Degradation mechanism of a Golgi-retained distal renal tubular acidosis mutant of the kidney anion exchanger 1 in renal cells

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Chu CY, King J, Berrini M, Rumley AC, Apaja PM, Lukacs GL, Alexander RT, Cordat E. Degradation mechanism of a Golgi-retained distal renal tubular acidosis mutant of the kidney anion exchanger 1 in renal cells. Am J Physiol Cell Physiol 307: C296–C307, 2014. First published June 11, 2014; doi:10.1152/ajpcell.00310.2013.—Distal renal tubular acidosis (dRTA) can be caused by mutations in the SLC4A1 gene encoding the anion exchanger 1 (AE1). Both recessive and dominant mutations result in mistrafficking of proteins, preventing them from reaching the basolateral membrane of renal epithelial cells, where their function is needed. In this study, we show that two dRTA mutants are prematurely degraded. Therefore, we investigated the degradation pathway of the kidney AE1 G701D mutant that is retained in the Golgi. Little is known about degradation of nonnative membrane proteins from the Golgi compartments in mammalian cells. We show that the kidney AE1 G701D mutant is polyubiquitylated and degraded by the lysosome and the proteasome. This mutant reaches the plasma membrane, where it is endocytosed and degraded by the lysosome via a mechanism dependent on the peripheral quality control machinery. Furthermore, we show that the function of the mutant is rescued at the cell surface upon inhibition of the lysosome and incubation with a chemical chaperone. We conclude that modulating the peripheral quality control machinery may provide a novel therapeutic option for treatment of patients with dRTA due to a Golgi-retained mutant.

Golgi; quality control; kidney; transporters; membrane protein; epithelium; trafficking; lysosome; proteasome; acidosis; bicarbonate; pH

AN INHERITED INABILITY to acidify urine is a hallmark of distal renal tubular acidosis (dRTA), a rare disease that can be dominantly or recessively inherited (3). dRTA patients typically present with metabolic acidosis, hypokalemia, nephrocalcinosis, and failure to thrive in childhood. Mutations in the gene SLC4A1 encoding the kidney anion exchanger 1 (kAE1) can cause dRTA (13). The kAE1 protein, an 846-amino acid membrane glycoprotein, is expressed in the basolateral membrane of type A intercalated cells in the cortical and medullary collecting ducts of the kidney (17, 24). It contains a long (~350-residue) cytosolic amino terminus and 12–14 transmembrane domains that carry a single N-glycosylation site in the fourth extracellular loop (34, 50). In renal epithelial cells, newly synthesized kAE1 proteins cotranslationally acquire a high-mannose oligosaccharide that is converted to a complex oligosaccharide when the protein reaches the Golgi (14, 41).

The short cytosolic carboxy-terminal domain of kAE1 is involved in physical interaction with carbonic anhydrase II (45–47). Cytosolic carbonic anhydrase II catalyzes the production of bicarbonate and protons from water and CO2. The cytosolic carboxy-terminal domain of kAE1 also interacts with adaptor protein 1A (37), an adaptor protein complex that is ubiquitously expressed. The presence of this adaptor is essential to kAE1 stability and is involved in the direct cell surface trafficking of kAE1 in renal epithelial cells (2). kAE1 exchanges one chloride anion for one bicarbonate anion, thereby participating in acid-base homeostasis of the body. Interestingly, although mutations in the same gene can also result in the most common form of inherited hemolytic anemia, hereditary spherocytosis, the presence of both diseases in the same patient is rare. This is likely due to different stringency of quality control machineries or the presence of cell-specific chaperones that assist folding and trafficking of the protein to its final residence.

Mutations in the SLC4A1 gene that cause dRTA are located within the transmembrane domain or within the short carboxy-terminal domain of kAE1. There are no known dRTA mutations in the amino-terminal cytosolic domain. kAE1 proteins naturally form dimers at the plasma membrane (33). In epithelial Madin-Darby canine kidney (MDCK) cells, dominant dRTA mutants can form heterodimers with kAE1 wild-type (WT), but they are predominately retained intracellularly or mistargeted to the apical membrane (14, 15, 41). In contrast, the heterodimeric form of kAE1 WT and a recessive dRTA mutant can traffic to the basolateral membrane (14).

Interestingly, some dRTA mutants are partially functional, on the basis of observations from patients’ red blood cells or when these mutant proteins are expressed in Xenopus oocytes (7, 40). The recessive kAE1 G701D dRTA mutant is common in Southeast Asia, either in the homozygous state or in the compound heterozygous state with S773P, E522K, or the Southeast Asian ovalocytosis mutant (10, 23, 42, 44). Recently, the G701D mutation was also found in a patient with no known Asian background, in the compound heterozygous state with the C479W mutation (11). This mutant is predominantly retained in the Golgi of renal epithelial cells (14, 23). However, it is functionally detected at the surface of Xenopus oocytes when coexpressed with the erythroid-specific, chaperone-like glycoporin A (8, 40). Recent work has started to identify strategies to overcome intracellular retention of partially active mutated membrane proteins (30, 43). Basolateral membrane targeting of the endoplasmic reticulum (ER)-retained kAE1 dRTA R901X and R589H mutants was observed after disruption of their interaction with lectin chaperones in MDCK cells (31). Incubation of cells expressing the Golgi-retained kAE1 G701D dRTA mutant with chemical chaperones also proved sufficient to partially restore its functional activity at the

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surface of MDCK cells (12). However, the functional rescue was only 50% of that of kAE1 WT.

We hypothesized that kAE1 dRTA mutants may be prematurely degraded, which would limit the ability of chemical chaperones to fully rescue the function of the mutant. There are two predominant degradation machineries in mammalian cells: proteosomes and lysosomes. Newly synthesized membrane proteins that are perceived as misfolded by the ER quality control machinery are typically polyubiquitylated, retrotranslocated to the cytosol, and degraded via the proteosome (35).

Surface membrane proteins can be endocytosed and then traffic to early endosomes and subsequently to recycling endosomes. Endocytosed proteins are then recycled to the plasma membrane or sorted to late endosomes, multivesicular bodies, and lysosomes, where they are degraded (35). The sorting of endocytosed membrane proteins involves a peripheral quality control machinery, the structure and function of which are starting to be elucidated (28). This machinery is composed of four types of endosomal sorting complexes required for transport: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. The main components of ESCRT-0 are the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal-transducing adaptor molecule (STAM) subunits (26), which recruit ESCRT-I components. ESCRT-I contains several mammalian homologs of yeast vacuolar protein sorting, including the mammalian tumor susceptibility gene 101 (Tsg101) (27). Together, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III function sequentially to recruit ubiquitylated proteins to the endosomal membrane, where they deform the endosomal membrane to allow the budding off of the endosome from the plasma membrane (35).

In this study, we investigated the fate of kAE1 dRTA mutants, the cell surface trafficking of which was either resuable or not by incubation with the chemical chaperone dimethyl sulfoxide (DMSO) in renal epithelial MDCK cells. We focused on the Golgi-retained kAE1 G701D mutant because its function can be partially restored in cells upon incubation with the chemical chaperone DMSO and the nonrescueable kAE1 C479W mutant as a control. Our results obtained for kAE1 WT and the Golgi-retained kAE1 G701D mutant will serve as a model to investigate degradation pathways of other Golgi-retained membrane proteins in mammalian cells. They could also provide therapeutic options for the treatment of other diseases caused by inappropriate Golgi retention of membrane proteins.

MATERIALS AND METHODS

*kAE1 mutant constructs.* cDNAs encoding WT, G701D, C479W, and R589H kAE1 were cloned into the viral vector pFB-Neo (Stratagene), as described previously (11, 14). These cDNA constructs contain the sequence encoding a hemagglutinin (HA) epitope in the third extracellular loop of kAE1 at position 557, previously shown to have no effect on normal folding, trafficking, or function of kAE1 (11, 14). Hereafter, this construct is referred to as kAE1. cDNAs encoding kAE1 WT or G701D cloned into the pcDNA3 plasmid were gifts from Dr. Reinhart Reithmeier (University of Toronto).

*Cell lines, transfections, and viral infections.* MDCK (CCL-34) and human embryonic kidney (HEK) 293 (CRL-11268) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in DMEM/F12 and DMEM, respectively, supplemented with 10% (vol/vol) fetal bovine serum, 0.5% penicillin, and 0.5% streptomycin under 5% CO2 at 37°C. Ts20 cells were maintained in DMEM/F12 supplemented with 10% (vol/vol) fetal bovine serum, 0.5% penicillin, and 0.5% streptomycin under 5% CO2 at 30–32°C. A 2-h incubation of these cells at 40°C followed by activation of the ubiquitin-activating enzyme E1, as previously reported (6, 25). Viral infections of MDCK cells were performed as previously described (14). Briefly, HEK 293 cells were transiently transfected with p-VIPack-GP, p-VIPack-VSV-G, and pFB-Neo kAE1 WT or mutant plasmids (Stratagene) using X-tremeGENE (Roche Applied Science). The supernatant containing the virus was collected and added to subconfluent MDCK cells with 8 μg/ml hexadimethrine bromide (Polybrene; Sigma). At 24 h after infection, cells were selected with 1 mg/ml Geneticin. We previously reported that expression levels of kAE1 in infected MDCK cells decrease over a 3- to 4-wk period (on average, 6 passages) (12, 14); therefore, infected cells were not used for periods longer than 4 wk. Alternatively, cells were transiently transfected using the Neon Transfection system as previously described (2).

Knockdown of endogenous ESCRT proteins was carried out as previously described (29) by incubation of HeLa cells with doxycycline as follows: shHrs, 300 ng/ml for 5 days; shSTAM, 300 ng/ml for 3 days; shTsg101, 50 ng/ml for 3 days; and shNT, 50 ng/ml for 3 days. At 24 h before initiation of the experiment, HeLa cells were transiently transfected with cDNA encoding kAE1 WT or the G701D or C479W mutant cloned into the pcDNA3 plasmid using X-tremeGENE transfection reagent according to the manufacturer’s instructions, and 16 h prior to lysis, cells were incubated with 1% DMSO directly added to the culture medium to rescue trafficking and function of the mutant.

To evaluate the contribution of the ubiquitin machinery to kAE1 stability, E36 and ts20 cells were transiently transfected with cDNA encoding kAE1 WT or the G701D or C479W mutant in the pcDNA3 plasmid using X-tremeGENE transfection reagent and grown at 37°C (E36 cells) or 30°C (ts20 cells). After 24 h, cells were transferred to 40°C for 2 h to inactivate the E1 ubiquitin-activating enzyme and lysed, and samples were prepared for immunoblotting following the protocol described below.

*Lysate preparation, immunoprecipitations, and immunoblots.* MDCK, HeLa, ts20, or E36 cells expressing WT or mutant kAE1 were lysed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4) containing 1% Triton X-100 and protease inhibitors [leupeptin, aprotinin, pepstatin, and PMSF (Sigma)]. Protein concentrations in cell lysates were quantitated by bicinchoninic acid assay [using bovine serum albumin (BSA) as standard; Sigma]. Proteins diluted in 2× Laemmli sample buffer (Bio-Rad) were resolved on 8% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose or PVDF membranes (Bio-Rad). The membranes were blocked with 3% skim milk in TBST (5 mM Tris base, 15 mM NaCl, and 0.1% Tween 20) and incubated with mouse anti-HA primary antibody (Covance) followed by anti-mouse secondary antibody coupled to horseradish peroxidase. Probed proteins were detected with enhanced BM chemiluminescence blotting substrate (POD; Roche) or ECL Prime Western blotting detection system (Amersham, GE) on film (Kodak). Intensities of the bands were compared using ImageJ software. For assessment of ubiquitylation of kAE1, the anti-ubiquitin FK2 antibody (Millipore) was used for immunoblotting after immunoprecipitation with anti-HA antibody.

*Immunocytochemistry.* Subconfluent HeLa cells expressing WT or mutant kAE1 were grown on glass coverslips, fixed with 4% paraformaldehyde (Canemco Supplies) in PBS, and incubated with 100 mM glycine in PBS (pH 8.5) to quench nonspecific fluorescence. After blocking with 1% BSA, the kAE1 proteins at the cell surface were detected with a mouse anti-HA primary antibody (Covance) followed by an anti-mouse antibody coupled to Cy3 (Jackson Immunoresearch Laboratories). Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 1% BSA, and incubated with mouse anti-HA primary antibody again followed by a secondary Alexa 488
antibody (Invitrogen Molecular Probes) to detect intracellular kAE1. Nuclei were stained with DAPI.

To evaluate colocalization of kAE1 WT or G701D with the lysosomal marker lysosomal-associated membrane protein-1 (Lamp-1), MDCK cells expressing kAE1 WT or G701D mutant were transiently transfected with cDNA encoding Lamp-1 fused to the Venus fluorescent protein (gift from Dr. Nevin Lambert, Georgia Regents University). At 24 h posttransfection, intact living cells were incubated with the mouse anti-HA antibody for 2 h at 37°C. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, and incubated with a Cy3-coupled anti-mouse secondary antibody. To determine colocalization with endogenous transferrin receptor, intact, living MDCK cells expressing kAE1 WT were starved for 30 min in Opti-MEM (Invitrogen) and then incubated with transferrin coupled to Alexa 488 (Molecular Probes), as well as mouse anti-HA antibody, at 37°C for 3 h. To improve the detection of internalized kAE1 proteins, the antibodies remaining at the cell surface were removed by incubation of the cells in a low-pH citrate buffer (“acid wash”: 40 mM citric acid, 100 mM KCl, and 135 mM NaCl, pH 1.5) for 8 min at 4°C (16). After they were washed, the cells were fixed and permeabilized, and the primary antibody was detected by incubation with anti-mouse antibody coupled to Cy3.

Imaging by conventional or confocal immunofluorescence microscopy. High-resolution images were acquired using the Angstrom illumination system (Quorum Technologies) equipped with OptiGrid structured illumination (Qioptiq), excitation and emission filter wheels (Ludl Electronic Products), and a Flash 4.0 camera (Hamamatsu). The Angstrom illumination system and associated hardware are mounted to the 100% side port of a fully motorized inverted microscope (model DMi6000, Leica Microsystems). All hardware was controlled with MetaMorph software (Molecular Devices). Alternatively, samples were examined using an Olympus IX81 microscope equipped with a Nipkow spinning disk optimized by Quorum Technologies (Guelph, ON, Canada), and a ×63 objective.

Bicarbonate transport assay. MDCK cells expressing WT or mutant kAE1 were grown on 11 × 7.5 mm glass coverslips in 6-cm² dishes until 70% confluent. Prior to the assay, cells were kept in control conditions, incubated with 1% DMSO for 16 h at 37°C, incubated with 2 mM leupeptin at 37°C for 2 h, or incubated with 1% DMSO for 16 h at 37°C and 2 mM leupeptin at 37°C for the last 2 h of DMSO incubation. The complete procedure is described elsewhere (39). Briefly, after they were washed with serum-free OptiMEM (Gibco), the coverslips were incubated with 10 μM BCECF-AM (Sigma) for 10 min at 37°C. The coverslips were then placed in fluorescence cuvettes, and the cells were perfused at room temperature with Ringer buffer (5 mM glucose, 5 mM potassium glutonate, 1 mM calcium glutonate, 1 mM magnesium sulfate, 10 mM HEPES, 2.5 mM NaH₂PO₄, and 25 mM NaHCO₃) containing 140 mM chloride and then with a chloride-free medium containing 140 mM gluconate to induce intracellular alkalization. BCECF intracellular fluorescence was calibrated with buffers at pH 6.5, 7.0, or 7.5 containing 100 μM nigericin sodium salt. The Ringer buffers were continuously bubbled with air-CO₂ (19:1), providing 5% CO₂. We used a fluorometer (Photon Technologies International, London, ON, Canada) to read the fluorescence fluctuations produced from the samples. Excitation wavelengths were 440 and 490 nm and emission wavelength was 510 nm (calibrated to the fluorometer). Transport rates of the cells were determined by linear regression of the initial fluorescence variations (first 30 s), normalized to pH calibration measurements. All measurements were done using Felix software. The initial resting pH values (means ± SE, measured for the initial 80 s) were as follows: 7.14 ± 0.07 for MDCK cells without DMSO, no leupeptin (n = 4); 7.61 ± 0.19 for MDCK cells with DMSO, no leupeptin (n = 3); 7.44 ± 0.01 for MDCK cells with DMSO and leupeptin (n = 3); 7.48 ± 0.01 for MDCK cells without DMSO, no leupeptin (n = 3); 7.37 ± 0.01 for kAE1 WT cells without DMSO, no leupeptin (n = 3); 7.81 ± 0.33 for kAE1 WT cells with DMSO, no leupeptin (n = 3); 7.09 ± 0.06 for kAE1 G701D cells without DMSO, leupeptin (n = 3); 7.45 ± 0.31 for kAE1 G701D cells with DMSO, no leupeptin (n = 3); 7.48 ± 0.06 for kAE1 G701D cells with DMSO and leupeptin (n = 5); and 7.70 ± 0.12 for kAE1 G701D cells without DMSO, with leupeptin (n = 4). ANOVA showed no statistically significant difference between initial resting intracellular pH values from MDCK with no DMSO, no leupeptin condition, and all the other values.

Statistical analysis. All the experiments were independently repeated a minimum of three times. Experimental results are summarized as means ± SE. All statistical comparisons were made using unpaired Student’s t-test or one-way ANOVA as indicated. P < 0.05 was considered statistically significant.

RESULTS

Golgi- and ER-retained kAE1 dRTA mutants are prematurely degraded. We first investigated the fate of kAE1 dRTA mutants in renal epithelial cells. We employed a previously described kAE1 cDNA construct carrying a HA epitope inserted in the third extracellular loop (kAE1 HA, hereafter called kAE1) (12, 14). This insertion does not affect expression, trafficking, or activity of kAE1 (14). MDCK cells expressing kAE1 WT, the recessive, partially active Golgi-retained G701D mutant, or the recessive, inactive ER-retained C479W mutant (11, 12) were incubated with the protein synthesis inhibitor cycloheximide for up to 24 h prior to lysis. In polarized MDCK cells, kAE1 WT is functional and targeted to the basolateral membrane, as occurs in type A intercalated cells (14), supporting the concept that this cell line is a good model for kAE1 trafficking studies. After cycloheximide incubation, the amount of kAE1 proteins remaining in the cell lysates at the various incubation times was quantified by immunoblotting. In cell lysates from MDCK cells, kAE1 migrates as two main bands (Fig. 1): the bottom band corresponds to protein carrying high-mannose oligosaccharides and the top band to kAE1 protein with complex oligosaccharides (14, 41). Figure 1 indicates that, in contrast to the 22-h half-life of kAE1 WT, the half-lives of the Golgi-retained G701D and ER-retained C479W mutants were 2 and 5 h, respectively. These results are in agreement with previously published results (31) and indicate that the cellular quality control machinery quickly detects and degrades these two kAE1 dRTA mutants. We next investigated the cellular mechanisms responsible for degradation of the kAE1 dRTA mutants.

Golgi-retained kAE1 G701D mutant is degraded by the proteasome and the lysosome. To investigate the degradation pathways for the ER-retained C479W and Golgi-retained kAE1 G701D dRTA mutants, we inhibited the proteosomal or lysosomal degradation pathway in cells expressing kAE1 WT, G701D, or C479W by incubating the cells for 2 h with 10 μM epoxomycin or 2 mM leupeptin, respectively. As seen on the immunoblots in Fig. 2, analysis of lysates from cells expressing kAE1 G701D protein revealed a marked stabilization of the mutant after either of these treatments. By comparing the intensity of the kAE1 bands (top and bottom), we observed 55 ± 13% (n = 7) and 48 ± 15% (n = 6) increases in the amount of kAE1 G701D in the lysates after epoxomycin and leupeptin incubations, respectively. In contrast, kAE1 C479W protein expression was not significantly altered by the same conditions. The stabilization of the Golgi-retained mutant by
both treatments resulted in an upward molecular weight shift of kAE1 bands, possibly corresponding to an increased amount of polyubiquitylated kAE1 protein (Fig. 3A), in addition to stabilization of the amount of kAE1 protein remaining in the lysates (Fig. 2). Interestingly, leupeptin treatment seemed to increase polyubiquitylation of kAE1 G701D more prominently, while epoxomycin treatment resulted in a denser smear of ubiquitylated kAE1 C479W. Importantly, the ubiquitylation experiment immunoprecipitations were not performed under denaturing conditions; therefore, it is possible that, in cell lysates, kAE1 remains associated with polyubiquitylated proteins that are detected on the blots shown in Fig. 3. This hypothesis is consistent with a lack of anti-HA-reactive protein bands of molecular higher than that of kAE1 (anti-HA immunoblots in Fig. 3).

To confirm our findings, we expressed kAE1 WT, G701D, or C479W in control E36 cells or ts20 cells, where the ubiquitin-activating enzyme E1 is knocked down at the non-permissive temperature (25). Incubation of ts20 cells for 2 h at 40°C results in downregulation of the endogenous ubiquitin-activating enzyme E1 (6, 25). Ts20 cells were transiently transfected with kAE1 WT, G701D, or C479W, and after a 2-h incubation at 40°C, lysates were loaded on SDS-polyacrylamide gels prior to analysis by immunoblotting with a mouse

epoxomycin or leupeptin is consistent with participation of the lysosome and the proteosome in degrading the Golgi-retained kAE1 mutant in renal epithelial cells and explains the premature degradation of this mutant within 2 h of protein synthesis inhibition.

**ER- and Golgi-retained dRTA mutants may be ubiquitylated upon inhibition of degradation machineries.** Proteins that are destined for degradation are often polyubiquitylated in cells (29). MDCK cells expressing kAE1 wild-type (WT) protein, the Golgi-retained mutant G701D, or the endoplasmic reticulum (ER)-retained mutant C479W were incubated for 0–24 h with 10 μg/ml cycloheximide (CHX) prior to cell lysis. The same amount of protein was loaded and resolved by immunoblotting using a mouse anti-HA antibody. Position of protein carrying complex oligosaccharides; ◊, position of protein carrying high-mannose oligosaccharides. B: intensities of bands were compared using ImageJ software and normalized to the intrinsic control actin. Values are means ± SE (error bars) from ≥3 independent experiments.

Fig. 2. The lysosome and the proteosome participate in degradation of Golgi- and ER-retained kAE1 mutants. A: MDCK cells expressing kAE1 WT, G701D, or C479W were untreated or treated with 10 μM epoxomycin (a proteasome inhibitor) or 2 mM leupeptin (a lysosome inhibitor) for 2 h at 37°C. Proteins in cell lysates were immunoprecipitated and resolved on SDS-polyacrylamide gels. Immunoblots (IB) show eluted kAE1 proteins detected using an anti-HA antibody. ◊, position of protein carrying complex oligosaccharides; ◊, position of protein carrying high-mannose oligosaccharides. Note more “smeary” appearance of kAE1 G701D and C479W mutants in epoxomycin-treated than control cells. B: percentage of kAE1 proteins in cell lysates after epoxomycin treatment vs. control. Intensity of the bands was analyzed using ImageJ software. Values are means ± SE (error bars) from ≥3 independent experiments. *P < 0.05 vs. control (by 1-way ANOVA).
These experiments indicate that a functional ubiquitin machinery is necessary for degradation of kAE1 WT or dRTA mutants and support the notion that dRTA mutants are more heavily polyubiquitylated than kAE1 WT, consistent with their premature degradation. As little is known about the degradation machinery of membrane proteins in the Golgi, we next examined the degradation mechanisms of the Golgi-retained kAE1 G701D mutant.

Golgi-retained kAE1 G701D mutant transiently reaches the plasma membrane prior to degradation. As the kAE1 G701D dRTA mutant is predominantly retained in the Golgi, we investigated several mechanisms by which the mutant could be prematurely degraded. The mutant could be loaded into intracellular vesicles trafficking from the Golgi 1) directly to lysosomes, 2) to the lysosomes via the plasma membrane, or 3) back to the ER for proteosomal degradation. Although knowledge of these mechanisms in mammalian cells is limited, experimental evidence for all three mechanisms, mostly obtained in yeasts, has been reported (5). To discriminate between hypotheses 1 and 2, we determined whether the kAE1 G701D dRTA mutant ever reaches the plasma membrane of intact, living MDCK cells by taking advantage of the extracellular HA epitope in the third extracellular loop of kAE1. After preincubation with 1% DMSO for 16 h or no preincubation period, we incubated live, untransfected MDCK cells or MDCK cells expressing kAE1 WT, G701D, or C479W with an anti-actin antibody as a control or with an anti-HA antibody for 1 h at 37°C. DMSO incubation is known to improve trafficking of the kAE1 G701D mutant to the cell surface (12). In non-permeabilized cells, the anti-HA antibody detects the HA epitope located in the third extracellular loop of kAE1 when the protein is targeted to the plasma membrane. After a 1-h incubation, the cells were fixed and permeabilized, and primary antibodies were detected using a secondary Cy3 (red)-coupled antibody. Red staining is thus only detectable if kAE1 has reached the plasma membrane, even transiently. As the cells are continuously incubated with the antibody for 1 h, kAE1 proteins at the cell surface and endocytosed kAE1 will labeled at the cell surface and intracellularly, respectively. Surprisingly, Fig. 4C shows specific staining of kAE1 in intracellular vesicles of kAE1 G701D-expressing cells, with no DMSO preincubation. Upon partial rescue of cell surface trafficking of the mutant with DMSO treatment, we observed that kAE1 G701D was still predominantly endocytosed within 1 h, while kAE1 WT was predominantly located at the plasma membrane (Fig. 4A, arrowheads). In contrast, neither intact MDCK cells incubated with mouse anti-HA antibody nor MDCK cells expressing kAE1 G701D incubated with anti-actin antibody displayed similar intracellular or plasma membrane staining (Fig. 4, D and E). These results indicate that, although predominantly retained in the Golgi at the steady state, the kAE1 G701D mutant transiently reaches the plasma membrane, even in the absence of the chemical chaperone DMSO, but is endocytosed in intracellular vesicles within 1 h. By comparing the density of kAE1 staining (red) at the plasma membrane in WT (Fig. 4A, arrowheads) with the absence of cell surface staining in G701D (Fig. 4C), we hypothesize that kAE1 G701D is prematurely endocytosed compared with kAE1 WT.

These results indicate that, although predominantly retained in the Golgi, the kAE1 G701D mutant transiently reaches the plasma membrane, even in the absence of a stabilizing chemical chaperone; from the plasma membrane, it is quickly
Endocytosed and predominantly accumulated in intracellular vesicles within 1 h.

**Endocytosed kAE1 G701D mutant colocalizes with lysosomes.** To investigate the fate of the endocytosed kAE1 G701D mutant, we transiently transfected MDCK cells expressing kAE1 WT or G701D with Lamp-1 cDNA fused to the Venus fluorescent protein. MDCK cells expressing kAE1 WT or G701D and transiently transfected with Lamp-1 were incubated with mouse anti-HA antibody for 2 h at 37°C prior to fixation, permeabilization, and detection of primary antibody with anti-mouse antibody coupled to Cy3. Examination of the cells revealed that endocytosed kAE1 G701D partially colocalized with the lysosomal marker Lamp-1 in a perinuclear location (Fig. 5, D, E, and F, arrowheads). In contrast, there was minimal overlap of the staining between endocytosed kAE1 WT and Lamp-1 proteins (Fig. 5, A–C). Instead, colocalization of kAE1 WT was observed with the recycling endosome marker transferrin after a 3-h incubation of intact, living cells with the mouse anti-HA antibody (Fig. 5, G–I). Endocytosed kAE1 G701D did not show an obvious colocalization with transferrin (Fig. 5, J–L). Although it is possible that overexpression of the lysosomal marker or the presence of Venus fluorescent protein slightly alters the final location of the lysosomal protein, these results support the idea that, after endocytosis, the kAE1 G701D mutant is targeted to the lysosome for degradation.

**Knocking down components of the peripheral quality control machinery stabilizes rescued kAE1 G701D mutant.** Recent studies have dissected the role of individual components of the peripheral quality control ESCRT machinery (28, 35). ESCRT machinery comprises a number of essential components for peripheral quality control, including Hrs, STAM, and Tsg101 (35). As we have shown in Fig. 4 that rescued and nonrescued Golgi-retained kAE1 G701D transiently reach the plasma membrane, we next asked whether these components of the peripheral quality control machinery are involved in premature degradation of kAE1 G701D. HeLa cells that are knocked down for Hrs, STAM, and Tsg101, or express nontargeting (NT) shRNA (HeLa NT) (29) were transiently transfected with kAE1 WT, G701D, or C479W and incubated with 1% DMSO for 16 h, and the relative amount of kAE1 WT or mutant proteins was analyzed 24 h posttransfection by immunoblotting in the various HeLa cell lines.

We first performed an immunofluorescence experiment to confirm that kAE1 reached the surface in these cells. Using the extracellular HA epitope, we detected cell surface kAE1 prior to cell permeabilization (Fig. 6a, red). After cell permeabilization, total kAE1 is shown in green. As shown by the red staining in Fig. 6a, kAE1 WT reaches the plasma membrane in NT HeLa cells. Less kAE1 G701D protein reaches the cell surface, shown by the less intense red staining compared with kAE1 WT. After permeabilization, staining of total kAE1 G701D shows a perinuclear localization, reminiscent of the Golgi localization of this mutant in MDCK and LLC-PK1 cells (12, 14, 23). No surface (red) staining was seen on cells transfected with the ER-retained kAE1 C479W mutant, indicating that, overall, the kAE1 WT or mutants trafficked in a similar way in HeLa and MDCK cells. Immunoblot and quan-
Fig. 5. Endocytosed kAE1 G701D mutant partially colocalizes with the lysosomal marker lysosomal-associated membrane protein-1 (Lamp-1). MDCK cells expressing kAE1 WT (A, B, C, G, H, and I) or G701D (D, E, F, J, K, and L) transiently transfected with cDNA encoding Lamp-1 fused to Venus fluorescent protein (A–F) were incubated with mouse anti-HA antibody for 2 h at 37°C. After fixation, kAE1 proteins were detected with an anti-mouse antibody coupled to Cy3. Arrowheads in F point to yellow staining, corresponding to colocalization of kAE1 G701D and Lamp-1. For colocalization with the transferrin receptor (G–L), MDCK cells expressing kAE1 WT or G701D were starved for 1 h before addition of transferrin coupled to Alexa 488 and mouse anti-HA antibody to the medium for 3 h at 37°C. After an acid wash to remove remaining cell surface antibody, cells were fixed and permeabilized, and primary antibody was detected with anti-mouse antibody coupled to Cy3. Arrowheads in I point to yellow staining, corresponding to colocalization of kAE1 WT and transferrin receptor. Samples were examined by fluorescence microscopy using a ×63 objective. Nuclei were stained with DAPI. Scale bars, 10 μm.
Identification results in Fig. 6, with 1% DMSO. Knocking down components of the peripheral quality control machinery stabilizes kAE1 G701D. Control [nontargeting (NT)] HeLa cells or HeLa cells knocked down for Hrs, STAM, or Tsg101 proteins were transiently transfected with kAE1 WT, G701D, or C479W. At 16 h prior to lysis, cells were incubated with a permeabilization in red and total kAE1 after permeabilization in green. Samples were examined using a Leica microscope coupled to an Angstrom illumination system with a ×40 oil objective. Scale bar, 10 μm. B: steady-state amount of kAE1 was investigated by immunoblotting using a mouse anti-HA antibody. In HeLa cells, kAE1 migrates as a single band at 96 kDa. C: intensities of kAE1 bands from the various cell lines were compared using ImageJ software, and percentage of kAE1 in treated vs. control conditions is shown. NT corresponds to HeLa cells treated with nontargeting shRNAs. In these HeLa cells, kAE1 is resolved as a single band, in contrast with the two bands seen in MDCK cells, suggesting that kAE1 glycosylation is different from that in MDCK cells. Values are means ± SE (error bars) from ≥3 independent experiments. *P < 0.05 vs. NT conditions (by ANOVA followed by Bonferroni’s t-test for multiple comparisons).

Inhibiting the lysosome restores the function of the kAE1 G701D mutant. As incubating cells expressing kAE1 G701D with a lysosomal inhibitor markedly increased the total amount of the mutant in MDCK cells (Fig. 2), we next examined whether inhibition of the lysosome with leupeptin would rescue the function of kAE1 G701D at the surface of MDCK cells. The cells were incubated under control conditions, in medium containing 1% DMSO for 16 h to enhance kAE1 G701D surface trafficking, in medium containing 2 mM leupeptin for 2 h at 37°C, or in medium containing 1% DMSO for 16 h, which was then complemented with 2 mM leupeptin for the last 2 h of incubation to inhibit the lysosome. We used the ratiometric fluorescence-based pH-sensitive dye BCECF-AM to perform a fluorescence-based functional assay by monitoring intracellular pH variations in control MDCK cells or cells expressing kAE1 WT or G701D (39). After preloading the cells with BCECF-AM in the presence of chloride, we perfused them with a chloride-free solution in the presence of extracellular bicarbonate (12, 39). If kAE1 is functional and present at the cell surface, entry of bicarbonate through kAE1 results in an increase in intracellular pH and subsequently alters the intracellular BCECF fluorescence. The bicarbonate-chloride transport rate in the initial 30 s of activity was measured and compared with nontreated conditions. As seen in Fig. 7, treatment with leupeptin significantly increased the functional activity of kAE1 G701D from 0.031 ± 0.002 mM/min (n = 7) to 0.144 ± 0.018 mM/min (n = 6) and eliminated a significant difference in transport rate between kAE1 WT and the kAE1 G701D mutant. There was no significant difference between transport rates of kAE1 G701D-expressing cells that were treated with leupeptin alone and cells treated with leupeptin and DMSO. These results support the idea that inhibition of lysosomal degradation promotes increased functional activity of the kAE1 G701D mutant in renal epithelial cells.

DISCUSSION

There is limited knowledge about Golgi quality control degradation machinery in mammalian cells. Most of our current knowledge is based on studies performed in yeast. In this study we demonstrate that a disease-causing mutant of kAE1 that is retained in the Golgi is prematurely degraded by the...
Possible polyubiquitylation (Fig. 3), and degradation by the proteasome (Fig. 2). ER-associated degradation has been implicated in the premature degradation of a number of misfolded proteins, causing numerous diseases, including cystic fibrosis, Huntington’s disease, and various cancers (18, 20, 22, 48).

The kAE1 G701D mutant that is predominantly retained in the Golgi is also more rapidly degraded than the kAE1 WT protein, in agreement with previous findings (31). As little is known about degradation mechanisms of Golgi-retained membrane proteins in mammalian cells, we subsequently focused on the Golgi-retained kAE1 G701D dRTA mutant. We first assessed the effect of individually inhibiting the activity of the two cellular degradation pathways on kAE1 stability, specifically the proteosome and the lysosome. Inhibition of the proteosome with the irreversible inhibitor epoxomycin or the lysosome with leupeptin for 2 h significantly increased the amount of kAE1 G701D in these cells at steady state (Fig. 2). We observed a similar stabilizing effect on kAE1 G701D abundance after incubating kAE1 G701D-expressing MDCK cells with another lysosomal inhibitor, chloroquine (100 μM), for 2 h at 37°C (data not shown). These results indicate that both cellular degradation pathways are involved in premature degradation of this Golgi-retained mutant. At the steady state, the kAE1 G701D mutant is predominantly located in the Golgi but also shows diffuse staining throughout the cell, likely corresponding to the ER (14). It is thus reasonable to hypothesize that the processing of this mutant from the ER to the Golgi is inefficient, as reflected by the larger amount of protein carrying high-mannose oligosaccharide (Fig. 2) (14). Alternatively, this mutant may traffic from the Golgi back to the ER, as is the case for some ER proteins that have escaped to the Golgi (32). In either case, it may be detected as misfolded by the ER quality control machinery and degraded by the proteosome. In agreement with this finding, our results are consistent with kAE1 being polyubiquitylated or being closely associated with polyubiquitylated protein(s) upon inhibition of the proteosome or the lysosome, as seen by the appearance of a high-molecular-weight smear detected by the anti-ubiquitin antibody (Fig. 3).

Our study shows that the lysosome is also involved in degradation of this mutant. In yeast, there are a few examples of mutant proteins that reach the Golgi, where they are directly targeted to lysosomal degradation or to the plasma membrane prior to their internalization and degradation. The yeast plasma membrane H^+ -ATPase Pma1-7 mutant escapes the ER quality control machinery and reaches the Golgi, where it is directly targeted to the vacuole (9). Alternatively, membrane proteins reach the plasma membrane prior to endocytosis and lysosomal degradation, as was seen for a portion of the ΔF508 CFTR mutant (4, 38). To further investigate the degradation pathway of this Golgi-retained mutant, we tested whether the kAE1 G701D mutant could reach the plasma membrane from the Golgi, with or without preincubation of the cells with the chemical chaperone DMSO, which can partially rescue trafficking of this mutant to the cell surface. Addition of anti-HA antibody to nonpermeabilized MDCK cells expressing this mutant protein for 1 h at 37°C could only show staining if the kAE1 G701D mutant, carrying an extracellular HA epitope, reaches the plasma membrane, even transiently. Interestingly, this strategy showed specific, perinuclear staining, indicating that kAE1 G701D reaches the plasma

![Graph](https://example.com/graph.png)
membrane prior to endocytosis, even in the absence of DMSO incubation (Fig. 4C).

After a 1-h incubation with anti-HA antibody, kAE1 WT remains abundantly located at the plasma membrane (Fig. 4A, arrowheads) while kAE1 G701D was predominantly endocytosed with or without preincubation with the chemical chaperone DMSO (Fig. 4, B and C). We therefore hypothesize that the presence of kAE1 G701D at the plasma membrane is a transient phenomenon. We previously failed to detect cell surface kAE1 G701D at the plasma membrane (12, 14), while our present study indicates that the mutant trafficked to the cell surface. Our controls indicate that the staining is specific for kAE1 G701D and that the cells were neither nonspecifically engulfing the antibody nor permeabilized. Our ability to detect the kAE1 G701D mutant at the cell surface under these conditions but not previously is likely due to the following factors: 1) only a small portion of kAE1 G701D is able to escape the Golgi and reach the plasma membrane, and the presence of kAE1 G701D at the cell surface is transient; 2) endocytosed kAE1 G701D accumulates in a perinuclear organelle, where the fluorescent signal is intensified compared with staining at the cell surface; and 3) in this study we used a new imaging and hardware system, which may be more sensitive than the imaging system used in our previous study. In agreement with these considerations, Patterson and Reithmeier (31) showed a low level of cell surface expression of kAE1 G701D by flow cytometry, a method with higher sensitivity than indirect immunostaining.

Our data were obtained in nonpolarized MDCK cells grown on glass coverslips. A recent study has elegantly demonstrated that, in polarized MDCK cells grown on semipermeable filters, newly synthesized kAE1 WT protein directly reaches the basolateral membrane without trafficking to the apical membrane (19, 49). We have shown that, in nonpolarized MDCK cells, direct targeting of kAE1 WT to the cell surface is dependent on ubiquitous adaptor protein-1A (2). Furthermore, carboxy-terminal kAE1 mutations result in mistargeting of the mutants to apical and basolateral membranes, from which they are constitutively endocytosed (19, 49). It would be interesting to determine whether a small amount of kAE1 G701D is also targeted to and endocytosed from the basolateral membrane in polarized cells grown on semipermeable filters.

Importantly, we also found that, upon inhibition of the lysosomal degradation machinery with leupeptin, the function of the exchanger at the surface of MDCK cells was restored to an extent equivalent to the WT protein (Fig. 7). This result highlights the finding that decreasing kAE1 G701D premature degradation is sufficient to restore the full function of the exchanger in renal cells. It is thus possible that identification of small molecules with such an effect on kAE1 degradation may provide alternative treatment for dRTA patients carrying this mutation. In red blood cells, expression of erythrocyte-specific chaperone-like glycophorin A likely explains the absence of anemia in patients carrying this mutation. Coexpression of glycophorin A and kAE1 G701D proteins in Xenopus oocytes results in chloride fluxes that are indistinguishable from that of kAE1 WT coexpressed with glycophorin A (8, 21, 40). If glycophorin A was expressed in renal epithelial cells, it may act in a manner similar to that of a lysosomal inhibitor, i.e., by stabilizing the kAE1 G701D mutant at the cell surface, thereby diverting it from premature degradation. However, this chaperone-like protein is exclusively expressed in red blood cells and is absent from renal intercalated cells.

In this study we also show that the peripheral quality control machinery, particularly the ESCRT-0 components Hrs and STAM and the ESCRT-1 component Tsg101, is involved in degradation of kAE1 G701D and WT proteins (Fig. 6). Although DMSO incubation partially rescues the function of the kAE1 G701D mutant (12), the mutant is endocytosed and recognized by the peripheral quality control machinery that targets it for degradation, as knockdown of some components of the machinery stabilizes this mutant in HeLa cells. Such an effect of peripheral quality control machineries on stability of cell surface membrane proteins is becoming well documented (1, 36). The most recent and best-characterized example of downregulation of a mutated membrane protein by the peripheral quality control machinery comes from the study of the ΔF508 CFTR mutant (29, 38). Trafficking of the ΔF508 CFTR mutant to the plasma membrane is rescued by incubation of cells expressing this mutant with the pharmacological chaperones VX-770 and VX-809. However, even in the presence of these chaperones, this mutant is rapidly endocytosed from the cell surface and prematurely degraded via a peripheral quality control machinery. Therefore, the kAE1 G701D and ΔF508 CFTR mutants have a similar fate in mammalian cells. Whether the same peripheral quality control machinery is present in the human kidney intercalated cells remains to be determined, but our study brings evidence that, in mammalian immortalized cells, premature degradation of this mutant, possibly recognized as misfolded, occurs.

In conclusion, our study provides novel evidence that the kAE1 G701D mutant, which accumulates predominantly in the Golgi and partially in the ER, is prematurely degraded by the lysosome and the proteasome. Previously, we showed that plasma membrane trafficking and function of this mutant can be partially rescued by incubation of renal epithelial cells expressing this mutant protein with the chemical chaperone DMSO. Here, we examined further the cellular mechanisms that degrade this mutant even after its partial rescue. We found that this mutant reaches the plasma membrane but is prematurely endocytosed via a peripheral quality control machinery and degraded by a lysosome-dependent pathway. Inhibition of the lysosome restored the function of the mutant to the same extent as the WT protein. Therefore, a therapy that slows down the lysosomal degradation machinery could be targeted to dRTA patients carrying the Golgi-retained kAE1 G701D mutant.

In this study we showed that, in mammalian cells, Golgi-retained membrane proteins can spontaneously escape and reach the plasma membrane prior to their detection and endocytosis by the peripheral quality control machinery. These data suggest that the escape from the Golgi and partial cell surface targeting of mutated membrane proteins occur not only in yeast, but also in mammalian cells. This finding may be applicable to other membrane proteins inappropriately retained in this organelle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.Y.C., J.K., M.B., A.C.R., and P.M.A. performed the experiments; C.Y.C., M.B., and E.C. analyzed the data; C.Y.C., M.B., R.T.A., and E.C. interpreted the results of the experiments; C.Y.C. and E.C. drafted the manuscript; C.Y.C., J.K., M.B., A.C.R., P.M.A., G.L.L., R.T.A., and E.C. edited and revised the manuscript; C.Y.C., J.K., M.B., A.C.R., P.M.A., G.L.L., R.T.A., and E.C. approved the final version of the manuscript; G.L.L. and E.C. are responsible for conception and design of the research; E.C. prepared the figures.

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