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Ubiquitination-independent endoplasmic reticulum-associated degradation of an AE1 mutant associated with dominant hereditary spherocytosis in cattle

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Running header: ERAD of an AE1 mutant leading to dominant HS

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Summary

Various mutations in the AE1 (anion exchanger 1, band 3) gene cause dominant hereditary spherocytosis, a common congenital hemolytic anemia associated with deficiencies of AE1 of different degrees and loss of mutant protein from red cell membranes. To determine the mechanisms underlying decreases in AE1 protein levels, we employed K562 and HEK293 cell lines and *Xenopus* oocytes together with bovine wild-type AE1 and an R664X nonsense mutant responsible for dominant hereditary spherocytosis in order 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 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 AE1 employed endoplasmic reticulum-associated degradation through the proteasomal pathway. Proteasomal degradation of R664X protein appeared to be independent of both ubiquitination and N-glycosylation, and aggresome formation was not observed following proteasome

inhibition. These findings indicate that AE1 R664X protein, which is associated with dominant hereditary spherocytosis, has a dominant negative effect on the expression of wild-type AE1.

Introduction

Hereditary spherocytosis (HS) is a common congenital hemolytic anemia characterized by spherocytic and osmotically fragile red 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 AE1, all of which lead to mechanical instability of the red cell membrane.

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 Cl^-/HCO_3^- exchange across the plasma membrane (Alper, 1991).

Various mutations of the human *AE1* (*SLC4A1*) gene, including missense, nonsense, and frameshift mutations, have been reported to cause dominant HS, which is associated with a 20-40% reduction in 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 AE1 deficiency in cattle in the homozygous state for a nonsense mutation R664X, corresponding to a premature termination at residue Arg^{646} in human AE1, also showed atypical spherocytosis with marked membrane instability (Inaba et al., 1996; Inaba, 2000). Previous studies in patients or animals with *AE1* mutations failed to demonstrate the presence of mutant AE1 protein in their red cell membranes. This finding was partly explained, particularly in the case of the nonsense mutations, by the absence of mutant transcripts in patients' reticulocytes (Jarolim et al., 1996; Jenkins et al., 1996; Dhermy et al., 1997). However, although HS with 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 *AE1* gene disruption 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 apparatus (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 since 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., 1995; Gelman et al., 2002) and certain AQP2 mutants. In the case of AQP2 mutants underlying NDI, degradation of the ER-retarded AQP2 mutant T126M in recessive NDI required proteasomal activity, while degradation of an E258K mutant in dominant NDI occurred in a proteasome- and lysosome-dependent manner (Hirano et al., 2003). However, the contribution of proteasomal ERAD to the degradation of one AE1 mutant (R760Q) is negligible (Quilty and Reithmeier, 2000) and not entirely clear in the case of other dominant HS-associated mutants. Therefore, the precise mechanisms underlying dominant inheritance of red cell phenotypes and the loss of mutant AE1 protein still remain unknown.

To determine potential involvement of proteasomal ERAD in dominant HS with AE1 deficiency caused by the R664X mutation in bovine *AE1* (band $3^{Bov.Yamagata}$) (Inaba et al., 1996; Inaba, 2000), we evaluated 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, since 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). Densitometric scanning of membrane proteins separated on SDS-gels revealed that red cell membranes from animals heterozygous for the R664X mutation showed significantly reduced AE1 content (69.2% ± 6.4% at the per cell level, $n = 4$, $p < 0.01$ versus control). This observation suggests that typical spherocytosis with increased osmotic fragility in heterozygous animals is due to partial deficiency of AE1. Quantitative RT-PCR using bone marrow cells showed that levels of AE1 mRNA in animals heterozygous or homozygous for the R664X mutation were increased approximately 3 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 major 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 (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). In contrast, R664X AE1 showed a rapid turnover with a half-life ($t_{1/2}$) 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 if 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 major 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 to that seen in K562bebWT cells (Fig. 2C, upper panels). Similar findings were obtained with the tmb3-29 antibody (data not

shown). In contrast, tmb3-26 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 tmb3-26 (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 (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 tmb3-26 but not with tmb3-29 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 tmb3-26 was seen only when translation of wild-type and R664X AE1 was carried out in a single reaction (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'-diisothiocyano-2,2'-stilbene disulfonate (DIDS)-sensitive Cl^- transport, which reached a

maximal level 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 wild-type AE1 uptake activity. Significant reduction in Cl^- transport was also observed in oocytes co-injected with 0.5 or 1.0 ng of R664X RNA plus 0.5 ng of wild-type RNA. It was unlikely that observed decreases in Cl^- uptake were due to non-specific suppressive effects due to high concentrations of RNA injected, since 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 4 times that amount of R664X RNA (4.0 ng/oocyte) rendered transport activity negligible, while 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. Figure 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 ($t_{1/2} \simeq$ about 10 hours) was increased by more than 2-fold compared to 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 ubiquitinated AE1 in cells treated with proteasome inhibitors. While lactacystin remarkably enhanced accumulation of ubiquitinated 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 ubiquitinated 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 ubiquitination, although the subcellular localization of R664X AE1 in K562 cells is not clear.

Degradation of AE1 by the ERAD without apparent ubiquitination 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. In 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 apparatus 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 about 10 hours in transfected HEK293 cells (Fig. 6B), suggesting that wild-type AE1 is less stable in HEK293 cells than it is in 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 if proteasomal degradation of AE1 proteins in HEK293 cells was independent of ubiquitination, as suggested by results in K562 cells. To do so we employed the ΔF508-CFTR as a positive ubiquitination control (Fig. 7). When HEK293 cells were transiently transfected with EGFP-ΔF508-CFTR, a major 170-kDa polypeptide and higher molecular weight species were detected by immunoblotting with the anti-CFTR antibody, and the 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 slowly 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-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 immunoprecipitation probed with an anti-ubiquitin antibody demonstrated the presence of ubiquitinated species of EGFP-ΔF508-CFTR larger than 170 kDa. However, no ubiquitin immunoreactivity signals were detected in immunoprecipitates using the anti-AE1 antibody 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, suggesting 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), as was observed for 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, while 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 ubiquitinated AE1.

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-linked glycosylation site, Asn-X-Ser/Thr (X = any amino acid residue except Pro) (Gavel and von Heijne, 1990), exhibiting Asn^{660}-Pro-Thr rather than the corresponding Asn^{642}-Ser-Ser 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 a 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 generated deglycosylated polypeptides of canine and human AE1 (supplementary material, Fig. S2). In addition, in vitro translation product in the presence of pancreatic microsomes of the P661S AE1, in which the wild-type sequence Asn-Pro^{661}-Thr has been changed to Asn-Ser^{661}-Thr 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 cell-free translation system (data not shown). These observations demonstrated 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 (Ellgaad and Helenius, 2003) through the proteasome pathway in transfected K562 and HEK 293 cells. Furthermore, misexpression 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 animals, since, 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 between 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 is likely to occur 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 apparatus and/or lysosomes (Mulders et al., 1998; Tamarappoo et al., 1999; Marr et al., 2002; Hirano et al., 2003) or mistargeting 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), since 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 both 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, while 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 the cells where they are endogenously expressed, they are processed quite differently through the secretory pathway in HEK293 cells (Ruetz et al., 1993). Our data shows 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 likely due to 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., 1995; Gelman et al., 2002; Johnston et al., 1998). CFTR and ΔF508-CFTR require ubiquitination 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 ubiquitination at steady state levels in cells or

in cells showing marked accumulation of R664X or wild-type AE1 (Figs. 4 and 7). It is unlikely that lack of ubiquitinated AE1 in cell lysates is due to high activity of deubiquitinating enzymes, since in parallel experiments, we observed remarkable accumulation of ubiquitinated EGFP-ΔF508-CFTR (Fig. 7), as has been reported previously (Gelman et al., 2002). Our findings suggest rather that ubiquitination 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 retrotranslocation 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 retrotranslocation 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 may 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, since 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. Ito and M. Inaba, unpublished observations). These observations are compatible with a recent finding that N-glycosylation is important in calnexin-mediated folding and overall stability of wild-type CFTR but that the major degradation pathway of ΔF508-CFTR is independent of calnexin and occurs at an early checkpoint during biosynthesis in the ER (Farinha and Amaral, 2005). It will be of particular interest to examine whether calnexin plays a role in quality control of AE1 proteins through polypeptide-based interactions, as previously demonstrated for various substrates (Ihara et al., 1999; Danilczyk and Williams, 2001; Leach and Williams, 2004).

In conclusion, our present study provides evidence for a principal role of ERAD in quality control of a polytopic membrane protein AE1 and in the pathogenesis of dominantly inherited HS. Using different cell lines we show that AE1 exhibits novel characteristics, including independence of ubiquitination 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 flanking the mutation site as described (Inaba et al., 1996). Resultant 107-bp fragments were digested with Dra III for genotyping.

Total RNA was collected from bone marrow cells and reverse transcribed using oligo(dT)$_{12\text{-}18}$ primers and SuperScript II reverse transcriptase (Invitrogen). AE1 and protein 4.1 mRNAs were quantitated by RT-PCR combined with a 5'-nuclease assay using TaqMan chemistry on the GeneAmp 5700 sequence detection system (Perkin-Elmer Applied Biosystems). TaqMan primers and probes used were as follows: primers 5'-ATGCTGCGCAAGTTCAAGAA-3' (nt 1,813 to 1,832) and 5'-AGAGATAGGAACCCCGAAGTCC-3' (nt 1,893 to 1,872), and the probe 5'-TACTTCCCTGGCAAGCTGCGACGAAT-3' (nt 1,840 to 1,865) for bovine erythroid AE1 (GenBank accession number NM_181036) and primers 5'-GAAAAAGAGAGAGAGACTAGATGG-3' (nt 1,362 to 1,385) and

5’-GAGTGAGTGGATAAGCGTTTA-3’ (nt 1,553 to 1,533), and the probe 5’-AACTTCATGGAATCCGTACCAGAACCAC-3’ (nt 1,489 to 1,516) for bovine erythroid protein 4.1 (GenBank accession number AF222767). Copy numbers were estimated from standard 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-^{32}P]dCTP (NEN Life Technologies) using primers p17 and p14. Labeled fragments were digested with Dra III and separated on 10% polyacrylamide gels. The relative intensity of digested fragments against the 107-bp PCR product was determined by densitometric scanning of X-ray films assuming that the PCR products amplified from genomic DNA of heterozygotes contained 50% mutant sequence.

Antibodies

Murine monoclonal antibodies cdb3-64, tmb3-26, and tmb3-29 to 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 Glu^{261}-Met^{303} for cdb3-64, Leu^{650}-Lys^{708} for tmb3-26, and the region adjacent to the extracellular trypsin-cleavage site Lys^{598} for tmb3-29, using the numbering of bovine red cell AE1. Thus, the antibodies cdb3-64 and tmb3-29 but not the tmb3-26 recognize R664X AE1 protein. The antibody to the N-terminal cytoplasmic domain of bovine AE1 (anti-38K) has been described (Inaba et al., 1996). Bovine glycophorin C (GPC) antibody was raised in rabbits using a recombinant C-terminal cytoplasmic peptide (Leu^{62}-Ile^{109}-COOH) 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-GM130 (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 AlexaFluor 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'-GCGGCCGCTCAGATCACTGCA-3') and BEBrevp2 (5'-GTCGACCCATGTCCCCGGGAC-3'). Primers contained mismatches (underlined) to create Not I and Sal I restriction sites. Fragments were cloned into the pCRII vector (Invitrogen) to generate pCRbebWT and pCRbebRX. Subsequently, cDNAs were inserted into the retroviral vector pLNCX2 (Clontech) to generate pLNCXbebWT and pLNCXbebRX. The Not I-Cla I fragments of these clones were transferred into the pLPCX vector (Clontech). Likewise, cDNA inserts were subcloned into pcDNA3.1 (Invitrogen) and pEGFP-C3 vectors (Clontech) resulting in pcbebWT, pcbebRX, pEGFP-bebWT, and pEGFP-bebRX. Nucleotide sequences of clones were confirmed using an 8800 CEQ DNA sequencer (Beckman Coulter).

cDNA containing the entire coding region of bovine GPC cDNA (GenBank accession number DQ324373) was obtained by PCR amplification of bone marrow cDNAs using the primers bGPCp5 (5'-GACACAGGTGCCGCTTCCTCC-3') and bGPCp10 (5'-ATTTCCAATCAGAGCTGCATCTAC-3'). The amplified fragment was cloned into pCRII and then subcloned into pcDNA3.1 to generate pcbGPC.

The plasmid clone pEGFP-ΔF508-CFTR (Loffing-Cueni et al., 2001) was a generous gift from Dr. Bruce A. Stanton (Dartmouth Medical School, Hanover, NH, USA).

Cell culture and transfection

Cell lines were purchased from Human Science Research Resources Bank (Osaka, Japan). Cells were grown in RPMI1640 medium (K562) or minimum essential Eagle medium (HEK293) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 5% CO_2 at 37˚C. K562 cells were infected with pLNCXbebWT or pLNCXbebRX retroviruses followed by selection in G418 (2 mg/ml, Wako Pure Chemical Industries, Osaka, Japan). Several independent clones stably expressing wild-type (K562bebWT) or R664X (K562bebRX) AE1 were established. Packaging of plasmids into viruses was carried out using the Retro-X system with RetroPack PT67 cells (Clontech). pLPbebRX was introduced into some clones of K562bebWT cells to generate K562bebWT/RX cells by the same procedure except that selection was in G418 (1.5 mg/ml) and puromycin (2 µg/ml, Wako). Transient transfection of stable K562 transfectants with pcbGPC was carried out in serum-free OptiMEM (Invitrogen) using Lipofectamine 2000 (Invitrogen) 24 hours before assay.

HEK293 cells were transiently transfected with the plasmids described above in serum-free OptiMEM using TransIt-LT1 (Mirus) 20 hours before assay. In some experiments,

cells were treated with lactacystin or MG132 (both from Peptide Institute, Inc., Osaka, Japan) or with aprotinin, leupeptin, or ammonium chloride (all from Wako).

Analyses of proteins

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

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

K562 and HEK293 cells expressing WT and/or R664X AE1 were pulse-labeled for 20-45 minutes with [^{35}S]methionine (EXPRE^{35}S^{35}S, NEN Life Science Products) in methionine-free Dulbecco's modified Eagle's medium (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 NutrAvidin beads (Pierce) after immunoprecipitation as described (Tamahara 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 as 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 5 to 10 oocytes were incubated in ND-96 medium (15 µl/oocyte) containing 7-10 µCi/ml Na^{36}Cl (Amersham) for 1 hour at 19°C in the presence or absence of 10 µM DIDS (Dojin Chemical Laboratories, Kumamoto, Japan). After incubation, oocytes were quickly washed 4 times with ND-96 containing 10 µM

DIDS and radioactivity was measured.

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 appropiate 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 ProLong antifade reagent (Molecular Probes) and examined under a Nikon ECLIPSE microscope or a Zeiss confocal laser microscope LSM5 PASCAL.

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Legends for Figures

Fig. 1. Expression of wild-type and R664X AE1 in K562 cells. A, expression of bovine AE1 was examined by immunofluorescence with the monoclonal antibody cdb3-64 in K562bebWT, K562bebRX, and control (K562C) cells. Bars = 10 μm. B, K562bebWT and K562bebRX cells were pulse-labeled with [^{35}S]methionine for 45 minutes and chased for the indicated periods. After cell-surface biotinylation, AE1 proteins were immunoprecipitated using tmb3-29 with (Surface) or without (Total) isolation on NutrAvidin beads. Migration of protein standards is indicated in kDa. Intensities of the bands at the indicated times for wild-type AE1 (WT, 105 kDa, ○ and □) and R664X AE1 (R664X, 75 kDa, ● and ■) in total (○ and ●) and cell-surface (□ and ■) fractions were determined by densitometric scanning and plotted (right). Data are the mean values of duplicate samples at each time point except for time zero (n = 3, means ± s.d.). This procedure always produced a non-specific 77-kDa band (*) even in immunoprecipitates from control cells.

Fig. 2. Dominant negative effect of R664X AE1 on cell surface expression of wild-type AE1. A, wild-type (○) proteins in K562bebWT cells and wild-type (●) and R664X (■) proteins in K562bebWT/RX cells were pulse-labeled and chased for the indicated periods as in Fig. 1. The left figure shows immunoprecipitates from each sample after labeling without

chase. The asterisk indicates a non-specific band. Data are the means of duplicate samples at each time point. B, K562bebWT, K562bebRX, K562bebWT/RX, and K562C cells were transfected with bovine GPC and stained for AE1 and GPC with cdb3-64 and the anti-GPC antibody, respectively. Bars = 5 μm. C, AE1 proteins in K562bebWT (WT), K562bebRX (RX), and K562bebWT/RX (WT/RX) cells were immunoprecipitated with monoclonal antibodies cdb3-64 or tmb3-26 (upper panels). Lane WT + RX contains immunoprecipitates from K562bebWT and K562bebRX cells mixed prior to solubilization for immunoprecipitation. The asterisk indicates what is likely a ≃ 70-kDa degradation product of wild-type AE1. Likewisse, AE1 proteins synthesized in cell-free translation system in the presence of canine pancreatic microsomes (Synthesized) were immunoprecipitated with tmb3-26 (lower right panel). Reactions contained wild type alone (WT), R664X alone (R664X), or both wild-type and R664X AE1 (WT/RX). WT and RX reactions were combined prior to solubilization (WT + RX).

Fig. 3. Dominant negative effect of R664X AE1 on functional expression of wild-type AE1 in *Xenopus* oocytes. A, $^{36}Cl^-$ uptake into 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 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 means ± s.d. ($n \geq 10$). *; $p < 0.05$, and **; $p < 0.01$ versus oocytes injected with 0.5 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 ubiquitinated 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 ubiquitinated polypeptides but not proteins recognized with the AE1 antibody (data not shown). Blots were reacted 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.

Fig. 5. Intracellular localization of wild-type and R664X AE1 in HEK293 cells. HEK 293 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 reacted with anti-calnexin (Calnexin), anti-GM130 (GM130), and anti-Lamp2 (Lamp2) followed by detection with a secondary antibody labeled with AlexaFluor 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 NutrAvidin 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).

Fig. 7. The absence of ubiquitinated AE1 in transfected HEK293 cells. A and 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. Ubiquitinated 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.

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 to that of calnexin (A, B) and ubiquitin (C).

Legends for Supplementary Materials

Fig. S1. Relative abundance of mRNA encoding R664X mutant AE1 in bone marrow cells from mutant animals. A, total amounts of mRNA for AE1 (closed squares) and protein 4.1 (open squares) were determined for normal (N, n = 3), heterozygous (He, n = 2), and homozygous (Ho, n = 2) animals by quantitative PCR and described as copy number per 1,000 copies of GAPDH mRNA. Data are expressed as means ± s.d. for N and as mean values for He and Ho. The number at the right indicates the relative value of AE1 mRNA/protein 4.1 mRNA. B and C, relative abundance of mRNA encoding mutant protein. PCR amplification of genomic DNA (gDNA) or bone marrow cell cDNA (cDNA) showed exponential increases of ^{32}P-labeled products of the 107-bp fragment from the 22nd through 28th cycles. A typical autoradiogram of ^{32}P-labeled PCR fragments obtained after 26 cycles of PCR amplification followed by digestion with Dra III is shown (B). When gDNAs from heterozygous animals were amplified, in which the mutant allele comprised 50% of the template, densitometric intensities of the 62-bp fragment generated by digestion of the products from the mutant allele with Dra III were 23-25% of the total. The intensity of the 62-bp fragments generated from amplified cDNAs ranged from 14% to 19% at PCR cycles 22 through 30. Therefore, in heterozygotes the relative abundance of AE1 mRNA with the R664X mutation ranged from 28% to 38% of the total amount. This method also showed that in heterozygotes levels of AE1

mRNA relative to protein 4.1 were decreased to 55% of normal, in agreement with the estimation described above (A). The amounts of the R664X mutant (closed squares) and wild-type (open squares) mRNA in bone marrow cells from heterozygotes (He) and homozygotes (Ho) relative to that in normal (N) cells are shown (C).

Fig. S2. Absence of N-glycan modification in bovine AE1. A, membranes from bovine, canine, and human red blood cells were incubated in the presence (PNGase) or absence (Mock) of peptide: N-glycosidase F and analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue. AE1 (band 3), protein 4.1 (a and b), and protein 4.2 along with positions of protein standards are indicated. Asterisks indicate deglycosylated polypeptides of canine and human AE1. B, in vitro translation in the presence of canine pancreatic microsomes of bovine AE1 wild type (bebWT) and a P661S mutant (bebP661S). P661S AE1 protein contained both N-glycosylated and non-glycosylated forms.