants P661S, P661S/R664X, and P661S/W680X gave higher molecular weight bands that were different in size by ≃3 kDa (Fig. 2B), indicating the addition of an *N*-glycan to the mutant protein. This increase in size was not observed in the translation products of wild-type and R664X AE1 lacking the *N*-glycosylation consensus site and in those obtained in the absence of microsomes (-*CPM*) of the mutants bearing P661S mutation, supporting that the P661S/R664X mutant, as well as P661S and P661S/W680X, was glycosylated. The efficiency of glycosylation (the abundance of glycosylated polypeptide relative to the total synthesized that was determined by densitometric scanning of signal intensities) for P661S/R664X AE1 was 45%. This lower efficiency was probably due to the placement of the glycosylation site (Asn^{660}) very close to the end of the polypeptide (Glu^{663}). Actually, the glycosylation efficiency of 60% for P661S/W680X mutant AE1 containing the C-terminal extension by 16 amino acid residues (Fig. 1B) was slightly but significantly higher than that for P661S/R664X and was comparable to that for P661S AE1 (61%). Similar observations were seen for an artificial truncation mutant of human AE1 very

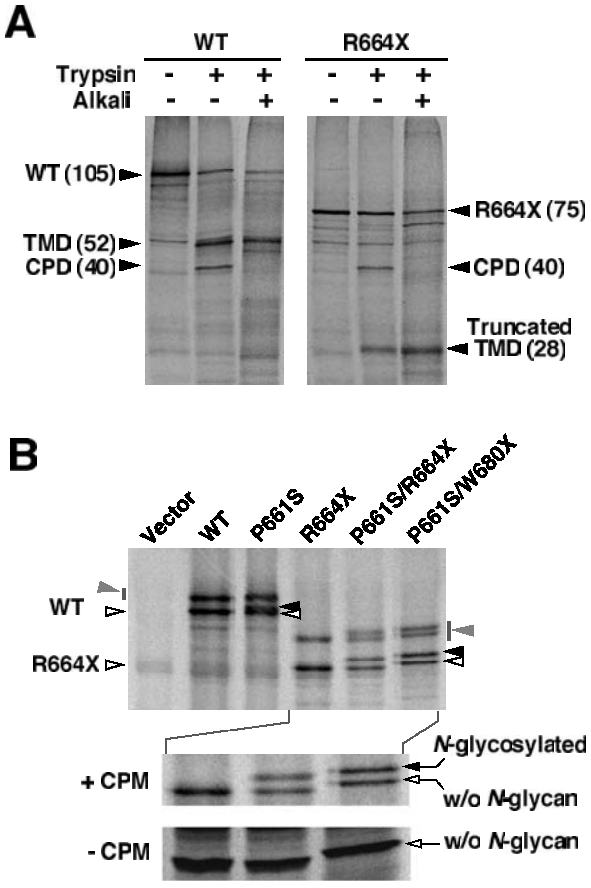

Fig. 2 Membrane orientation of the R664X mutant AE1 and its C-terminal tail in cell-free translation system. A, cDNAs encoding the wild-type (*WT*) and R664X AE1 (*R664X*) were translated in reticulocyte lysates in the presence of [^{35}S]methionine and canine pancreatic microsomes as reported previously[6]. Microsomes were collected by centrifugation and were placed on ice for 1 h in the presence (+) or absence (−) of 3 μg/ml of TPCK-trypsin (*Trypsin*) followed by the addition of difluorophosphate. To one of the reactions was added 500 mM NaOH to yield a final concentration of 50 mM and incubated for 30 min at ambient temperature (*Alkali*), followed by centrifugation to precipitate microsomes. Proteins were separated by SDS-PAGE followed by autoradiography. Bands presenting the wild-type transmembrane domain (*TMD*), cytoplasmic domain (*CPD*), and truncated transmembrane domain from R664X protein (*Truncated TMD*) are indicated with their sizes in kDa. B, AE1 mutants possessing a potential *N*-glycosylation site (*P661S, P661S/R664X, and P661S/W680X*) were generated by site-directed mutagenesis as described[6], and the products of those constructs in cell-free translation were analyzed as described above. Lower panels show magnified area of the gels containing the products of R664X, P661S/R664X, and P661S/W680X mutants in the presence (+*CPM*) or absence (-*CPM*) of microsomes. Migrating positions of *N*-glycosylated (*N-glycosylated*) and unglycosylated (*w/o N-glycan*) mutants are indicated. Higher molecular weight bands indicated with grey arrowheads are likely the products translated from a possible translation initiation codon in the 5' upstream region.

similar to R664X mutant[14] and for rabies virus glycoprotein[13]. These results indicate that, in the cell-free translation system, the C-terminal region of the R664X mutant AE1 is translocated into and localized in the lumenal side of the ER.

The increase in electrophoretic mobility of R664X AE1 due to the additional P661S mutation was also observed in immunoblotting of the lysates from the transfected HEK293 cells (Fig. 3), indicating the *N*-glycan modification of the mutant. Moreover, the immunoblotting

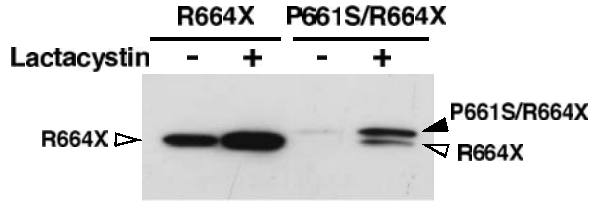

Fig. 3 Effect of lactacystin on steady state expression of R664X AE1 and its *N*-glycosylated forms. HEK293 cells expressing R664X or P661S/R664X AE1 were incubated for 8 h in the presence (+) or absence (−) of 10 μM lactacystin and were analyzed for AE1 protein contents by immunoblotting as described previously[6]. Migrating positions of unglycosylated (*R664X*) and *N*-glycosylated (*P661S/R664X*) mutants are indicated.

showed that a proteasome inhibitor lactacystin caused a remarkable increase in the steady state levels of P661S/R664X AE1 in transfected HEK293 cells as observed for the R664X mutant. These observations demonstrate that the C-terminal region of the R664X mutant AE1 is localized in the ER lumen and that the P661S/R664X mutant is degraded by the proteasome as observed for the R664X mutant in transfected cells.

Taken together, the present study demonstrates that the C-terminal tail containing the sequence Lys^{649}-Glu^{663}-COOH in R664X AE1 has no apparent roles, at least as the PEST sequence, in recognition and degradation of the mutant by the proteasome pathway. Our data also suggests that *N*-glycosylation at the 4th extracellular loop has no significant effect on proteasomal degradation of the mutant AE1, supporting our previous finding that the ERAD of R664X AE1 occurs principally in a manner independent of *N*-glycosylation[6]. However, our observations do not rule out a possible function of molecular chaperones such as calnexin in the quality control of the AE1 protein through either of or both *N*-glycan-based and/or polypeptide-based association[7].

The present study also suggests that the truncated R664X mutant AE1 is inserted into the membrane with correct orientation for N-terminal seven TMs (TM1-TM7) preceding the site of translation termination at residue Glu^{663} (Fig. 1). The lumenal localization of the C-terminus of R664X mutant demonstrated by the addition of core *N*-glycan (Fig. 2B) is of particular interest, since it indicates that the preceding TM (TM7) alone has topogenic function for proper translocation of the 4th loop sequence even in the absence of TM8. Topogenic functions of TMs in human AE1 have been studied extensively in the reticulocyte lysate system supplemented with microsomes as in the present study, and suggested that each TM has somewhat different topogenic properties during the synthesis and co-translational insertion into the ER membrane[10,14]. However, topogenic functions of each TM have not been fully understood. Tam *et al.*[14] reported that TM7 of human AE1 has the topogenic properties of an internal signal sequence, whereas Ota *et al.*[10] suggested that signal-anchor function of TM7 in human AE1 is not sufficient for translocation of the 4th loop and that TM8 with strong type I signal-anchor and stop-transfer functions is required to form correct membrane topology of this region. Our study first describes, using both cell-free translation system and a cell line, that TM7 has signal-anchor activity enabling the following sequence to be translocated into the ER lumen.

In conclusion, the present study demonstrates that R664X mutant AE1, $band3^{Bov.Yamagata}$ has nearly normal membrane topology with localization of the C-terminal end in the ER lumen and that the truncated C-terminal region, therefore, is not accessible to machineries in the cytosol involved in the proteasomal degradation of this mutant. Our study also demonstrates that TM7 of bovine AE1 pos-

sesses signal-anchor function sufficient for translocation of the following hydrophilic sequence.

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