Autosomal dominant and recessive distal renal tubular acidosis (dRTA) can be caused by mutations in the anion exchanger 1 (AE1 or SLC4A1) gene, which encodes the erythroid chloride/bicarbonate anion exchanger membrane glycoprotein (eAE1) and a truncated kidney isofrom (kAE1). The biosynthesis and trafficking of kAE1 containing a novel recessive missense dRTA mutation (kAE1 S773P) was studied in transiently transfected HEK-293 cells, expressing the mutant alone or in combination with wild-type kAE1 or another recessive mutant, kAE1 G701D. The kAE1 S773P mutant was expressed at a three times lower level than wild-type, had a 2-fold decrease in its half-life, and was targeted for degradation by the proteasome. It could not be detected at the plasma membrane in human embryonic kidney cells and showed predominant endoplasmic reticulum immunolocalization in both human embryonic kidney and LLC-PK1 cells. The oligosaccharide on a kAE1 S773P N-glycosylation mutant (N555) was not processed to the complex form indicating impaired exit from the endoplasmic reticulum. The kAE1 S773P mutant showed decreased binding to an inhibitor affinity resin and increased sensitivity to proteases, suggesting that it was not properly folded. The other recessive dRTA mutant, kAE1 G701D, also exhibited defective trafficking to the plasma membrane. The recessive kAE1 mutants formed dimers like wild-type AE1 and could hetero-oligomerize with wild-type kAE1 or with each other. Hetero-oligomers of wild-type kAE1 with recessive kAE1 S773P or G701D, in contrast to the dominant kAE1 R589H mutant, were delivered to the plasma membrane.

The kidney plays an essential role in maintaining the acid-base balance of the body by reabsorbing bicarbonate in the proximal tubule and secreting acid into the urine. Impairment in acid secretion by α-intercalated cells of the distal nephron leads to the development of distal renal tubular acidosis (dRTA), which is inherited in both an autosomal dominant (AD dRTA) (1–7) and autosomal recessive manner (AR dRTA) (4, 8–10). Patients with AD dRTA usually remain asymptomatic until adulthood, whereas AR dRTA patients are severe cases and early onset (11–13). Recently, a number of missense, nonsense, and deletion mutations in the anion exchanger 1 (AE1 or SLC4A1) gene resulting in both AD and AR dRTA have been identified and characterized (1–7). Human AE1 is a red blood cell membrane glycoprotein containing 911 amino acids (14, 15) with a large N-terminal cytoplasmic domain of about 400 amino acids and a C-terminal domain consisting of 500 amino acids, which forms 12 transmembrane spans. A short C-terminal cytoplasmic tail (16) provides a binding site for carbonic anhydrase II (17) and is important for the proper trafficking of the protein to plasma membrane (18). Kidney AE1 (kAE1) is transcribed from an alternative promoter (19, 20) resulting in the production in human kidney of an isoform that is missing the N-terminal 65 amino acids found in erythroid isoform (eAE1) (21). KAE1 is located in the basolateral membrane of acid-secreting α-intercalated cells where it mediates the transport of bicarbonate into the blood (22). The first reported AD dRTA AE1 mutations were nucleotide substitutions causing an amino acid change of residue 589 from arginine to histidine (R589H) (1–3), to cysteine (R589C) (1, 2), or to serine (R589S) (2). The dominant kAE1 R589H mutant was functional as assessed in transport assay in Xenopus oocyte (3) but was retained in the endoplasmic reticulum in transfected HEK cells (23) and in both nonpolarized and polarized MDCK cells (24). Similarly, a dominant dRTA mutant (kAE1 R901Stop) missing the C-terminal 11 amino acids had normal chloride transport activity in Xenopus oocytes but was impaired in acid secretion. AR dRTA patients with mutations in the kAE1 gene are usually born with normal or mild acidosis that worsens with age, whereas AD dRTA patients are usually asymptomatic until adulthood (4, 11–13).
Characterization of a Recessive dRTA kAE1 Mutant

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Queen's University of Toronto. Both kAE1 S773P and kAE1 S773P targeted at their C terminus with His6 were generated using the Quick-Change™ site-directed mutagenesis kit from Stratagene (La Jolla, CA) and oligonucleotide primers from ACGT Corp. (Toronto, Canada). The mutations were confirmed by sequencing performed by ACGT Corp. (Toronto, Canada). Plasmid DNA for transfections was purified with Qiagen (Valencia, CA) Plasmid Midi columns.

Cell Culture—HEK-293 (human embryonic kidney) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 0.5% penicillin and streptomycin (Invitrogen) in 5% CO2 at 37 °C. The day before transfection the cells were trypsinized and plated in either 6-well or 35-mm plates. Either DEAE-dextran method (34) or LipofectAMINE 2000 (Invitrogen) was used to transiently transfect the cells (1 μg of DNA per well of a 6-well dish; 10 μg of DNA per well of a 100-mm dish) grown in DMEM/F-12 with 10% calf serum and 0.5% penicillin and streptomycin. At 15000 dilution of mouse anti-GAPDH (Chemicon, Temecula, CA) was used to detect the level of endogenous GAPDH expression in HEK cells. BM Chemiluminescence Blotting Substrate (Roche Applied Science) and exposure to BioMax MR film (Eastman Kodak Co.) were used to detect AE1 protein bands. Densitometric analysis to determine the relative amounts of AE1 was performed by using NIH Image 1.62 software (National Institutes of Health).

Pulse-Chase Assay—The day after transfection, cells (~60–80% confluent, in 35-mm dishes as described previously) were starved in methionine-free DMEM (Invitrogen) for 30 min and pulsed with 100 μCi/ml of L-[35S]methionine (PerkinElmer Life Sciences) for 20 min to label newly synthesized protein. After labeling, the medium was replaced with fresh medium supplemented with 10% calf serum and 0.5% penicillin and streptomycin. In some cases, the proteasome inhibitor, MG262 (Biomol, Hamburg, Germany) (1 μM), was added to the cells for 30 min before pulsing. At each chase time point, the cells were lysed in 1 ml of RIPA buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 0.15 μM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) containing protease inhibitors: 100 μM aprotinin (Roche Applied Science), 1 μM leupeptin (Roche Applied Science), 1 μM pepstatin (Roche Applied Science). The wild-type and mutant proteins were detected with a antibody raised against the C-terminal 15 amino acids of AE1 and goat anti-rabbit antibody. The blot was also incubated with mouse anti-tubulin antibody. The membranes were exposed to film. The relative amounts of kAE1 were determined using NIH Image 1.62 software as mentioned above.

Processing of the N-Linked oligosaccharide was determined using kAE1 N555 construct (33). After immunoprecipitation using anti-Ct AE1 antibody, the high mannose and complex oligosaccharides were distinguished by endo H digestion (33).

Cell Surface Biotinylation—The cells were washed with borate buffer (10 mM boric acid, 154 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, pH 9.0) and treated with 1 ml of 0.5 mg/ml EZ-Link NHS-SS-Biotin (Pierce) in borate buffer for 15 min at room temperature (36). Unreacted reagent was then quenched by rinsing the cells with 0.192 mM glycine, 25 mM Tris, pH 8.3. Cells were lysed with 1 ml of RIPA buffer with protease inhibitors, and an aliquot of the lysate was taken for Western blotting. Immunopure (Pierce) immobilized streptavidin (100 μl) was added to the lysate for 1 h at 4 °C to bind the biotinylated proteins. The supernatant was removed, and an aliquot (unbound fraction) was analyzed for Western blotting. The streptavidin beads were washed three times with RIPA buffer. SDS sample buffer (containing 5% β-mercaptoethanol) was added to the beads (bound fraction), and the samples were incubated for 1 h at room temperature to cleave the disulfide bond in the biotinylating reagent and release the captured proteins. Samples were resolved by SDS-PAGE and blotted as described above. The mAb was used as an antibody against the carboxyl terminus of kAE1. The blot was also incubated with mouse anti-tubulin antibody to confirm that biotinylation was solely restricted to the cell surface.

Immunofluorescence—HEK and LLC-PK1 were grown on glass coverslips. The transiently transfected HEK or LLC-PK1 cells were rinsed...
Western blotting using anti-Ct AE1 antibody. The total and bound fractions were analyzed for kAE1 content by fixing with 3.7% paraformaldehyde for 10 min, washed with 100 mM citrate buffer, pH 7.1, with or without 1 mM free anion transport inhibitors (PMSF, leupeptin, pepstatin A, and aprotinin) at 4 °C for 30 min. The lysates were centrifuged (13,000 × g) to remove insoluble material. 25 μl of SITS-Affi-Gel 102 prepared as described previously (37) and 100 μl of lysate were combined in 1% C4E6 (Nikkol, Tokyo, Japan) and protease inhibitors (PMSF, leupeptin, pepstatin A, and aprotinin) at 4 °C for 30 min. The reaction was centrifuged (8000 × g) for 5 min and washed three times with 0.1% C4E6, 228 mM sodium citrate buffer, pH 7.1. To elute bound proteins from the SITS-Affi-Gel, 75 μl of 2× sample buffer was added to the SITS-Affi-Gel beads. The total and bound fractions were analyzed for kAE1 content by Western blotting using anti-Ct AE1 antibody.

**Proteolysis Assay**—Transfected HEK cells expressing either kAE1, KAE1 S773P, kAE1 R589H, or kAE1 SAO were lysed in 1% C4E6 without any protease inhibitors at 4 °C for 30 min. The 2 μl of 1, 10, or 100 μg/ml dilution of trypsin or chymotrypsin were added to 20 μl of protein samples. All samples were digested at 4 °C for 1 h, and the enzyme was inhibited by addition of 2 μl of 40 mM PMSF protease inhibitor. To visualize all fragments generated from the enzyme digestion, SDS-PAGE and Western blot were used. Molecular weight markers were β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp981 (25.0 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).

**Transport Assay**—KAE1 WT and kAE1 S773P were tested for the chloride/bicarbonate exchange activity in transfected HEK cells (38). Briefly, HEK-293 cells were grown on poly-L-lysine-coated coverslips. Two days after transfection, the coverslips were incubated with serum-free DMEM containing 2 μM 2,7' -bis-carboxyethyl-5,6-carboxyfluorescein-AM at 37 °C for 30 min. Coverslips were mounted in the fluorescence cuvette, which was perfused with Ringer buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO4, 2.5 mM NaH2PO4, 25 mM NaHCO3, 10 mM Hepes, pH 7.4) with 140 mM NaCl or 140 mM sodium gluconate continuously bubbled with air/CO2. The transport rates were measured by linear regression of the initial H+ equivalent flux using Kaleidagraph software (Synergy Software, Reading, PA) (38).

**Copurification of kAE1 Mutants with His-tagged kAE1**—In order to determine whether mutant kAE1 can oligomerize with wild-type or mutant kAE1 proteins, a C-terminal His-tagged version of kAE1 (kAE1 His) was used. Cells were either transfected with kAE1 His alone or cotransfected with kAE1 His in combination with kAE1, the dominant mutant kAE1 S773P, recessive S773P, or dominant G701D mutants. Transfected cells were lysed in 500 μl of PBS, pH 7.4, containing 1% C4E6, 5 mM imidazole, and protease inhibitors at 4 °C. After removing the insoluble material by centrifugation, 400 μl of cell lysate was incubated with 40 μl of ProBond34 nickel beads (Invitrogen) at 1 h at 4 °C. Beads were washed three times with 0.5 ml of PBS, pH 7.4, with 5 mM imidazole. Bound proteins were eluted using the same buffer containing 500 mM imidazole. KAE1 in the eluant was detected by Western blotting as described above. KAE1 His is not detected by Western blotting as described above. KAE1 His is not detected by Western blotting as described above. KAE1 His is not detected by Western blotting as described above. KAE1 His is not detected by Western blotting as described above. KAE1 His is not detected by Western blotting as described above.

**Biotinylated Histidine-tagged Copurification**—HEK cells were co-transfected with eAE1 His and either kAE1, recessive S773P, G701D, or dominant R589H. To biotinylate the cell surface proteins, the cells were treated twice with 1 ml of 0.5 mg/ml EZ-Link NHS-LC-Biotin (Pierce) in borate buffer for 15 min at 4 °C following by quenching buffer as described above (36). Cells were lysed in 1% C4E6 in PBS containing 5 mM imidazole, centrifuged, and incubated with ProBond34 nickel beads (Invitrogen) for 1 h at 4 °C to capture eAE1 His and any associated proteins as described previously. Erythroid AE1 and any associated KAE1 proteins were eluted in 500 mM imidazole and detected by Western blotting. Anti-Nt eAE1 and KAE1 antibodies were used to detect AE1 in both erythroid and kidney isoforms in total cell lysate, whereas anti-biotin horseradish peroxidase (Cell Signaling Technology, Beverly, MA) was used to visualize biotinylated AE1. The anti-Ct AE1 antibody detected kAE1 or mutant kAE1 that was associated with AE1 His. The erythroid version of AE1 His was used in these experiments to allow detection of both eAE1 and kAE1 in the immunoblot.

**Fluorescence Activated Cell Sorting**—Transfected HEK cells expressing either KAE1 HA557 or kAE1 S773P HA557 alone and in combination with wild-type or S773P, G701D, and dominant R589H mutants were trypsinized for 5 min at 4 °C, centrifuged to collect the cells, and resuspended in Hanks’ solution with 1% BSA. The cells were incubated with 1:1,000 dilution of mouse anti-HA antibody in Hanks’ solution with 1% BSA for 15 min, and then the cells were stained with goat anti-mouse Alexa488 (1:1,000) (Molecular Probes, Eugene, OR) for 15 min on ice. The samples were washed and analyzed using Beckman-Coulter Epics Elite (BD Biosciences), and the percent of positive cells was determined to quantify the level of the cell surface expression.

**RESULTS**

Fig. 1A shows the location of mutations that are characterized as causing autosomal dominant (filled star) or recessive (open star) distal renal tubular acidosis (AD and AR dRTA) in a model of human AE1. The dRTA mutations are distributed throughout the membrane domain of kAE1 but are absent from the N-terminal cytosolic domain (1–10). Here we characterized a novel recessive kAE1 S773P mutant found as a compound heterozygote with kAE1 G701D in a patient from northeastern Thailand. This mutation is located within the tenth transmembrane segment in the C-terminal region of the protein. Most interestingly, serine 773 is highly conserved among the amino exchanger family members, and introduction of a proline residue within a transmembrane segment may be expected to alter the structure of this region of the protein.

**Expression of kAE1 S773P in Transfected HEK Cells**—To determine the effect of the S773P mutation on the biosynthesis of kAE1, we expressed kAE1 and kAE1 S773P in transiently transfected HEK cells. KAE1 was expressed as a 96-kDa band as shown in Fig. 1B. The kAE1 S773P mutant was expressed at a much lower level (34 ± 5%, n = 3 ± S.D.) relative to normal kAE1 (Fig. 1B, lane 2). The level of expression was normalized to endogenous GAPDH expressed in HEK cells (Fig. 1B, lower panel).

**Biosynthesis and Turnover of kAE1 S773P**—In order to investigate the rates of biosynthesis and turnover of kAE1 S773P, pulse-chase experiments using [35S]methionine were performed (Fig. 2). The kAE1 protein persisted over 24 h and exhibited a half-life of ~11 h, whereas mutant kAE1 S773P demonstrated a shorter half-life of about 5 h. Hence, the reduction in kAE1 S773P expression as shown by immunoblot (Fig. 1B) was likely caused by the more rapid turnover of kAE1 S773P. This is in contrast to the AD dRTA mutant R589H, which exhibited a normal half-life and expression level in transfected HEK cells (23).

Mutant membrane proteins are often misfolded and targeted to the proteasome where they are degraded (39). To investigate the degradation pathway of kAE1 S773P, pulse-chase experiments were performed in the presence and absence of the proteasome inhibitor MG262 (1 μM) (Fig. 2C). At the 5-h chase time point, about 81% of kAE1 WT remained. There was a small effect of the proteasome inhibitor on the amount of kAE1 WT remaining at this time point (85%) compared with the
untreated sample. In contrast, only 60% of kAE1 S773P remained at the 5-h chase point, and this protein was protected from degradation by treatment of cells with MG262 since 79% of the protein remained at the 5-h chase time. This indicates that degradation of the kAE1 S773P mutant is mediated by the proteasome.

**Fig. 1. Position and expression of a novel mutation kAE1 S773P.** A, schematic depiction of the folding of human kAE1 indicating the location of mutations causing autosomal dominant (filled star) and recessive (open star) dRTA. The folding model of kAE1 consists of 12 transmembrane segments with cytosolic N and C termini. The endogenous N-glycosylation site is located at acceptor site Asn-642. The position of the introduced N-glycosylation site at residue 555 in AE1 N555 and the epitope HA tagged at the residue 557 are indicated. The designations of the known dominant mutants are R589H, G609R, S613F, A858D, A→L899X, and R901X, and the recessive mutants are G701D, ΔV850, R602H, and S773P. Residue numbering is based on the full-length erythroid protein. Human kAE1 is missing residues 1–65 and begins at Met-66. B, immunoblot analysis of total lysates of HEK cells transiently transfected with 1 μg of cDNA of kAE1 wild-type (lane 1), kAE1 S773P (lane 2), kAE1 N555 (lane 3), or kAE1 S773P N555 (lane 4) was revealed by using an anti-C-terminal AE1 (anti-Ct AE1) antibody. Samples were treated with endoglycosidase H to better separate the high mannose (open circle) from complex oligosaccharide form of kAE1 N555 (filled circle). The endogenous protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize kAE1 protein expression in HEK cells. The levels of the relative mutant protein kAE1 or kAE1 N555 expression were determined in three independent experiments and are shown below the gel.

As mentioned above, kAE1 N555 can be used to monitor the rate of glycoprotein processing and trafficking by pulse-chase experiments. At the beginning of the chase period, both kAE1 N555 and kAE1 N555 with the S773P mutation showed a single endo H-sensitive band indicating a high mannose oligosaccharide (Fig. 3A, open circle). After a 2-h chase, kAE1 N555 of the high mannose to a higher molecular weight complex form that was resistant to endo H digestion was apparent (Fig. 3A, filled circle). The time course of conversion of the high mannose to the complex form (Fig. 3B) indicates that ~50% of the radioactivity in pulse-labeled kAE1 was present in the upper complex band by 5 h of chase time. This means that kAE1 acquired complex oligosaccharide and had moved from the ER to the Golgi. On the other hand, the mutant kAE1 S773P N555 did not acquire complex oligosaccharide.
over the same chase period (Fig. 3, A and B). This indicates that kAE1 S773P N555 did not traffic from the ER to the medial Golgi. The kAE1 S773P N555 mutant was more rapidly degraded than kAE1 N555, with no detectable protein remaining after the 24-h chase period.

Cell Surface Biotinylation of kAE1 S773P—To determine the cell surface expression of kAE1 in transfected HEK cells, biotinylation of intact cells was performed. KAE1 expressed in transfected cells could be biotinylated by NHS-SS-biotin (Fig. 4, lane B). Moreover, the diminished unbiotinylated fraction (Fig. 4, lane U) compared with total fraction (lane T) indicated the presence of kAE1 at the cell surface, determined to be 21 ± 2% (n = 5 ± S.D.) of the total kAE1 expression. We could not detect either the recessive kAE1 S773P or G701D mutants in the biotinylated fraction (2.5 ± 2 and 0.5 ± 0.4%, respectively, n = 5 ± S.D.) (Fig. 4, lane B). There was no protein in the bound fraction of control cells expressing kAE1 that were not treated with biotinylating reagent, indicating no binding of the unbiotinylated protein to the resin. No biotinylation of intracellular tubulin was detected indicating that the labeling was restricted to the cell exterior (data not shown).

Immunofluorescence Localization—We next determined the subcellular location of the recessive kAE1 S773P and G701D mutants in transfected HEK and LLC-PK1 cells by immunolocalization. Confocal images of transiently transfected HEK cells showed that kAE1 was present at the cell membrane, as well as in intracellular compartments (Fig. 5A) as described previously (23). In contrast, kAE1 S773P did not show plasma

Fig. 2. Turnover of wild-type and mutant S773P kAE1. A, autoradiograph showing the rapid turnover of mutant kAE1 S773P. Transfected cells were immunoprecipitated with anti-Ct AE1 antibody followed by protein G-Sepharose after pulsing with 100 μCi/ml [35S]methionine and chasing at various time points. Radiolabeled kAE1 proteins were separated by SDS-PAGE and then visualized by autoradiography. Arrow indicates position of kAE1. B, the amount of radiolabeled kAE1 and kAE1 S773P present during the chase period in three independent experiments was quantitated by scanning the autoradiographs. The pixel density was analyzed by using NIH Image 1.62f. Error bars represent mean ± S.D. C, autoradiograph showing the effect of proteasome inhibitor (MG262) on the degradation of kAE1 and kAE1 S773P. The transfected HEK cells were preincubated with 1 μM of MG262 for 30 min and then pulsed with 100 μCi/ml [35S]methionine for 30 min. The cells were chased for 5 h in the absence or continued presence of 1 μM MG262. The percent protein remaining was the average of three independent experiments.
The intracellular protein colocalized with calnexin, an ER marker (data not shown). The intracellular retention of kAE1 S773P in the ER was confirmed in transiently transfected nonpolarized LLC-PK1 cells, which exhibited a better defined cell morphology than HEK cells (Fig. 5D). The kAE1-transfected LLC-PK1 cells showed a clear plasma membrane staining (Fig. 5B) that was absent in cells transfected with kAE1 S773P (Fig. 5D). The kAE1 G701D recessive mutant was also localized intracellularly in both HEK (Fig. 5E) and nonpolarized LLC-PK1 cells, with a perinuclear staining pattern suggesting Golgi localization (Fig. 5F).

**Transport Activity and Inhibitor Binding**—Measurements of the anion transport activity were carried out in the transfected HEK cells to determine the Cl⁻/HCO₃⁻ transport rate of kAE1 at the plasma membrane (38). Whereas normal kAE1 showed...
robust transport activity (31 ± 6 mM H+/min, n = 3 ± S.D.), the recessive S773P mutant had no significant transport activity (5 ± 2 mM H+/min, n = 3 ± S.D.) over pCDNA3-transfected cells (4 ± 2 mM H+/min, n = 3 ± S.D.), which is consistent with a lack of expression at the cell surface. We also found that the recessive G701D mutant showed no transport activity (5 ± 3 mM H+/min, n = 3 ± S.D.) in transfected HEK cells. These results confirm the lack of functional expression of the recessive dRTA mutants at the level of the plasma membrane in transfected HEK cells.

To test whether kAE1 S773P and G701D were properly folded, we employed an inhibitor affinity resin, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS)-Affi-Gel (37, 40). This binding assay provides a measure of the integrity of the inhibitor binding site of the expressed kAE1 protein, including the intracellular pool present in solubilized cell extracts. The immunoblot showed that the majority of wild-type kAE1 bound to SITS-Affi-Gel (85 ± 3%, n = 5 ± S.D.), under the conditions employed, and that this binding was efficiently blocked by the free inhibitor H2DIDS (Fig. 6A, lane D). On the contrary, the kAE1 SAO mutant, which does not bind inhibitors (40), bound very poorly to SITS-Affi-Gel relative to kAE1 (15 ± 2%, n = 5 ± S.D.). The amount of mutant kAE1 S773P bound to SITS-Affi-Gel was lower than wild type (35 ± 8%, n = 5 ± S.D.). The kAE1 G701D also bound to SITS-Affi-Gel less well (63 ± 11%, n = 4 ± S.D.) than the wild-type protein. The results suggest that the recessive mutations induce a change in the inhibitor-binding site of the protein, resulting in poorer binding to the affinity resin, although binding is not completely eliminated.

In order to further examine the folding of the mutant proteins, we determined their sensitivity to proteolysis by trypsin and chymotrypsin in detergent extracts of transfected cells. Both kAE1 S773P and G701D were more sensitive to proteolytic cleavage than the wild type and generated a different fragment pattern (data not shown). The impaired binding to an inhibitor affinity matrix and increased protease sensitivity suggest that the recessive mutants kAE1 S773P and G701D were not properly folded. This is in contrast to dominant dRTA mutant (kAE1 R589H), which bound to SITS affinity gel at the same level as wild-type kAE1 (23) and had an unaltered protease sensitivity compared with wild-type kAE1 (data not shown).

Native Gel Electrophoresis—AE1 exists as dimers in erythrocyte cells (41) and in transfected HEK cells (23, 42). Native gel electrophoresis using the detergent PFO was used to determine the oligomeric state of kAE1 and kAE1 mutants in transfected cells. It was shown previously that PFO preserves the oligomeric state of native AE1 (43) and in transfected HEK cells (23, 42). Native gel electrophoresis using the detergent PFO was used to determine the oligomeric state of kAE1 and kAE1 mutants in transfected cells. It was shown previously that PFO preserves the oligomeric state of native AE1 (43) and in transfected HEK cells (23, 42). Native gel electrophoresis using the detergent PFO was used to determine the oligomeric state of kAE1 and kAE1 mutants in transfected cells. It was shown previously that PFO preserves the oligomeric state of native AE1 (43) and in transfected HEK cells (23, 42). Native gel electrophoresis using the detergent PFO was used to determine the oligomeric state of kAE1 and kAE1 mutants in transfected cells. It was shown previously that PFO preserves the oligomeric state of native AE1 (43) and in transfected HEK cells (23, 42). Native gel electrophoresis using the detergent PFO was used to determine the oligomeric state of kAE1 and kAE1 mutants in transfected cells. It was shown previously that PFO preserves the oligomeric state of native AE1 (43) and in transfected HEK cells (23, 42).
Mutant kAE1 S773P but not kAE1 S773P-His in the bound fraction can be detected by anti-Ct AE1 antibody as shown in the lower panel of the immunoblot. This is representative of five independent experiments.

The 6-histidine tag interferes with the recognition epitope of anti-Ct AE1; therefore, kAE1 His, in contrast to kAE1, is unable to be detected by the antibody against the C terminus. All proteins expressed in the bound fraction (kAE1 S773P His and coexpressed protein) probed by anti-Nt AE1 antibody. The associated kAE1 and mutant proteins were then copurified by nickel-affinity resin and eluted in 500 mM imidazole. Immunoblots probed by both anti-Nt eAE1 and anti-Nt kAE1 antibodies revealed the AE1 proteins coexpressed in the bound fraction. The anti-biotin-horseradish peroxidase was used to detect the surface-biotinylated proteins in the bound fraction as shown in the lower panel of the immunoblot. This is representative of three independent experiments.

To understand the mechanism of dRTA in this patient, who is compound heterozygous for recessive S773P and G701D, we also studied the interaction between kAE1 S773P and G701D by using histidine-tagged copurification (Fig. 7B). kAE1 S773P with a C-terminal 6-histidine tag was created. We found that kAE1 S773P His can form a hetero-oligomer not only with kAE1, confirming the data presented above, but also with kAE1 G701D. Thus, the mutant proteins were not only capable of interacting with the wild-type protein but also with each other, indicating that heterodimers of kAE1 S773P and G701D can form.

Cell Surface Expression of Hetero-oligomer by Cell Surface Biotinylation and Histidine-tagged Copurification—The dominant R589H mutant retained wild-type kAE1 proteins intracellularly because of hetero-oligomerization (23). In order to elucidate whether the recessive S773P and G701D mutants had the same effect on trafficking of kAE1 to the plasma membrane, we used a biotinylation histidine-tagged copurification assay. Wild-type eAE1 His and mutant kAE1 were coexpressed, and cell surface protein was biotinylated. KAE1 His and associated proteins were then purified by nickel-affinity resin and eluted in 500 mM imidazole. Immunoblots probed by both anti-Nt eAE1 and anti-Nt kAE1 antibodies revealed the AE1 proteins coexpressed in the bound fraction. The anti-biotin-horseradish peroxidase was used to detect the surface-biotinylated proteins in the bound fraction as shown in the lower panel of the immunoblot. This is representative of five independent experiments.
His was detected by immunoblot, and the biotinylated protein was detected by streptavidin blotting. Most interestingly, we found that kAE1 and the recessive kAE1 S773P and G701D mutants that copurified with eAE1 His were biotinylated but dominant kAE1 R589H was not (Fig. 8). This suggested that the wild-type protein could rescue the trafficking of recessive mutants, allowing the movement of the mutant protein from the ER to the plasma membrane, because of hetero-oligomerization. In contrast, the dominant dRTA mutant exhibited a dominant-negative effect, retaining the wild-type protein intracellularly.

Confocal images (Fig. 9) of LLC-PK1 cells cotransfected with eAE1 containing an external HA epitope (eAE1 HA557) with either dominant or recessive kAE1 confirmed that the dominant mutant could retain eAE1 HA557 intracellularly. The anti-HA antibody detected eAE1 at the cell surface in nonpermeabilized cells cotransfected with kAE1, recessive kAE1 S773P, and recessive kAE1 G701D. In contrast, cell surface staining of eAE1 was decreased in cells cotransfected with the dominant mutant kAE1 R589H. The recessive kAE1 S773P or G701D proteins were detected at the plasma membrane when coexpressed with the wild-type protein, whereas the dominant mutant kAE1 R589H was localized intracellularly.

**FACS Analysis**—We also examined the expression of kAE1 at the cell surface by fluorescence-activated cell sorting (FACS) using kAE1 with an hemagglutinin (HA) epitope inserted in the third extracellular loop (position 557). The effect of coexpression of dominant and recessive dRTA mutations on the cell surface expression of kAE1 HA557 was determined. For this experiment, cells were transfected with 0.25 μg of kAE1 HA557 plus an equal amount of mutant DNA. At 0.25 μg of kAE1 HA557 DNA alone, 19 ± 4% (n = 3 ± S.D.) of cells showed higher than background staining (2 ± 1%, n = 3 ± S.D.), indicating the population of cells that expressed kAE1 at the plasma membrane. Coexpression of wild-type kAE1 with kAE1 HA557 did not affect the percentage of cells (18 ± 4%) expressing the tagged protein at the plasma membrane. There was little effect on the surface expression of kAE1 HA557 by coexpression with either recessive kAE1 S773P (17 ± 8%, n = 3 ± S.D.) or G701D (14 ± 5, n = 3 ± S.D.) mutants compared with coexpression with wild-type kAE1 (Fig. 9). In contrast, coexpression with dominant R589H caused a decrease (8 ± 4%, n = 3 ± S.D.) in the percentage of cells showing cell surface expression.

The cell surface expression of the recessive S773P mutant containing an external HA epitope was also determined directly by FACS analysis of transfected HEK cells (Fig. 10). KAE1 S773P HA557 transfected alone showed a low signal (8 ± 5%, n = 3 ± S.D.) revealing poor cell surface expression. Furthermore, HA epitope-tagged kAE1 S773P showed a 2-fold
increased number of cells showing cell surface expression when coexpressed with kAE1 WT (15 ± 5, n = 3 ± S.D.). Coexpression of the recessive S773P mutant with G701D resulted in background number of cells showing cell surface expression (3 ± 2, n = 3 ± S.D.). The results indicate that the dominant dRTA mutant retains the wild-type protein intracellularly, whereas the wild-type protein can rescue the recessive mutant proteins and allow their expression at the cell surface. Coexpression of the two recessive mutants resulted in no cell surface expression of these mutants.

**DISCUSSION**

**SLC4A1** mutations can lead to the molecular pathogenesis of both AD (1–7) and AR dRTA (4, 8–10). In this paper, the effect of a novel missense recessive mutation (S773P) in the **SLC4A1** gene on the biosynthesis of kAE1 was characterized in transfected cells. KAE1 S773P was localized to the endoplasmic reticulum in both HEK cells and in nonpolarized LLC-PK1 cells. Whereas kAE1 N555 acquired complex oligosaccharide, kAE1 S773P contained solely high mannose oligosaccharide through 24 h of chase time. This indicates that the recessive kAE1 S773P mutant is impaired in its trafficking from the ER to the medial Golgi. As well, KAE1 S773P could not be biotinylated at the cell surface, was not detected by FACS analysis of nonpermeabilized cells, and showed no transport activity confirming its intracellular localization in HEK cells. The kAE1 S773P mutant is found as a compound heterozygous in the patient with another recessive mutant, kAE1 G701D. KAE1 G701D was also impaired in its trafficking to the plasma membrane in transfected HEK cells. Intracellular retention of recessive mutants was observed previously in other membrane proteins, such as cystic fibrosis transmembrane conductance regulator, aquaporin, and pendrin proteins causing human diseases (44–47).

KAE1 S773P showed a reduced level of expression compared with wild-type kAE1, because of a decrease in its stability. An inhibitor binding assay and protease sensitivity showed that the kAE1 S773P was improperly folded. The effect of this mutation in the folding of the protein might be expected since the mutation leads to the replacement of a highly conserved serine to proline within a putative transmembrane segment. Studies of proline substitution in the transmembrane domain concluded that the introduction of proline in transmembrane segment tends to have a strong helix-breaking property (48). Moreover, the proline residue may induce the kink formation in transmembrane segment and subsequently be the reason for misfolding. Misfolded membrane proteins are often retained in the ER by cellular quality control involving interactions with chaperones such as calnexin (49). Although only one amino acid is changed in the protein structure, kAE1 S773P is recognized by the quality control system of the cell, which leads the defective protein to be degraded by the proteasome (50–52). Protein misfolding because of the presence of a missense mutation is a common mechanism in the pathogenesis of human diseases such as cystic fibrosis, nephrogenic diabetes insipidus and long QT syndrome (44, 45, 53).

Mutations in the **SLC4A1** gene causing the autosomal dominant form of dRTA such as R589H, S613F, G609R, and R901X are impaired in trafficking to the cell surface and are mistargeted in polarized epithelial cells (7, 27). R589H and R901X exert a dominant effect because of hetero-oligomerization with the normal protein, resulting in intracellular retention of the normal protein (23, 26). In contrast, kAE1 could traffic to the plasma membrane after forming hetero-oligomers with the recessive kAE1 S773P and G701D mutants. This is similar to AE1 SAO, a mutant that forms heterodimers with the normal protein that are present at the red cell surface (54). The normal...
AE1 protein retains transport function, although it is associated with an inactive SAO subunit (28).

In conclusion, the defective trafficking of kAE1 S773P in a compound heterozygous state with kAE1 G701D provides an explanation for the dysfunction found in dRTA (Fig. 11). Both kidney AE1 S773P and G701D recessive mutants are impaired in trafficking to the plasma membrane in transfected HEK cells. The recessive S773P mutant was misfolded and targeted to the proteasome for degradation. The kAE1 S773P mutant can form a homo-oligomer, as well as a hetero-oligomer with kAE1 G701D. With the lack of the functional expression of either mutant protein at the basolateral membrane of the α-intercalated cells, the cells would not be capable of transporting bicarbonate into the blood. In the heterozygous state with wild-type AE1, the mutant can form hetero-oligomers, but this does not prevent trafficking of the wild-type protein to the plasma membrane. This is in contrast to the dominant dRTA mutant (kAE1 R589H) that retains the wild-type protein in the ER. Also, the more rapid degradation observed for the mutant protein may limit the amount of hetero-oligomer formation leaving the wild-type protein relatively free to homo-dimerize and traffic normally to the cell surface. Thus, in the heterozygous state, sufficient kAE1 would be present in the basolateral membrane of α-intercalated cells to retain adequate bicarbonate transport into the blood. The expression of dominant and recessive dRTA mutants in MDCK cells is currently under investigation in order to determine the effects of the mutations on kAE1 trafficking to the basolateral membrane in polarized epithelial cells.

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