Ubiquitylation-independent ER-associated degradation of an AE1 mutant associated with dominant hereditary spherocytosis in cattle

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Summary

Various mutations in the \(\text{AE1}\) (anion exchanger 1, band 3) gene cause dominant hereditary spherocytosis, a common congenital hemolytic anemia associated with deficiencies of \(\text{AE1}\) of different degrees and loss of mutant protein from red blood cell membranes. To determine the mechanisms underlying decreases in \(\text{AE1}\) protein levels, we employed K562 and HEK293 cell lines and \(\text{Xenopus}\) oocytes together with bovine wild-type \(\text{AE1}\) and an R664X nonsense mutant responsible for dominant hereditary spherocytosis to analyze protein expression, turnover, and intracellular localization. R664X-mutant protein underwent rapid degradation and caused specifically increased turnover and impaired trafficking to the plasma membrane of the wild-type protein through hetero-oligomer formation in K562 cells. Consistent with those observations, co-expression of mutant and wild-type \(\text{AE1}\) reduced anion transport by the wild-type protein in oocytes. Transfection studies in K562 and HEK293 cells revealed that the major pathway mediating degradation of both R664X and wild-type \(\text{AE1}\) employed endoplasmic reticulum (ER)-associated degradation through the proteasomal pathway. Proteasomal degradation of R664X protein appeared to be independent of both ubiquitylation and N-glycosylation, and aggresome formation was not observed following proteasome inhibition. These findings indicate that \(\text{AE1}\) R664X protein, which is associated with dominant hereditary spherocytosis, has a dominant-negative effect on the expression of wild-type \(\text{AE1}\).

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Introduction

Hereditary spherocytosis (HS) is a common congenital hemolytic anemia characterized by spherocytic and osmotically fragile red blood cells (Tse and Lux, 2001). The principal cellular lesion in HS is loss of membrane surface area relative to intracellular volume. Most forms of HS are inherited in an autosomal dominant mode with various red-cell and clinical phenotypes. The molecular bases for HS phenotypes are defects in major red-cell membrane proteins, such as spectrin, ankyrin, and \(\text{AE1}\), all of which lead to mechanical instability of the red-cell membrane.

\(\text{AE1}\) (anion exchanger 1, band 3), with apparent molecular masses of 95-105 kDa, is the most abundant transmembrane protein in mammalian red blood cells, accounting for about 25\% of the total red-cell membrane proteins (Reithmeier et al., 1996; Tanner, 1997). The N-terminal cytoplasmic domain functions in maintaining mechanical properties of red-cell membranes by attaching the spectrin-actin membrane skeleton to the lipid bilayer through association with ankyrin (Low et al., 1991; Michaely and Bennet, 1995; Zhang et al., 2000). The C-terminal half consists of the transmembrane domain which mediates rapid \(\text{Cl}^-\) and \(\text{HCO}_3^-\) exchange across the plasma membrane (Alper, 1991).

Various mutations of the human \(\text{AE1}\) (\(\text{SLC4A1}\)) gene, including missense, nonsense and frameshift mutations, have been reported to cause dominant HS, which is associated with a 20-40\% reduction in \(\text{AE1}\) protein levels in the red-cell membrane (Jarolim et al., 1994; Jarolim et al., 1996; Alloisio et al., 1996; Alloisio et al., 1997; Dhermy et al., 1997; Jenkins et al., 1996; Tanner, 2002). Total \(\text{AE1}\) deficiency in cattle in the homozygous state for a nonsense mutation R664X, corresponding to a premature termination at residue R646 in human \(\text{AE1}\), also showed atypical spherocytosis with marked membrane instability (Inaba et al., 1996; Inaba, 2000). Previous studies in patients or animals with \(\text{AE1}\) mutations failed to demonstrate the presence of mutant \(\text{AE1}\) protein in their red-cell membranes. This finding was partially explained, particularly in the case of the nonsense mutations, by the absence of mutant transcripts in the reticulocytes of patients (Jarolim et al., 1996; Jenkins et al., 1996; Dhermy et al., 1997). However, although HS with \(\text{AE1}\) deficiency is usually homogeneous with regard to the clinical and biochemical
features within a given family, several investigators report that the extent to which AE1 protein levels are reduced differs depending on the mutation (Alloisio et al., 1996; Alloisio et al., 1997; Dhermy et al., 1997). Moreover, mice heterozygous for a disrupted AE1 gene exhibit only a mild (20%) deficiency of AE1 protein (Peters et al., 1996), possibly reflecting the amount of the protein produced from the normal allele. Several possibilities may therefore account for the absence of mutant AE1 protein, including abnormal biosynthesis, incorrect insertion into the endoplasmic reticulum (ER) or altered trafficking and processing.

Some proteins associated with dominant inheritance of a disease state function as part of a multimeric complex. For example, the E258K mutant of aquaporin-2 (AQP2) has been reported to be retained in the Golgi complex (Mulders et al., 1998; Tamarappoo et al., 1999) and to form tetramers with wild-type AQP2, thereby causing dominant nephrogenic diabetes insipidus (NDI). By contrast, R187C AQP2, a mutant seen in recessive NDI, cannot heterotetramerize with wild-type AQP2 (Kamsteeg et al., 1999). If mutant AE1 forms oligomers with the wild-type protein, the dominant inheritance of HS could be explained by impaired trafficking to the plasma membrane followed by degradation of the wild-type AE1. Actually, previous studies demonstrated that several distinct AE1 missense mutations, including R760Q, caused defective trafficking and protein-folding in HEK293 cells (Quilty and Reithmeier, 2000). Others suggest that the presence of the AE1 mutant R490C, which is retained in the ER, reduces cell surface expression of wild-type AE1 (Dhermy et al., 1999).

In a previous study, we supposed that the AE1 R664X mutant was totally degraded after synthesis and insertion into the ER membrane because bone marrow cells from affected animals expressed mRNA of the same size as that of the wild-type controls (Inaba et al., 1996). The most likely mechanism is proteolytic degradation by ER-associated degradation (ERAD) (Ellgaard and Helenius, 2003) via the proteasome pathway, as has been observed for the misfolded cystic fibrosis transmembrane-conductance regulator (CFTR) (Ward et al., 2000). Metabolic labeling with [3H]biotin indicated that an amount of mutant protein was detected at the plasma membrane throughout the chase period (Fig. 1B). By contrast, R664X AE1 showed a rapid turnover with a half-life (t1/2) of less than 6 hours, and insignificant levels, indicating that mutant AE1 protein should be synthesized in those cells.

To determine potential involvement of proteasomal ERAD in dominant HS with AE1 deficiency caused by the R664X mutation in bovine AE1 (band 3Bov; Yamagata) (Inaba et al., 1996; Inaba, 2000), we examined intracellular distribution and degradation of stably expressed wild-type and mutant proteins in K562 erythroleukemia cells. K562 cells constitute a suitable expression system for AE1 because they do not express endogenous AE1 and show higher levels of expression of functional exogenous human AE1 at the cell surface (Beckmann et al., 1998) than do HEK293 cells (Ruetz et al., 1993). We also asked how R664X and wild-type AE1 were processed in both K562 and HEK293 cells, and found that the mechanism underlying proteasomal degradation of AE1 differed from that of transmembrane proteins such as CFTR.

Results

Relative abundance of mutant AE1 transcripts in cattle heterozygous for the R664X mutation

We previously described a mutation in AE1 (R664X) arising in cattle with dominantly inherited spherocytosis (Inaba et al., 1996; Inaba, 2000). Quantitative reverse transcriptase (RT)-PCR using bone marrow cells showed that levels of AE1 mRNA in animals heterozygous or homozygous for the R664X mutation were increased approximately three times over control animals (supplementary material, Fig. S1), presumably reflecting elevated erythropoiesis. However, the level of AE1 transcripts relative to that encoding protein 4.1, the only main component of membranes not altered in mutant animals, was reduced to 61% in heterozygotes and 13% in homozygotes, demonstrating reduced levels of AE1 mRNA in mutant animals.

The relative abundance of AE1 mRNA from bone marrow cells in two heterozygous R664X mutants was evaluated by PCR–restriction-fragment-length polymorphism of cDNAs reverse-transcribed from RNAs. That abundance ranged from 28% to 38% of the total amount (supplementary material, Fig. S1). These results show that erythroid precursor cells express AE1 mRNA encoding the R664X mutation at reduced but not insignificant levels, indicating that mutant AE1 protein should be synthesized in those cells.

Rapid degradation of R664X mutant protein without trafficking to the plasma membrane in K562 cells

Immunofluorescent detection of wild-type AE1 protein showed prominent signals at the plasma membrane in stably transfected K562 cells, whereas the R664X mutant showed a dispersed membranous localization within the cells (Fig. 1A). Metabolic labeling and immunoprecipitation or immunoblotting showed that K562 cell clones expressed wild-type or R664X AE1 with predicted sizes of 105 kDa or 75 kDa, respectively. One stably transfected clone of each respective construct, K562bebWT and K562bebRX, was used throughout this study.

Pulse-labeling followed by chase and surface-labeling of K562bebWT cells demonstrated that wild-type AE1 was very stable: specifically, approximately 90% of the wild-type protein remained intact even after 24 hours and about 10% of that protein was detected at the plasma membrane throughout the chase period (Fig. 1B). By contrast, R664X AE1 showed a rapid turnover with a half-life (t1/2) of less than 6 hours, and a negligible amount of mutant protein was detected in the biotinylated membrane-surface fraction, indicating that R664X AE1 was rapidly degraded after synthesis without trafficking to the plasma membrane.
Dominant-negative activity of the R664X mutant in the membrane expression of wild-type AE1

To determine whether the R664X mutation has a dominant-negative effect on expression of wild-type AE1, we analyzed the stability and functional expression of the latter in the presence of R664X in both K562 cells and _Xenopus_ oocytes.

To do so, we established a clonal line of K562 cells (K562bebWT/RX) expressing both wild-type and R664X AE1 and analyzed expression of AE1 proteins in pulse-chase experiments. The amount of the pulse-labeled wild-type AE1 retained in K562bebWT/RX cells was markedly reduced during the chase period (with a $t_{1/2}$ of less than 12 hours), correlating with a decrease of R664X AE1 and contrasting with the stability of wild-type protein in K562bebWT cells (Fig. 2A). When K562bebWT, K562bebRX, or control K562C cells were transiently transfected with expression vectors encoding bovine glycophorin C (GPC), a main single-span sialoglycoprotein in red-cell membranes (Chasis and Mohandas, 1992), immunofluorescent signals for GPC were seen at the cell surface despite the presence or absence of AE1 proteins at the plasma membrane (Fig. 2B). Likewise, in K562bebWT/RX cells, GPC was detected at the plasma membrane, whereas immunofluorescent signals for AE1 were predominantly observed intracellularly as in K562bebRX cells (Fig. 2B), indicating a selective decrease in cell surface expression of wild-type AE1 in the presence of the R664X mutant.

Immunoprecipitation with the cdb3-64 antibody (epitopes for the anti-AE1 monoclonal antibodies are described in Materials and Methods) confirmed the presence of wild-type, R664X, and both wild-type and R664X AE1 proteins in K562bebWT, K562bebRX and K562bebWT/RX cells, respectively, although steady-state levels of wild-type protein in K562bebWT/RX cells were markedly reduced compared with those seen in K562bebWT cells (Fig. 2C, upper panels).

Similar findings were obtained with the tm3-29 antibody (data not shown). By contrast, tm3-26 reacted with the wild-type protein in K562bebWT but not with R664X AE1 in K562bebRX cells. However, the R664X AE1 signal was seen when the extract from K562bebWT/RX cells was immunoprecipitated with tm3-29 (Fig. 2C, lane WT/RX in right panel). R664X AE1 protein was not detected in the precipitate when K562bebWT and K562bebRX cells were mixed prior to solubilization for immunoprecipitation (Fig. 2C, lane WT + RX in right panel). These data indicate that multimers of R664X and wild-type AE1 form, and that this association does not arise during sample preparation. Co-immunoprecipitation of R664X AE1 and the wild-type protein with tm3-26 but not with tm3-29 or cdb3-64 was also seen when proteins were synthesized in a cell-free in vitro translation system in the presence of pancreatic microsomes (Fig. 2C, lower panels). In this case, immunoprecipitation of R664X AE1 with tm3-26 was seen only when translation of wild-type and R664X AE1 was carried out in a single reaction (Fig. 2C, lane WT/RX, right panel).

To further establish the effect of and quantitative requirement for the R664X mutant on functional expression of the wild-type AE1, we used _Xenopus_ oocytes to analyze anion transport mediated by the wild-type AE1 in the presence of co-injected R664X mutant at different expression levels. Oocytes injected with wild-type AE1 alone showed 4,4′- diisothiocyanato-2,2′-stilbene disulfonate (DIDS)-sensitive Cl$^-$ transport, which reached maximal levels when injected RNA was increased to approximately 2.5 ng/oocyte (Fig. 3A). When R664X mutant RNA was co-injected with 1.0 ng/oocyte of wild-type AE1 RNA, DIDS-sensitive Cl$^-$ uptake was markedly decreased in a dose-dependent manner (Fig. 3B). Co-injection of mutant RNA at half or even one-fourth of the amount of wild-type RNA (0.5 and 0.25 ng/oocyte of R664X versus 1.0 ng/oocyte of wild type), designed to mimic physiological conditions in bone marrow cells of heterozygotes as determined above (supplementary material, Fig. S1), caused significant reduction in the uptake activity of wild-type AE1. Significant reduction in Cl$^-$ transport was also observed in oocytes co-injected with 0.5 ng or 1.0 ng of R664X RNA together with 0.5 ng of wild-type RNA. It was unlikely that
observed decreases in Cl⁻-uptake were owing to non-specific suppressive effects due to high concentrations of RNA injected because the total amount of RNA injected was at most 2 ng/oocyte, less than levels required for saturation in functional assays of wild-type AE1 (Fig. 3A). In addition, co-injection of four times that amount of R664X RNA (4.0 ng/oocyte) rendered transport activity negligible, whereas transport activity comparable to or higher than that seen in oocytes injected with 1.0 ng of wild-type RNA was observed in oocytes injected with increasing amounts of wild-type AE1 RNA (10 ng/oocyte, Fig. 3A).

Degradation of R664X AE1 by the proteasome pathway in K562 cells

Accumulation of R664X AE1 in K562bebRX cells was observed when cells were treated with the proteasome inhibitors lactacystin or MG132 but not with several protease inhibitors, including aprotinin, leupeptin or the lysosomal enzyme-inhibitor ammonium chloride (Fig. 4A). Lactacystin and MG132 had no effect on steady-state levels of wild-type AE1. Fig. 4B shows that decreases in newly synthesized R664X AE1 were remarkably inhibited by lactacystin treatment. The turnover rate of R664X in the presence of lactacystin ($t_{1/2}$ ~10 hours) was increased by more than twofold compared with that seen in the absence of inhibitor. A slight but significant decrease in degradation of wild-type AE1 in the presence of lactacystin was also observed (Fig. 4B).

We next assayed for ubiquitylated AE1 in cells treated with proteasome inhibitors. Whereas lactacystin remarkably enhanced accumulation of ubiquitylated polypeptides with slow mobility in K562bebWT and K562bebRX cells, and caused marked accumulation of R664X AE1 in K562bebRX cells, no apparent changes in the migration of AE1 proteins were detected following lactacystin treatment (Fig. 4C, upper panels). Immunoprecipitation failed to detect AE1 polypeptides with slower mobility on SDS-PAGE gels (Fig. 4C, lower panels). Moreover, no signals for ubiquitylated polypeptides were detected with the anti-ubiquitin antibody in immunoblots of the same precipitates from K562bebWT and K562bebRX cells. These data suggest that R664X AE1 was retained in the ER and degraded principally by the proteasome pathway in a manner independent of ubiquitylation, although the subcellular localization of R664X AE1 in K562 cells is not clear.

Degradation of AE1 by the ERAD without apparent ubiquitylation in HEK293 cells

To further characterize mechanisms underlying AE1 degradation, we examined expression and degradation of wild-type and R664X protein in HEK293 cells. As shown in Fig. 5, EGFP-tagged wild-type AE1 was predominantly localized to the plasma membrane, and fluorescent signals merged with those of co-transfected GPC, with some fluorescence in the ER,
indicating normal protein synthesis and trafficking. By contrast, EGFP-R664X AE1 was not present at the cell surface but was localized intracellularly, and co-transfected GPC showed significant localization to the plasma membrane. The same observations of wild-type and R664X AE1 protein were made in HEK293 cells (data not shown). The intracellular localization of most R664X protein is similar to that of the ER marker calnexin but different from the respective Golgi complex and lysosome markers, GM130 and Lamp2, indicating that R664X AE1 accumulates primarily in the ER (Fig. 5).

Immunoblot analysis with anti-AE1 antibody of biotinylated cell surface proteins confirmed that wild-type but not R664X AE1 was transported to the plasma membrane (Fig. 6A). Interestingly, wild-type and R664X proteins showed no significant difference in turnover rates, both exhibiting a $t_{1/2}$ of ~10 hours in transfected HEK293 cells (Fig. 6B), suggesting that wild-type AE1 is less stable in HEK293 cells than it is in

![Fig. 3. Dominant-negative effect of R664X AE1 on functional expression of wild-type AE1 in Xenopus oocytes. (A) $^{36}$Cl–-uptake of oocytes injected with increasing amount of wild-type (○) or R664X (●) AE1 RNA. (B) $^{36}$Cl–-uptake into oocytes injected with wild-type AE1 RNA (WT) with or without various amounts of R664X RNA (R664X). Values indicate DIDS-sensitive $^{36}$Cl–-uptake calculated by subtracting the mean values in the presence of 10 μM DIDS (n=5-10) from values obtained in the absence of DIDS. Data are expressed as mean ± s.d. (n=10). *P<0.05, **P<0.01 in oocytes injected with 0.5 ng or 1 ng/oocyte of wild-type RNA alone.]

![Fig. 4. Increased stability of R664X AE1 in K562 cells in the presence of proteasome inhibitors. (A) K562bebWT and K562bebRX cells were incubated for 8 hours in the absence (None) or presence of the reagents indicated, and then wild-type and R664X protein levels were analyzed by immunoblotting with the tmb3-29 antibody. (B) K562bebWT and K562bebRX cells were pulse-labeled and chased for the indicated periods followed by immunoprecipitation of wild-type (WT) or R664X (R664X) AE1 in the presence (Lactacystin) or absence (None) of 10 μM lactacystin, as described in the legend for Fig. 1. (C) K562bebWT (WT) and K562bebRX (RX) cells were incubated for 8 hours in the absence (−) or presence (+) of 10 μM lactacystin and lysed in IP-buffer, followed by immunoprecipitation with cdb3-64. AE1 proteins and ubiquitylated proteins in detergent-soluble whole cell lysates (Whole, upper panels) or immunoprecipitates (IP, lower panels) were detected with the anti-AE1 (left) and anti-ubiquitin (right) antibodies, respectively. The detergent-insoluble fraction contained various ubiquitylated polypeptides but not proteins recognized with the AE1 antibody (data not shown). Blots were incubated with tmb3-29 (Whole) or anti-38K (IP) antibodies. Asterisks indicate non-specific signals observed in lysates from mock-treated cells (not shown). Migrating positions of the protein standards are shown in kDa.]

K562 cells, as described above. Thus, the presence of the R664X mutant or GPC had no apparent effect on wild-type AE1 turnover. However, decreased turnover of both wild-type and R664X protein in the presence of lactacystin (Fig. 6B) clearly demonstrates that in both HEK293 and K562 cells AE1 degradation requires the proteasome pathway.

We next asked whether proteasomal degradation of AE1 proteins in HEK293 cells is independent of ubiquitylation, as suggested by results in K562 cells. We therefore employed the ΔF508-CFTR as a positive ubiquitylation control (Fig. 7).

When HEK293 cells were transiently transfected with EGFP-ΔF508-CFTR, one main 170-kDa polypeptide and several high-molecular mass species were detected by immunoblotting with the anti-CFTR antibody; levels of these polypeptides were increased in cells treated with a proteasome inhibitor (Fig. 7A), consistent with previous observations (Gelman et al., 2002). By contrast, no obvious slow-migrating species of AE1 or its EGFP-tagged forms were seen with the anti-AE1 antibody cdb3-64, although more intense signals for wild-type and R664X AE1 were evident in transfected cells treated with lactacystin. Insoluble fractions from the cell lysates after centrifugation at 18,000 g for 15 minutes gave no signals for AE1 proteins when analyzed by immunoblotting (data not shown). Immunoprecipitation with cdb3-64 or anti-CFTR antibodies followed by immunoblotting with anti-AE1 (anti-

**Fig. 5.** Intracellular localization of wild-type and R664X AE1 in HEK293 cells. HEK293 cells were transiently co-transfected with the EGFP-wild-type (EGFP-WT) or EGFP-R664X (EGFP-R664X) AE1 and GPC (EGFP-WT/GPC or EGFP-RX/GPC) and stained for GPC with the anti-GPC antibody. HEK293 cells expressing EGFP-wild-type or EGFP-R664X AE1 (EGFP-WT or EGFP-RX) were also incubated with anti-calnexin (Calnexin), anti-GM130 (GM130), and anti-Lamp2 (Lamp2) followed by detection with a secondary antibody labeled with Alexa-Fluor-568. Bars, 10 μm.

**Fig. 6.** Turnover of wild-type and R664X AE1 proteins in HEK293 cells. (A) Cell-surface proteins in HEK293 cells transiently transfected with wild-type (WT) and R664X (RX) AE1 were biotinylated and polypeptides in total cell lysate (T) were separated into bound (B) and unbound (U) fractions on NutriAvidin beads, followed by detection of AE1 proteins by immunoblotting. Each lane contained proteins from the equivalent volume of cell lysate. (B) HEK293 cells transiently transfected with wild type (WT), R664X (RX), wild type and GPC (WT/GPC), or wild-type and R664X AE1 (WT/RX) were pulse-labeled for 20 minutes and chased for the indicated periods followed by immunoprecipitation of AE1 as described for Fig. 1. Lanes 10+ contained immunoprecipitates from the cells chased for 10 hours in the presence of 10 μM lactacystin. The lower panel shows wild-type and R664X proteins remaining after the chase in the presence (■) or absence (○) of lactacystin. Data are the mean values of duplicate samples. Pulse-labeled wild-type and R664X AE1 in WT/GPC and WT/RX HEK293 cells showed stability similar to that observed in HEK293 cells transfected with wild type or R664X alone (data not shown).
3608 Journal of Cell Science 119 (17)

Fig. 7. The absence of ubiquitylated AE1 in transfected HEK293 cells. (A,B) HEK293 cells transiently transfected with wild-type (WT) and R664X (RX) AE1, their EGFP-tagged forms (EGFP-WT and EGFP-RX), EGFP-ΔF508-CFTR (EGFP-ΔF508), or empty vectors (Mock) were incubated for 8 hours in the absence (−) or presence (+) of 10 µM lactacystin. AE1 and EGFP-ΔF508-CFTR were immunoprecipitated with cdb3-64 and the anti-CFTR, respectively. AE1 and EGFP-ΔF508-CFTR in whole-cell lysates (A) and immunoprecipitates (B) were detected by immunoblotting with cdb3-64 and anti-CFTR (A) or anti-38K and anti-GFP (B, left panel), respectively. Ubiquitylated proteins in the immunoprecipitates were also reacted with the anti-ubiquitin (anti-Ub) antibody (B, right panel). Signals corresponding to high-molecular-weight species (HMW) were seen only on blots of EGFP-ΔF508-CFTR. The asterisk indicates a non-specific product.

Absence of N-glycan modification in bovine AE1

The amino acid sequence of bovine AE1 predicted from the cDNA sequence suggested that bovine AE1 lacks a potential N-glycan-linked glycosylation site of residues Nx[S/T] (with x being any amino acid residue except P) (Gavel and von Heijne, 1990), exhibiting residues NPT (at N660) rather than the corresponding NSS (at N642) of human AE1, to which an N-glycan chain is attached (Reithmeier et al., 1996). The absence of processing with increasing sizes of newly synthesized proteins during the chase period (Figs 1, 4 and 6) supported this idea. To verify this assumption, red-cell membrane proteins were treated with the deglycosylation enzyme PNGase and analyzed by SDS-PAGE. We observed no change in electrophoretic mobility of AE1 after PNGase treatment in bovine red-cell membranes, whereas PNGase did generate deglycosylated polypeptides of canine and human AE1 (supplementary material, Fig. S2). In addition, the in vitro translation product in the presence of pancreatic microsomes of the P661S AE1 (in which the wild-type sequence NPT (at P661) has been changed to NST (at S661) to enable N-glycosylation) contained both glycosylated and non-glycosylated forms (supplementary material, Fig. S2). An R664X mutant bearing the P661S mutation also showed an increase in size by ≈2 kDa in a cell-free translation system (data not shown). These observations demonstrate the lack of N-glycan modification of bovine AE1.

Discussion

The present study demonstrates that R664X AE1, the mutation underlying dominant HS in cattle (Inaba et al., 1996: Inaba, 2000), is principally degraded by the ERAD system (Ellgaard and Helenius, 2003) via the proteasome pathway in transfected K562 and HEK 293 cells. Furthermore, mis-expression of R664X AE1 significantly and specifically reduces the expression of co-expressed wild-type AE1 in the plasma membrane in both K562 cells and Xenopus oocytes. It is likely that the dominant-negative effect of R664X AE1 on functional expression of the wild-type protein occurs in vivo in mutant 38K or anti-GFP antibodies, respectively, gave results similar to those obtained for the cell lysates described above (Fig. 7B, left panel). A corresponding immunoblot of the immunoprecipitated sample probed with an anti-ubiquitin antibody demonstrated the presence of ubiquitylated species of EGFP-ΔF508-CFTR larger than 170 kDa. However, no ubiquitin immunoreactivity signals were detected with the anti-AE1 antibody in immunoprecipitates from HEK293 cells expressing either wild-type, or R664X AE1 or their EGFP-tagged forms (Fig. 7B, right panel).

Immunofluorescence indicated that lactacystin-treated HEK293 cells expressing EGFP-R664X AE1 showed accumulation of EGFP-positive material in several inclusions (Fig. 8B) as well as in the reticular distributions described above (Fig. 5). These inclusions, localized at perinuclear regions where condensed signals for calnexin were also observed, suggest accumulation of EGFP-R664X AE1 in compartments of the ER. No immunofluorescent inclusions were observed for GM130 or Lamp2 (data not shown). Such a distribution of EGFP-R664X was quite distinct from that of EGFP-ΔF508-CFTR. The latter was localized intracellularly (Fig. 8A), like the R664X protein, but was typically seen in large foci at the juxtanuclear region reminiscent of aggresomes (Jonston et al., 1998) when transfected cells were treated with lactacystin (Fig. 8B). Interestingly, the localization of EGFP-R664X inclusions was very different from that of ubiquitin immunofluorescence, whereas distribution of ubiquitin was similar to that of EGFP-ΔF508-CFTR in transfected cells (Fig. 8C). These findings were in agreement with those obtained in immunoblot analysis indicating the absence of ubiquitylated AE1.
animals because, as demonstrated in *Xenopus* oocytes, the effect was significant when the relative abundance of R664X mutant mRNA was as low as that estimated in bone marrow cells from heterozygous animals (Fig. 3 and supplementary material, Fig. S1).

The dominant-negative effect of mutant AE1 appears to be derived from hetero-oligomerization of mutant and wild-type AE1 (Fig. 2). AE1 forms homodimers at the dimerization arm within the cytoplasmic domain (Zhang et al., 2000), which then form tetramers associating with ankyrin (Michaely and Bennett, 1995). Since R664X protein contains the cytoplasmic domain and appears to be monomeric rather than aggregated on SDS-gels of immunoprecipitates from K562bebWT/RX cells (Fig. 2), the association of R664X and wild-type AE1 probably occurs through heterodimerization of the dimerization arm. Similar mechanisms, therefore, are plausible for some of the dominant HS mutations that produce a structurally abnormal AE1 protein that has nonetheless an intact cytoplasmic domain, such as the R490C mutant, which has been shown to exert a dominant-negative effect on the plasma membrane expression of the wild-type AE1 in transfected K562 cells (Dhermy et al., 1999).

Similar dominant-negative effects have been demonstrated for a missense (E258K) and several frame-shift mutants of human AQP2, leading to NDI with dominant phenotypes (Mulders et al., 1998; Kamsteeg et al., 1999; Marr et al., 2002; Kamsteeg et al., 2003; Asai et al., 2003). Mutant AQP2 molecules in dominant NDI can associate with wild-type AQP2, forming heterotetramers (Kamsteeg et al., 1999; Marr et al., 2002; Kamsteeg et al., 2003). This interaction causes retention in the Golgi complex and/or lysosomes (Mulders et al., 1998; Tamarappoo et al., 1999; Marr et al., 2002; Hirano et al., 2003) or mis-targeting of wild-type protein to the basolateral membrane (Kamsteeg et al., 2003; Asai et al., 2003), whereas AQP2 mutants in recessive NDI cannot associate with wild-type protein (Kamsteeg et al., 1999) and are retained in the ER (Deen et al., 1995; Mulders et al., 1997). Kamsteeg et al. therefore suggested that AQP2 assembly into tetramers occurs after exit from the ER (Kamsteeg et al., 1999). The striking difference in processing of R664X and wild-type AE1 is that AE1 hetero-oligomer formation appears to occur in the ER, as is commonly accepted for various transmembrane proteins (Hurtley and Helenius, 1989) because the association occurs in a cell-free translation system in the presence of pancreatic microsomes as well as in K562 cells (Fig. 2C). Consequently, mechanisms underlying subsequent degradation of the wild-type–mutant complexes of AQP2 and AE1 differ somewhat. Degradation of the dominant NDI-causing mutant E258K of AQP2 in clone 9 hepatocytes occurs rapidly in a proteasome- and lysosome-dependent manner (Hirano et al., 2003), whereas in cells transfected with the R664X mutant of AE1, the protein is principally degraded by the proteasomal pathway, as demonstrated in this study.

Interestingly, the dominant-negative effect of R664X AE1 seen in K562 cells and oocytes was not apparent in HEK293 cells co-expressing R664X and wild-type AE1. A previous study showed that most murine AE1 synthesized in HEK293 cells fails to mature and arrive at the cell surface and is restricted to the pre-Golgi compartment, most probably the ER, whereas a homologous anion exchanger AE2 is successfully targeted to the plasma membrane. These observations suggest that, although these two anion exchangers normally reside in the plasma membrane in cells where they are endogenously expressed, they are processed quite differently through the secretory pathway in HEK293 cells (Ruetz et al., 1993). Our data show that substantial amounts of wild-type bovine AE1 expressed in HEK293 cells reach the plasma membrane (Figs 5 and 6), as does human AE1 (Quilty and Reithmeier, 2000), but that turnover of the wild-type protein is more rapid than in K562 cells and indistinguishable from that of R664X AE1 in HEK293 cells (Fig. 6). Therefore, the lack of a dominant-negative effect by the R664X mutant in HEK293 cells is probably because of the manner in which wild-type AE1 is processed, which is undefined but different from the way the protein is processed in K562 cells. Such processing might involve an alternative lysosomal degradation route taken by protein transported from the plasma membrane.

Proteasomal ERAD (Ellgaard and Helenius, 2003) of transmembrane proteins in mammalian cells has been characterized primarily in studies of CFTR and its most common ΔF508-CFTR mutant (Jensen et al., 1995; Ward et al.,

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Fig. 8. Distinct intracellular localization of EGFP-R664X AE1 and EGFP-ΔF508-CFTR in HEK293 cells treated with lactacystin. Cells were transiently transfected with EGFP-R664X AE1 (EGFP-RX) or EGFP-ΔF508-CFTR (EGFP-ΔF508) and incubated with (B,C) or without (A) 10 μM lactacystin for 8 hours, and the intracellular localization of EGFP-tagged proteins was compared with that of calnexin (A,B) and ubiquitin (C). Bars, 10 μm.
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). Our data indicate that mechanisms underlying degradation of R664X AE1 differ significantly. The most notable difference is the absence of AE1 ubiquitylation at steady-state levels in cells, or in cells that show marked accumulation of R664X or wild-type AE1 (Figs 4 and 7). It is unlikely that lack of ubiquitylated AE1 in cell lysates is due to high activity of de-ubiquitylating enzymes because in parallel experiments, we observed remarkable accumulation of ubiquitylated EGFP-ΔF508-CFTR (Fig. 7), as has been reported previously (Gelman et al., 2002). Rather, our findings suggest that ubiquitylation is not required for proteasomal degradation of R664X or wild-type AE1, as has been reported for T cell receptor α chains (Yu et al., 1997) and some cytosolic proteins, including p21Cip1 (Sheaff et al., 2000) and α-catenin (Hwang et al., 2005).

Another difference between AE1 and CFTR degradation is that proteasome inhibition does not reduce retro-translocation of CFTR but rather, increasing levels of misfolded CFTR in the cytosol lead to aggresome formation (Johnston et al., 1998). 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 (Fig. 8), suggesting that retro-translocation of R664X AE1 is coupled to proteasome function, as has been reported for several other transmembrane proteins (Mayer et al., 1998; Saliba et al., 2002). Since enhanced ER retention was evident in specified perinuclear regions within the cell (Fig. 8), sequestration of R664X AE1 to a compartment of the ER might occur prior to retrotranslocation and degradation.

N-Glycosylation has been recognized to play a pivotal role in ER-retention of CFTR and mutant forms of the protein (Pind et al., 1994). By contrast, ER retention of R664X AE1 is independent of an N-glycan moiety, because both R664X and wild-type bovine AE1 lack N-glycan modification as demonstrated in the present study (supplementary material, Fig. S2). In addition, an R664X mutant bearing the P661S mutation had essentially the same profile of ER retardation in HEK293 cells (D.I. and M.I., unpublished observations). These observations are compatible with a recent finding that N-glycosylation and N-glycosylation in proteasomal ERAD. Mutations in the AE1 gene are known to cause various types of HS and renal tubular acidosis (Tse and Lux, 2001; Tanner, 2002). Understanding the mechanism by which AE1 mutants are recognized and degraded by the proteasome may reveal both the mechanism of intracellular processing of membrane proteins and the etiology of inherited membrane disorders.

Materials and Methods
Genotyping of the R664X mutation and quantification of AE1 transcripts
Genomic DNA from peripheral blood of animals was amplified by PCR using the primer pair p17 and p14, banking the mutation site, as described (Inaba et al., 1996). Resultant 107-bp fragments were digested with DraIII for genotyping.

Total RNA was collected from bone marrow cells and reverse transcribed using oligo(dT)12-18 primers and SuperScript II reverse transcriptase (Invitrogen). AE1 and protein 4.1 mRNAs were quantified by RT-PCR combined with a 5'-nuclease assay (TaqMan) (TaqMan chemistry kit; Perkin-Elmer Applied Biosystems). TaqMan primers and probes used were as follows: primers 5'-ATGGCTGCGCAAGTTCAAGAA-3' [nucleotides (nt) 1813-1832] and 5'-AGAGATAGAAACCCCCAGATGCC-3' [nt 1893-1872], and the probe 5'-TACTTCTCGGCAAGTCCGGCAAGA-3' [nt 1840-1865] for bovine erythroid AE1 (GenBank accession number NM_181036) and primers 5'-GAAGAACGAGAGACACTAGTG-3' [nt 1362-1385] and 5'-GAGTGAAGGTTAATACCTTGA-3' [nt 1553-1553], and the probe 5'-AACCTCCGATACCGG-3' [nt 1489-1516] for bovine erythroid protein 4.1 (GenBank accession number AF222767). Copy numbers were estimated by dilution curves obtained using plasmid clones of AE1 and protein 4.1.

To evaluate the relative abundance of mutant AE1 RNA, cDNA was amplified in the presence of [α-32P]dCTP (NEL Life Technologies) using primers p17 and p14. Labeled fragments were digested with DraIII and separated on 10% polyacrylamide gels. The relative intensity of digested fragments against the 107-bp PCR product was determined by densiometric scanning of X-ray films assuming that the PCR products amplified from genomic DNA of heterozygotes contained 50% mutant sequence.

Antibodies
Murine monoclonal antibodies cdh3-64, tm3-26, and tm3-29 against bovine AE1 were prepared by standard procedures using bovine red-cell membranes as antigen, and clones were isolated based on the recognition of bovine AE1 in immunoblotting. Epitopes recognized by these antibodies were roughly determined by N-terminal amino acid sequencing of bovine AE1 peptides generated by digestion with trypsin or chemical cleavage with cyanogen bromide, followed by probing with antibodies. Epitopes resided in residues E261 to M303 for cdh3-64, residues L650 to K708 for tm3-26, and the region adjacent to the extracellular trypsin-cleavage site at residue K598 for tm3-29, using the numbering of bovine red-cell AE1. Thus, the antibodies cdh3-64 and tm3-26, but not tm3-29 recognize R664X AE1 protein. The antibody against the N-terminal cytoplasmic domain of bovine AE1 (anti-38K) has been described previously (Inaba et al., 1996). Bovine glycophorin C (GPC) antibody was raised in rabbits using a recombinant C-terminal cytoplasmic peptide (residues L62 to C-terminal I109) as antigen and purified on a Protein G-Sepharose 4FF column (Amersham).

Other primary antibodies used were: anti-CFTR (clone M3A7, Upstate), anti-green fluorescence protein (GFP) and anti-GMI30 (both from BD Biosciences Clontech), anti-calnexin (Stressgen), and anti-Lamp2 (Santa Cruz Biotechnology). Anti-ubiquitin rabbit polyclonal (Santa Cruz Biotechnology) and mouse monoclonal (Zymed Laboratories Inc.) antibodies were also used. Secondary antibodies labeled with Alexa-Fluor-488 or -568 were obtained from Molecular Probes.

Construction of plasmids and mutations
cDNAs containing the entire coding region of wild-type bovine erythroid AE1 (bebWT) and the R664X mutant (bebRX) were obtained by PCR amplification of bone marrow cDNAs using the primer pair BEBrevp1 (5'-GGGCGGCGCCTCAATGCAGTGCAACGA-3') and BEBrevp2 (5'-GGTGACACATGTGCCCGGAC-3'). Primers contained mismatches (underlined) to create NorI and SstI restriction sites. Fragments were cloned into the pCR II vector (Invitrogen) to generate pbeBF and pCRbebRX. Subsequently, cDNAs were inserted into the retroviral vector pLNCX2 (Clontech) to generate pLNCXbebWT and pLNCXbebRX. The NorI-ClaI fragments of these clones were transferred into the pLPX vector (Clontech). Likewise, cdna inserts were subcloned into pcDNA 3.1 (Invitrogen) and pEGFP-C3 ( vectors (Clontech) resulting in pcbeBFWT, pbebRX, pEGFP-bebWT, pEGFP-bebRX. Nucleotide sequences of clones were confirmed using an 8800 CEQ DNA sequence (Beckman Coulter).

cDNA containing the entire coding region of bovine GPC DNA (GenBank accession number DQ234373) was obtained by PCR amplification of bone marrow cDNAs using the primers GPP35 (5'-GACACAGCGCGCCTCCCTCGC-3') and GPP30 (5'-ATTTCAAATCAGAGAGCTGCTCATTAC-3'). The amplified fragment was cloned into pCR II and then subcloned into pcDNA3.1 to generate pbeGPC. The plasmid clone pbeGPC-ΔF508-CFTR (Lotfing-Cuem et al., 2001) was a generous gift from Bruce A. Stanton (Dartmouth Medical School, Hanover, NH).
Cell culture and transfection

Cells were grown in RPMI1640 medium (K562) or minimum essential Eagle’s medium (HEK293) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 5% CO2 at 37°C. For experiments, cells were treated with lactacystin or MG132 (both from Peptide Institute, Inc., Osaka, Japan) or with aprotinin, leupeptin, or aprotinin chloride (all from Wako). Treatment was carried out using the Retro-X system with RetroPack PT67 cells (Clontech). Transfection of stable K562 transfectants with pCtG was performed using Lipofectamine 2000 (Invitrogen) 24 hours before assay.

Analyses of proteins

Cells were washed in phosphate-buffered saline (PBS) and lysed in IP buffer containing 20 mM Tris-HCl (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-(-aminomethyl)-benzenesulfonyl fluoride for 30 minutes on ice. After removal of cell debris by centrifugation at 18,000×g for 20-45 minutes with [35S]methionine (EXPRE 35S, NEN Life Science Products) in methionine-free Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 5% dialyzed fetal bovine serum and chased for the indicated periods. Cells were processed for immunoprecipitation and SDS-PAGE followed by autoradiography. When indicated, cell-surface proteins were labeled with NHS-S-S-biotin (Pierce Chemical Co.) and isolated on NutriAvidin beads (Pierce Chemical Co.) for immunoprecipitation (as described by Ihara et al., 2002). Preparation of red-cell ghosts, determination of membrane protein contents, deglycosylation of AE1 in red-cell membranes, and in vitro translation were carried out by methods described previously (Inaba and Maede, 1988; Inaba et al., 1996; Sato et al., 2000).

Chloride transport assay in Xenopus oocytes

In vitro transcription of RNA and microinjection into Xenopus oocytes were performed essentially as described (Sato et al., 2000). Groups of five to ten oocytes were pulse-labeled with [35S]methionine (EXPRESS 35S, NEN Life Science Products) in methionine-free Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 5% dialyzed fetal bovine serum and chased for the indicated periods. Cells were processed for immunoprecipitation and SDS-PAGE followed by autoradiography. When indicated, cell-surface proteins were labeled with NHS-S-S-biotin (Pierce Chemical Co.) and isolated on NutriAvidin beads (Pierce) after immunoprecipitation as described (Tamahara et al., 2002).

Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting.

Protein concentration was determined using a Bio-Rad protein assay kit.

Immunofluorescence microscopy

Cells were grown on collagen-coated coverslips (Iwaki Glass Co., Tokyo, Japan). After washing in PBS, cells 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 antibody in the same solution for 1 hour at ambient temperature, washed with PBS and then incubated with the secondary antibody. After washing with PBS, cells were mounted in Pro-Long antifade reagent (Molecular Probes) and examined under a fluorescence microscope.

Analyses of cell-surface protein oligomers were performed essentially as described (Farinha and Amaral, 2005). Most F508del-CFTR is targeted to the endoplasmic reticulum.

References


