Expression and interaction of two compound heterozygous distal renal tubular acidosis mutants of kidney anion exchanger 1 in epithelial cells

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Cordat, Emmanuelle, and Reinhart A. F. Reithmeier. Expression and interaction of two compound heterozygous distal renal tubular acidosis mutants of kidney anion exchanger 1 in epithelial cells. Am J Physiol Renal Physiol 291: F1354–F1361, 2006. First published July 18, 2006; doi:10.1152/ajprenal.00015.2006.—Kidney AE1 (kAE1) is a glycoprotein responsible for the electroneutral exchange of chloride for bicarbonate, promoting the reabsorption of bicarbonate into the blood by α-intercalated cells of the collecting tubule. Mutations occurring in the gene encoding kAE1 can induce defects in urinary acidification resulting in distal renal tubular acidosis (dRTA). We expressed two kAE1 dRTA mutants, A858D, a mild dominant mutation, and ΔV850, a recessive mutation, in epithelial Madin-Darby canine kidney (MDCK) cells. Individuals heterozygous with wild-type (WT) kAE1 either did not display any symptoms of dRTA (ΔV850/WT) or displayed a mild incomplete form of dRTA (A858D/WT), while compound heterozygotes (ΔV850/A858D) had dRTA. We found that the A858D mutant was slightly impaired in the endoplasmic reticulum (ER) exit but could target to the basolateral membrane of polarized MDCK cells. Despite an altered binding to an inhibitor affinity resin, anion transport assays showed that the A858D mutant was functional at the cell surface. The ΔV850 mutant showed altered binding to the affinity resin but was predominantly retained in the ER, resulting in undetectable AE1 expression at the basolateral membrane. When coexpressed in MDCK cells, the WT protein, and to a lesser extent the A858D mutant, enhanced the cell surface expression of the ΔV850 mutant. The ΔV850 mutant also affected the cell surface expression of the A858D mutant. Compound heterozygous (A858D/ΔV850) patients likely possess a decreased amount of functional anion exchangers at the basolateral membrane of their α-intercalated cells, resulting in impaired bicarbonate transport into the blood and defective acid transport into the urine.

IN THE KIDNEY, α-INTERCALATED cells in the collecting tubule play a crucial role in urine acidification. This process is ensured by the linked action of three proteins in these cells: cytosolic carbonic anhydrase II, converting CO₂ and H₂O into HCO₃⁻ and H⁺, apical H⁺-ATPase extruding the cytosolic H⁺ into the urine, and basolateral kidney anion exchanger 1 (kAE1), transporting HCO₃⁻ into the blood in exchange for Cl⁻ (12). Impairment in acid secretion due to genetic mutations in the genes encoding these three proteins leads to distal renal tubular acidosis (dRTA), a kidney disease characterized by metabolic acidosis that can be associated with nephrocalcinosis, metabolic bone disease, deafness and mental retardation (12).

Human anion exchanger 1 (AE1) is the major glycoprotein found in the plasma membrane of red blood cells. This 911-amino acid membrane protein is organized into two main domains: a large cytosolic domain involved in multiple interactions with red blood cell cytosolic proteins such as globin, cytoskeletal proteins, and glycolytic enzymes, and a COOH-terminal membrane domain crossing the lipid bilayer 12 to 14 times, responsible for the electroneutral exchange of chloride for bicarbonate (24, 25). A short COOH-terminal cytosolic tail provides a binding site for carbonic anhydrase II (30, 31), and is involved in the proper trafficking of the exchanger to the plasma membrane (5). Due to the use of an alternative promoter, kidney anion exchanger 1 (kAE1) is missing the first 65 amino acids found in the erythrocyte isoform (1, 15, 32). Both the erythrocyte and kidney isoforms of AE1 carry a single N-glycosylation site at asparagine 642 and both proteins exist as dimers.

An increasing number of mutations in the kAE1 gene have been reported to induce both dominant and recessive dRTA. Most of these mutations are located within the COOH-terminal half of the transmembrane domain, with two mutations resulting in COOH-terminal truncated proteins. The dominant R589H, C or S and S613F mutations did not affect the protein’s function in patients’ red blood cells or when expressed in Xenopus laevis oocytes (28). However, when expressed in HEK 293 (19) or Madin-Darby canine kidney (MDCK) cells, these dominant mutants were retained in the endoplasmic reticulum (ER), with some protein mistargeted to the apical membrane (4, 19, 28). The dominant mutants retained wild-type (WT) kAE1 in the ER due to heterodimer formation (18). Other dominant mutations, G609R (21) and R901X (7), also exhibited normal activity in patients’ red blood cells and in X. laevis oocytes. When transiently expressed in polarized MDCK cells, these mutant proteins were largely retained intracellularly but were also mistargeted to the apical membrane. However, when stably expressed in polarized MDCK I cells, R901X was found exclusively targeted to the apical membrane (28), indicating that the cytosolic COOH-terminal tail is involved in the basolateral membrane targeting of the transporter.

Recessive dRTA mutations in the gene encoding kAE1, seem to be restricted to South Asian population where a red blood cell condition, Southeast Asian ovalocytosis (SAO) is endemic; therefore, recessive dRTA patients are often compound heterozygotes with SAO. SAO is characterized by a 9-amino acid deletion within the first transmembrane segment of AE1 resulting in a nonfunctional anion transporter and an altered erythroid shape (10, 22). Generally, recessive dRTA mutants (G701D and S773P) induce a functional defect when expressed in X. laevis oocytes while patients do not present any
major red blood cell functional defect. In MDCK cells, despite its misfolding, the S773P mutant could exit the ER and reach the basolateral membrane of polarized cells. The G701D mutant also exited the ER but was largely localized to the Golgi (4). In X. laevis oocytes, the functional defect was corrected in the case of G701D, when a red blood cell-specific protein called glycopyrin A (GPA), known to promote eAE1 trafficking to the cell surface, was coexpressed with eAE1 (26, 29). No equivalent protein of GPA, potentially acting in a similar way has been so far described in the kidney (14). Thus the G701D equivalent protein of GPA, potentially acting in a similar way to the cell surface, was coexpressed with eAE1 (26, 29). No the case of G701D, when a red blood cell-specific protein encoding the entire human kidney anion exchanger carrying an external hemagglutinin (HA) tag inserted into position 557 (5) into the XhoI site of the retroviral expression vector pFBneo (Stratagene, La Jolla, CA). The pFBneo-kAE1HA557 dominant and recessive mutants were constructed using Stratagene QuickChange site-directed mutagenesis kit and confirmed by automated sequencing (ACGT, Toronto, Canada).

Cell culture. MDCK cells stably expressing kAE1 proteins were obtained as previously described (4). Briefly, HEK 293 cells were cotransfected with three retroviral plasmids pVpack-GP, pVpack-VSVG (vesicular stomatitis virus glycoprotein), and pFBneo-kAE1HA557 using FuGene 6 Transfection reagent (Roche Diagnostics, Indianapolis, IN). The virus-containing supernatant was collected 24–36 h later and added to 30–50% confluent MDCK cells, in presence of 8 µg/ml of polybrene (Sigma, St. Louis, MO). The infected cells were selected with 1 mg/ml geneticin (G418, Sigma). Polarized MDCK cells were obtained by growing confluent infected cells on Transwell polycarbonate filters (Corning Headquarters, Corning, NY) for 4 to 5 days. As previously described, the viral infection results in a heterogenous population of kAE1-expressing cells (4).

Western blotting, SDS-PAGE and Western blotting were performed as previously described (20). Briefly, infected MDCK cells were lysed in PBS containing 1% C$_4$E$_8$ detergent and protease inhibitors Leupeptin (1 µM), Aprotinin (1 µM), PMSF (200 µM), and pepstatin A (1 µM). After centrifugation to remove insoluble material, 15 µg of total protein per lane were loaded and resolved on a 8% SDS-PAGE and transferred to a nitrocellulose membrane. The blotted proteins were detected with a mouse anti-HA monoclonal antibody (Covance, Princeton, NJ), followed by an anti-mouse antibody coupled to horseradish peroxidase.

Deglycosylation experiments. MDCK cell lysates were centrifuged to remove the insoluble material and 100 µl of the supernatants were treated with 1,000 units of endoglycosidase H (Endo H; New England Biolabs, Ipswich, MA) or 500 units of peptide N-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA) at room temperature for 1 h, followed by the addition of 1 volume of 2× Laemmli sample buffer containing 4% SDS. kAE1 was detected by Western blots using a mouse monoclonal anti-HA antibody.

SITS-Affi-Gel binding assay. MDCK cells were lysed in 500 µl PBS containing 1% C$_4$E$_8$ detergent and 100 µl of cell lysate was incubated with 25 µl SITS-Affi-Gel with or without 1 mM free anion transport inhibitor H$_2$DIDS for 30 min at 4°C (17). After several washes, the bound proteins were eluted from the beads by adding 2× Laemmli sample buffer containing 4% SDS, resolved on an SDS-PAGE gel, transferred to nitrocellulose and detected by Western blotting with a mouse anti-HA antibody.

Anion exchange assay. To detect positive cells for kAE1 cell surface expression, MDCK cells grown on glass coverslips were first inoculated for 30 min on ice with 1/100 mouse anti-HA antibody. This first step was followed by a second incubation at 37°C with 1/1,000 goat anti-mouse antibody coupled to Alexa 488 and 2 µM of the pH-sensitive probe SNARF-5F (Molecular Probes). The coverslips were then mounted on Leiden chambers and placed on the stage of a Leica IRE microscope for ratio determinations of fluorescence using two emission wavelengths (590 nm and 640 nm), as described in (6). Image acquisition and selection of excitation filters were controlled through a lambda 10 filter wheel controller (Sutter Instrument, Novato, CA) by Metaphor software (Universal Imaging, West Chester, PA) running on a Dell computer interfaced with a Photometrics CCD camera via a 12-bit GPIB/IIA board (National Instruments, Foster City, CA). The NaCl-containing extracellular Ringer solution was then replaced by a NaCl-free Ringer solution. Under these conditions, intracellular Cl$^-$ exits while bicarbonate enters the cell via kAE1, resulting in intracellular alkalization, detectable by fluorescence ratio changes of SNARF-5F. To correct for the difference of cellular SNARF-5F loading, the analysis was performed by exciting the sample at 490 nm and recording the dual emission ratio with $\lambda_1 = 590$ nm and $\lambda_2 = 640$ nm. Each measurement was followed by an

METHODS

Construction of plasmids and mutations. Retroviral expression plasmid pFBneo-kAE1HA557 was constructed by shuttling the cDNA encoding the entire human kidney anion exchanger carrying an external hemagglutinin (HA) tag inserted into position 557 (5) into the XhoI site of the retroviral expression vector pFBneo (Stratagene, La Jolla, CA). The pFBneo-kAE1HA557 dominant and recessive mutants were constructed using Stratagene QuickChange site-directed mutagenesis kit and confirmed by automated sequencing (ACGT, Toronto, Canada).
intracellular pH calibration using 10 μM of the ionpore nigericin, to allow the pH to equilibrate across the cell membrane.

**Coimmunoprecipitation.** Coimmunoprecipitations were performed as previously described (4). Briefly, coinfected MDCK cells with either HA- or myc-tagged proteins were lysed in 0.5 ml of PBS containing 1% C₂H₄₂O₄ and centrifuged to remove insoluble materials. An aliquot was saved as total fraction (lane T) while the remaining supernatant was incubated with 10 μl rabbit anti-myc antibody followed by 40 μl protein G-Sepharose. After three washes, the protein was eluted with Laemmli buffer (lane IP), resolved by SDS-PAGE and blotted with a mouse anti-HA antibody followed by an anti-mouse antibody coupled to HRP.

**Flow cytometry analysis.** MDCK cells were detached from the flask by trypsin digestion, a procedure known to keep AE1 and the HA epitope intact (4). The cells were fixed with 3.8% formaldehyde, blocked with 1% BSA incubated with mouse anti-HA antibody (1/500) for 30 min followed by anti-mouse antibody coupled to Alexa 488 (1/500) for 30 min to detect the cell surface kAE1. Samples were analyzed using Beckman-Coulter EPICS Elite.

**Immunocytochemistry.** MDCK cells were grown on glass coverslips, or on semipermeable Transwell polycarbonate filters (Corning Headquarters, Corning, NY) to confluence for 4 to 5 days. Cells were fixed using 3.8% formaldehyde for 15 min, then washed once with 100 mM glycine, permeabilized with 0.2% Triton X-100 for 15 min and blocked for 30 min with 1% BSA. A 1/1,000 mouse anti-HA antibody (Covance) and 1/500 rabbit polyclonal anti-calnexin antibody (Stressgen Biotechnologies, San Diego, CA) in 1% BSA were added to the sample for 30 min. After several washes, 1/1,000 of Alexa 488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) or Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added for 30 min. After several washes, 1/1,000 of mouse anti-HA antibody followed by an Alexa 488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) or Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories). Samples were then examined using a Zeiss laser confocal microscope LSM 510.

**RESULTS**

**Dominant A858D and recessive ΔV850 mutants are impaired in ER exit.** To evaluate the effect of the mild dominant A858D and the recessive ΔV850 mutations on kAE1 expression in epithelial cells, MDCK cells were infected with recombinant MMLV viruses resulting in the individual expression of the two mutant kidney isoforms of AE1, carrying an HA epitope in the third extracellular loop. This situation therefore mimicked the homozygous state of dRTA. AE1 possesses only one N-glycosylation site in position N642 that is processed to the complex oligosaccharide (closed circle), resistant to digestion by Endo H but sensitive to PNGase F (Fig. 1, A, lane C), or with mannose oligosaccharides, while the N-glycosylation site in position N642 that is processed to the complex oligosaccharide (closed circle) resistant to digestion by Endo H but sensitive to PNGase F (Fig. 1, A, lane F) and were then loaded on a 8% SDS-PAGE gel before transfer to a nitrocellulose membrane. The proteins were then detected using an anti-HA antibody indicated that both mutants displayed at least two main bands (Fig. 1A, lane C), a lower band that corresponded to kAE1 carrying high mannose (open circle) and an upper band that corresponded to kAE1 with complex oligosaccharides (closed circle) resistant to digestion by Endo H but sensitive to PNGase F (Fig. 1A, lanes H and F, respectively). This is in contrast with kAE1 WT that displays predominantly complex oligosaccharide and only a small amount of the high-mannose form. Scans of multiple immunoblots of whole cell extracts showed that 76 ± 4% (n = 6) of kAE1 WT was in the complex form compared with 68 ± 5% (n = 6) and 47 ± 8% (n = 6) for the A858D and ΔV850 mutants, respectively. These data indicate that some A858D mutant and ΔV850 mutant moved from the ER to the medial Golgi. However, the higher relative intensity of the high-mannose band in A858D, and particularly ΔV850, indicates that these mutants have an impaired ER exit compared with the WT protein. This would result in a lower level of cell surface expression.

We next determined whether the mutants were targeted to the cell surface by flow cytometric analysis using a mouse anti-HA antibody followed by an Alexa 488-coupled anti-mouse antibody on intact cells. The same protocol was applied to permeabilized cells as a control experiment to ensure that the proteins were expressed (data not shown). The WT showed a broad distribution of fluorescence intensity, while above the level of control uninfected cells. Figure 1B shows that the mild dominant A858D displayed an intermediate fluorescence intensity consistent with a lower level of surface expression compared with the WT protein. The recessive ΔV850 showed a similar fluorescence intensity to the control sample, indicating a negligible level of cell surface expression (Fig. 1A). This is in agreement with the N-glycosylation analysis, which indi-
cates that the majority of this mutant protein was localized in the ER. The portion of the ΔV850 protein carrying complex oligosaccharide had exited the ER and moved to the Golgi but was not detected at the cell surface.

To confirm these results, we measured the intracellular pH variations associated with the functional expression of kAE1 at the cell surface of MDCK, using the pH-sensitive fluorescent probe SNARF-5F-AM (23). A significantly higher intracellular alkalinization was observed when kAE1-expressing cells, loaded in HCO₃⁻, were incubated in a Cl⁻-free extracellular medium, compared with the noninfected control MDCK cells. These results indicate that functional kAE1 proteins are present at the plasma membrane of kAE1-expressing MDCK cells. We observed a slight decrease of the initial transport rate of the mild dominant mutant A858D compared with the WT AE1 (78 ± 8% of the WT activity, n = 7, vs. 16 ± 1%, n = 3, for un-infected MDCK cells), consistent with lower level of protein expression at the cell surface. No transport activity measurements were made on MDCK cells expressing ΔV850, since this mutant could not be detected at the cell surface of intact cells.

Recessive ΔV850 mutant is localized to the ER while the dominant A858D mutant can reach the basolateral membrane in MDCK cells. To better investigate the trafficking defects of the mutant proteins, we determined their respective location in nonpolarized (Fig. 2A) and polarized (Fig. 2B) MDCK cells by immunofluorescence staining. We observed the relative location of the tagged proteins by immunofluorescence in nonpolarized (Fig. 3A) and polarized (Fig. 3B) MDCK cells. When the WT protein was coexpressed with the dominant A858D in the same cells, we observed that both proteins were predominantly localized to the cell surface. We

Fig. 2. Typical immunostaining of the ΔV850 and the A858D mutants in epithelial MDCK cells. Infected MDCK cells were either grown in a nonpolarized state on a glass coverslip (A) or until fully polarized on a semipermeable filter (B). Cells were then fixed, permeabilized and incubated with mouse anti-HA antibody (A and B) and rabbit anti-calnexin antibody (A only), followed by anti-mouse antibody coupled to the fluorophore Alexa 488 (A and B) and anti-rabbit antibody coupled to Cy3 (A only). The cells were then observed using a Zeiss LSM 510 confocal microscope. Bar represents 10 μm. X-Z corresponds to side view of the cells, X-Y shows middle section of the cells.
also observed a decreased staining of intracellular A858D (compare Fig. 2A and 3A), indicating that the WT protein improved the trafficking of the mutant to the cell surface. In contrast to other dominant dRTA mutants (R589H), coexpression of the mild dominant A858D mutant did not result in a major intracellular retention of the WT protein (4, 13). Consistent with previous results observed on other recessive dRTA mutants (4, 13), the recessive ΔV850, while predominantly located in the ER in the homozygous state, was partially found at the cell surface when coexpressed with the WT AE1 protein. This result suggests that the WT protein can rescue the recessive mutant’s trafficking to the plasma membrane.

When the two mutants were coexpressed in MDCK cells, mimicking the compound heterozygous state, we observed that while some A858D protein remained intracellular, this dominant mutant helped, although less efficiently than the WT protein, the recessive ΔV850 to reach the cell surface. Some of the A858D mutant also colocalized with the ΔV850 mutant within the cell, suggesting that the ΔV850 mutant affects the trafficking of the A858D mutant. The perinuclear staining suggests a Golgi or endosomal localization of the heterodimers although both proteins were also colocalized at the cell surface.

**Dominant A858D mutant interacts physically with the recessive ΔV850 mutant.** The trafficking improvement of the mutant ΔV850 when coexpressed with the dominant A858D mutant suggested that when present in the same cells, the dominant A858D mutant physically interacts with the recessive ΔV850 mutant. We confirmed this hypothesis by coimmunoprecipitating both proteins (Fig. 4). The two mutants carrying either the HA or the myc epitope were coexpressed in nonpolarized MDCK cells and were immunoprecipitated with an anti-myc antibody before detection of the interacting proteins using the anti-HA antibody. We found that each of the mutants is not only able to immunoprecipitate with the WT protein, but they are also found interacting with each other. The WT protein associates with both the complex and high-mannose form of the A858D mutant, consistent with cell surface expression of the heterodimers (complex form) and some ER retention (high mannose form) of the heterodimers. The WT protein associates predominantly with the complex

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**Fig. 3.** Typical immunostaining showing coexpression of the WT AE1 protein with the ΔV850 or the A858D mutants, and the A858D mutant with the ΔV850 mutant in nonpolarized and polarized MDCK cells. MDCK cells cocinfected with either WT AE1 and A858D or ΔV850, or cocinfected with the mutant A858D and ΔV850 were either grown at sub-confluency on a glass coverslip (A) or until fully polarized on a semipermeable filter (B). Cells were then fixed, permeabilized and incubated with mouse anti-HA antibody and rabbit anti-myc antibody, followed by anti-mouse antibody coupled to the fluorophore Alexa 488 and anti-rabbit antibody coupled to Cy3. The cells were then observed using a Zeiss LSM 510 confocal microscope. Bar represents 10 μm. X-Z corresponds to side view of the cells, X-Y shows middle section of the cells.
form of the mutant ΔV850, indicating the presence of heterodimers at the plasma membrane. Finally, the A858D mutant associates almost exclusively with the complex form of the ΔV850 mutant, suggesting that the ΔV850 mutant does not retain significant amounts of A858D mutant in the ER. The complex forms of coimmunoprecipitated A858D and ΔV850 may represent the proteins seen colocalized to the Golgi or endosomal compartment. It is, however, possible that the heterooligomer of the two proteins carrying high-mannose oligosaccharide is rapidly degraded in the ER and therefore not detected. These results confirm that it is via a physical interaction, and likely heterodimerization, that the dominant A858D mutant or the WT protein helps the recessive ΔV850 reach the plasma membrane of MDCK cells.

ΔV850 and A858D mutants do not bind efficiently a stilbene inhibitor affinity resin. It is known that the functional activity of the A858D mutant is significantly reduced (29% of the WT while ~50% of the protein was found at the cell surface) when expressed in X. laevis oocytes (2). We found that the A858D mutant had a lower level of protein expressed at the cell surface and seemed to have a slightly decreased transport activity in MDCK cells. To test whether the A858D mutation affects the structure of kAE1, the ability of native WT or mutant AE1 to bind an inhibitor affinity resin, SITS-Affi Gel was tested (Fig. 5, A and B). This method was previously used to detect folding defects of various mutants of AE1 (17, 20). We found that both A858D and ΔV850 mutants had a decreased binding to the AE1 inhibitor, similar to the level of binding of the severely misfolded SAO mutant.

These results indicate that the impaired binding to the stilbene likely reflects a local alteration in kAE1 structure since the overall transport properties of the A858D mutant are conserved, consistently with a mild phenotype. It is therefore likely that these individuals are able to acidify their urine in normal conditions, but in metabolic acidosis, the functional defect results in an impaired bicarbonate transport and lack of efficient urine acidification.

DISCUSSION

Autosomal recessive and dominant dRTA is a disease that can be caused by mutations within the gene SLC4A1 that encodes the anion exchanger 1 (AE1). Here, we describe the situation occurring in compound heterozygous dRTA patients from nine Papua New Guinean and Malaysian families carrying two mutations, A858D and ΔV850 (2). Due to its misfolding, the recessive mutant ΔV850 shows an impaired ER exit, a defect already observed for other recessive dRTA mutants of kidney isoforms of AE1 (4, 13). The mild dominant mutant A858D was slightly impaired in ER exit but was targeted to the basolateral membrane of polarized epithelial cells, in contrast to the other dominant dRTA mutants which were predominantly retained in the ER.

In the present study, we characterized a dRTA mutant with a recessive form of inheritance, ΔV850. We observed that this mutation induces the intracellular retention of kAE1 (Fig. 2), likely due to the protein’s misfolding (Fig. 5). However, when coexpressed with the WT or the dominant mutant A858D, this protein can be targeted to the basolateral membrane in polarized epithelial cells (Fig. 3). Our lab recently published data showing a similar phenotype for another recessive mutation, S773P (4, 13). When coexpressed with the WT protein, the WT helps the recessive mutant to reach the basolateral membrane of polarized epithelial cells.

Previous studies showed that dominant dRTA mutants of AE1 are usually properly folded but inappropriately retained by the quality control machinery in the ER and partially misrouted to the apical membrane of epithelial cells (4, 8, 21, 28). Since AE1 is a dimer, a proposed mechanism for dRTA in these cases is that the dominant mutant retains WT AE1 in the ER, via heterodimerization (18). Several recent papers exam-
in the effect of four different mutations inducing dominant dRTA. A truncation of the last 11 amino acids (R901X) induced a mistargeting of AE1 in polarized MDCK and LLC-PK1 cells as well as intracellular retention (8) or an exclusive apical targeting of the mutant (28). Since the kidney isoform of the anion exchanger 1 is normally targeted to the basolateral membrane of α-intercalated cells where it reabsorbs the bicarbonate into the blood, this mistargeting may prevent adequate acidification of the urine by intercalated cells. Similarly, a point mutation G609R, was also shown to induce a mistargeting to both apical and basolateral membranes in polarized MDCK cells (21). Two other dominant mutations, R589H and S613F, induced predominantly the intracellular retention of the proteins (28), with detectable mistargeting of the proteins to the apical membrane of some MDCK cells (4). But all these studied dominant mutants displayed a comparable folding to the WT protein. In this work, we observed that the A858D mutant, characterized as a mild dominant phenotype, is functional with a decreased expression to the plasma membrane of MDCK cells (Fig. 1B and 2). Previous work showed that when A858 was mutated to cysteine, the functional activity of the mutant protein was slightly altered (65% of the positive control) (33). Furthermore, the residual transport activity was inhibited by preincubation with a cysteine-directed compound MTSES (9). These two residues, A858 and ΔV850, are both located close to lysine 851, which reacts covalently with DIDS to inhibit the anion transport (16) and is implicated in anionic translocation (11, 33). Structural alterations in this region may account for the loss of inhibitor binding observed for the A858D mutant. However, our results indicate that this protein is partially functional and can traffic to the basolateral membrane of polarized epithelial cells (Fig. 2). A858D is a dominant dRTA mutant predominantly found at the basolateral membrane of polarized epithelial cells, in contrast to other dominant mutants that localize in the ER or are mistargeted to the apical membrane. This suggests that this mutant does not exhibit the intracellular retention properties of other dominant mutants, either when expressed alone or when with WT kAE1 in epithelial cells. The two other dominant dRTA mutants extensively studied, R589H and S613F, were retained in the ER (4, 28). The mild functional and trafficking defects of A858D are in agreement with the incomplete dRTA phenotype of the heterozygotes carrying this mutation. They were unable to acidify their urine only when tested with frusemide/fludrocortisone, but had no usual feature of dRTA (2), while heterozygotes patients carrying R589H, C or S displayed complete dRTA.

As well, the A858D and ΔV850 heterozygotes showed only a slight reduction (80% of normal) in the amount of AE1 at the red cell surface and the protein found at the plasma membrane displayed nearly normal anion transport (78–80 and 95% of control SO42− influx per AE1 molecule, respectively) (2). The normal activity of the ΔV850 mutant in red blood cells contrasts with our finding that this mutant displays an intracellular retention in MDCK cells likely due to impaired folding. This difference may be due to the presence of GPA in red blood cells, an erythrocyte specific protein known to promote AE1 trafficking to the cell surface, with no known equivalent in the kidney. A similar rescue of the recessive G701D mutant by GPA was observed in X. laevis oocytes (26). The fact that ΔV850 is predominantly retained intracellularly in MDCK cells likely reflects the fact that MDCK cells do not express GPA. When expressed in X. laevis oocytes, the A858D and ΔV850 mutants showed lower activity (28% and 14% relative to normal kAE1) due to a decrease in cell surface expression (approximately half of the WT). Coexpression with GPA enhanced somewhat the cell surface expression of the mutants and the resulting transport activity (43% and 64% for A858D and ΔV850 mutants, respectively when coexpressed with GPA) (2). The trafficking defect would be more pronounced in the kidney since they do not express GPA. On the basis of the following observations: 1) the dominant mutant A858D is able to physically interact with either the WT or the recessive mutant ΔV850 (Fig. 4), 2) the heterodimers are targeted to the basolateral membrane of epithelial cells (Fig. 3), and 3) the A858D mutant displays an altered inhibitor binding (Fig. 5) but only a slight functional impairment, we hypothesize that the weak dominance of the A858D mutation might be due to a subtle dominant negative effect of the mutant on the WT protein function when they heterodimerize, as is found for the SAO protein (22).

In conclusion, in this work, we characterized in polarized MDCK cells, the situation occurring in compound heterozygous patients carrying the dominant mutation A858D on one allele and the recessive deletion ΔV850 on the other allele, as well as the situation occurring in their heterozygous parents, carrying the WT AE1 protein on one allele and either A858D or ΔV850 on the other allele. We observed that the recessive mutant ΔV850 displayed a classic recessive dRTA phenotype (misfolding of the protein and intracellular retention, unless coexpressed with the WT AE1 protein). The dominant mutant A858D showed different characteristics compared with the other dominant dRTA mutants previously studied. Indeed, instead of being predominantly retained in the ER as was shown for R589H or S613F (19), this mild dominant mutant A858D can escape the ER and is targeted to the basolateral membrane of epithelial cells. While the A858D mutant retains some of the WT protein in the ER, the effect was not as pronounced as with other dominant mutants. The amount of functional protein present at the cell surface in heterozygotes (A858D/WT) is likely reduced, resulting in some impairment in transport function. The A858D mutant may also affect the activity of the WT protein in a heterodimer. In the case of the recessive mutant ΔV850, it did not impair the trafficking of the WT protein to the cell surface. Therefore, in heterozygotes (ΔV850/WT), an sufficient amount of functional protein is present at the cell surface to carry out anion transport. When expressed together in MDCK cells, despite a partial intracellular retention of both A858D and ΔV850 mutants, the A858D mutant could also promote some cell surface expression of the ΔV850 mutant. We hypothesize that compound heterozygous patients (ΔV850/A858D) develop dRTA because these patients do not have sufficient functional kAE1 present at the basolateral membrane of their α-intercalated cells. This could be due to an intracellular retention of the proteins, the lack of sufficient active proteins at the cell surface or an effect of the ΔV850 mutant on the A858D mutant in heterodimers.

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