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Rescue of Kidney Anion Exchanger 1 Trafficking Mutants

by

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Abstract

The kidney anion exchanger 1 (kAE1) is crucial in the regulation of physiological pH by facilitating Cl^-/HCO_3^- exchange. Inability to do so results in distal renal tubular acidosis (dRTA), which is more often due to mutations leading to mis-localization of kAE1, rather than complete abolishment of its functional activity. The purpose of this thesis is to understand the trafficking, turnover, and rescue of wildtype (WT) and trafficking mutants of the kAE1. Chemical chaperones and low temperature treatments were studied for their ability to rescue trafficking of G701D, C479W and R589H kAE1 mutants. G701D kAE1 was rescued by dimethyl sulfoxide (DMSO) and is functional at the membrane. The turnover of all kAE1 mutants studied was dependent on the proteasomal pathway, while G701D and WT kAE1 are also degraded via the lysosomal pathway. These studies provide essential information for developing rescue methods at the molecular level, targeting the source of the disease rather than the symptoms.

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List of Abbreviations

AE	anion exchanger
AQP	aquaporin
BCECF-AM	2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester
CA	carbonic anhydrase
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	carboxyl terminus of Hsc70-interacting protein
CHX	cycloheximide
Cl^-	chloride
CO_2	carbon dioxide
DIDS	4-4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DMSO	dimethyl sulfoxide
dRTA	distal renal tubular acidosis
e / kAE1	erythroid / kidney anion exchanger 1
E1	ubiquin activating enzyme
E3	ubiquitin ligase
ER	endoplasmic reticulum
ERAD	ER associated degradation
GPA	glycophorin A
H_2DIDS	4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonic acid
HA	hemagglutinin
HCO_3^-	bicarbonate
HEK	human embryonic kidney
HRP	horseradish peroxidase
HS	hereditary spherocytosis
IC	intercalated cell
MDCK	Madin Darby Canine Kidney
NBC(e/n)	sodium bicarbonate co-transporters (electrogenic / electroneutral)
NHE	sodium proton exchanger
pRTA	proximal renal tubular acidosis
RBC	red blood cell
SAO	Southeast Asian Ovalocytosis
SITS	4-Acetamido-4'-isothiocyano-2,2'-stilbene disulphonic acid
WT	wildtype

1. General Introduction

1.1 pH homeostasis and importance of buffers

Maintaining a balance of acids and bases is important for our body. In particular, this balance is crucial in circulating blood plasma. Potential Hydrogen, or pH, is defined as the measure of the acidity or basicity of an aqueous solution [1]. Resident proteins of different parts of the body require specific pH ranges within their environment for appropriate function, and the additive effect of normal protein performance is necessary for healthy body maintenance. Exposed amino acids of proteins, such as those at a gating site, can interact with excess protons or hydroxide ions, altering or inhibiting normal protein activity. A source that can influence plasma pH is our diet. For instance, a high protein meal (metabolism of sulphur-containing proteins produces protons as a breakdown product) results in increase in acidity of plasma [2]. Plasma pH has to be tightly maintained between 7.36 and 7.44 [3], and small deviations lead to metabolic imbalances if not corrected. One consequence of metabolic imbalance is calcium wasting due to stimulated calcium release from the bones, with a final repercussion of bone depletion.

Several factors play a role in alterations of plasma pH. Decrease in plasma pH can be due to an increase in plasma carbon dioxide (CO_2) from hypoventilation (respiratory acidosis), generation of non-volatile acids (acids that can dissociate into anions and protons, such as phosphoric acid, sulphuric acid, lactic acid, and organic acids) from protein catabolism (metabolic acidosis), or loss of bicarbonate from diarrhea and in urine (metabolic acidosis). In contrast, increases in plasma pH can be due to hyperventilation

(respiratory alkalosis), metabolism of organic anions, loss of protons when vomiting, or loss of protons in urine (metabolic alkalosis for the latter three) [4].

Therefore, buffers (molecules that require large amounts of acids / bases to disturb its stable equilibrium between its acidic/basic form and its conjugate form) are essential in maintaining a constant extracellular and intracellular pH. The amount of acids / bases needed to disturb the buffer's equilibrium is reflective of its buffering capacity. The dissociation constant, pKa, is a good indication of the pH range at which the buffer functions best; buffers typically are most effective within one pH value of its pKa.

The major buffer in extracellular fluid is bicarbonate (HCO_3^-), while intracellular buffers include phosphates and proteins [4]. More than one type of buffering molecules are required, as they serve to compensate for each other when the buffering capacity of one becomes saturated. For example, when filtered bicarbonate has reached its buffering capacity, filtered monohydrogen phosphates will bind up extra protons in the lumen, and the product dihydrogen phosphate is excreted (Figure 1.1). In addition, phosphate stores from the bones can be redistributed via resorption (breakdown of bone to release minerals), to increase the buffering capacity of blood in times of dire need.

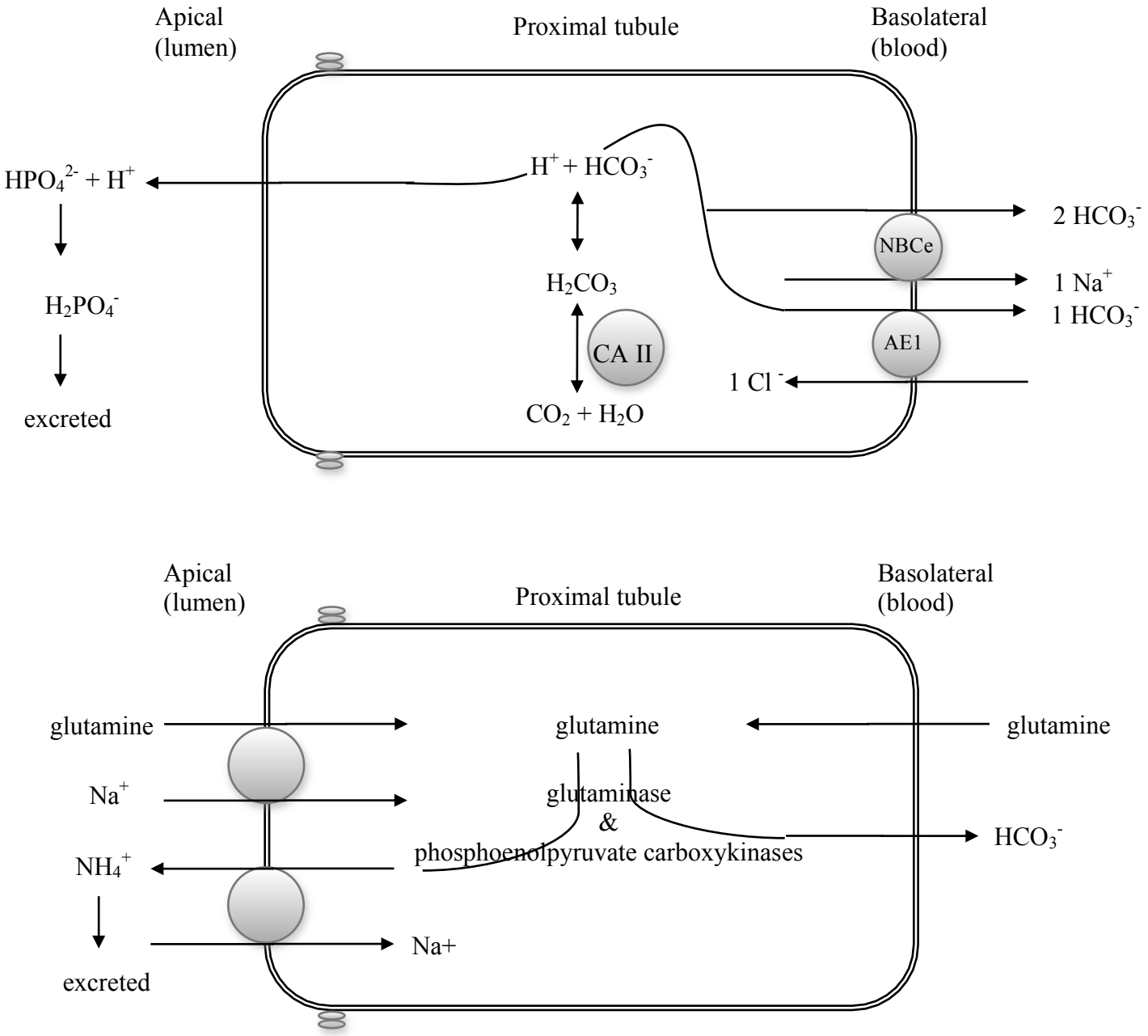

Figure 1.1 Schematic of excretion of phosphate and ammonium.

Phosphate and ammonium are excreted when buffering capacity of bicarbonate has reached its capacity. The double ovals close to the apical membrane (left) by the cell edges represent tight junctions.

The major extracellular buffer in our body is bicarbonate, an anion that binds the free acidic protons in our plasma, maintaining extracellular pH very close to 7.4 [4]. The buffering capacity of bicarbonate is illustrated by the following chemical reaction:

$$CO_{2\,(gas\ dissolved)} + H_2O \xrightarrow{A} H_2CO_3 \xrightarrow{B} HCO_3^- + H^+$$

This reaction occurs spontaneously, but the rate of reaction is greatly enhanced by carbonic anhydrase II (CA II) at step A. H_2CO_3 is an unstable intermediate, and not a predominant species found in our bodies. The forward reaction at step B has a pKa of 6.4. When plasma pH becomes more basic (alkalosis), the kidneys reabsorb less bicarbonate and newly synthesized bicarbonate can be excreted in the urine to restore plasma pH. Conversely, when plasma pH increases, more bicarbonate ions are generated in the kidney to buffer excess protons in the blood. In conjunction, secretion (movement of a molecule from the capillaries into the lumen) and excretion (expulsion of a substance from the lumen) of protons is increased, coupled to other buffers, like hydrogen phosphate, to result in a net gain of bicarbonate in the plasma to counter acidosis (Figure 1.1).

1.2 Role of respiratory and renal systems in pH regulation

There are two key organ systems that are involved in pH regulation – the respiratory system, and the renal system. The respiratory system is responsible for immediate response to changes in systemic pH (response rate is in the order of minutes), whereas the

kidneys are responsible for long-term maintenance of pH homeostasis (response rate is in the order of hours to days) [4]. Increased concentrations of plasma protons stimulate chemoreceptors of the respiratory system to increase smooth muscle contraction and ventilation, which then returns alveolar and arterial partial pressure of CO_2 and proton concentration to normal. In prolonged metabolic acidosis, increased excretion of protons and synthesis and reabsorption of bicarbonate (into the blood) is stimulated in the nephrons of the kidney to help counterbalance changes in acidity of the body. The urine acidity of a healthy individual alters in a way that is reflective of the changes in acidity of diet intake.

1.3 Bicarbonate transporters in the kidney

As bicarbonate is a charged molecule, its movement across the membrane from one compartment to another requires the help of transporters. The kidney's bicarbonate transporters belong to two major gene families – *SLC26A* and *SLC4A*. There are many ways of classifying these bicarbonate exchangers. In this thesis, we chose to classify them based on their electrogenicity (electrogenic and electroneutral transporters) which helps understand their role in changing the electrochemical gradient inside the cell, and the consequences this has on driving transport of other ions. Electrogenic transport results in a positive or negative net charge, whereas electroneutral transporters produce no net charge. For example, if a transporter carries 2 negatively charged ions into the cell while transporting 1 out of the cell, the net charge is negative 1 inside the cell. If it

transports one negatively charged ion in, while transporting one positively charged ion out, then no net charge on either side of the membrane is generated (electroneutral).

In the kidney, the electrogenic bicarbonate transporters include NBCe1 [5], SLC4A11 [6], SLC26A7 [7, 8], and SLC26A9 [8]. Electroneutral bicarbonate transporters in the kidney are AE1/2/4 [6, 9-12], NBCn1 [13-16], and SLC26A4/6 [17-20]. These different bicarbonate transporters are distributed at different locations along the kidney and some have not been very well studied. Although we have highlighted these bicarbonate transporters in the context of the kidney, it is important to realize that bicarbonate transporters are found in other parts of the body as well. For example, AE1 is found in the kidneys, erythrocytes and the heart [9]. AE2 is ubiquitous [10], and AE3 is found in the brain, heart, retina, pituitary, and the adrenal gland [21]. Since the topic of this thesis revolves around kAE1, the focus will remain on the kidney.

1.4 Bicarbonate handling in the nephron

Ion reabsorption and secretion is differentially regulated in different nephron segments (Figure 1.2). The reabsorption of bicarbonate demonstrates this point: 80 % of filtered bicarbonate is reabsorbed by the proximal tubules; 10 % by the thick ascending limb of the Loop of Henle; 10 % by distal tubules and the cortical collecting duct [4]. The distinction in ion handling is explained by a different distribution of regulatory proteins along the epithelial cells of the nephron, as described below.

1.4.1 Bicarbonate handling in the glomerulus

The glomerulus is the site where the blood gets filtered into the pro-urine. Here, the podocytes (special epithelial cells that help with the filtration selectivity) have low levels of kAE1 expression [22]. Although these exchangers are also capable of carrying out anion exchange, their predominant role here is likely to interact with other proteins, such as nephrin and to maintain the structure of the podocytes and slit diaphragms, which is essential for proper glomerular filtration [22].

1.4.2 Bicarbonate handling in the proximal tubule

The proximal tubule is where the bulk of the filtered substances are reabsorbed. Membrane proteins along the apical (the side of the cell that faces the lumen) and basolateral (the side of the cell that faces the interstitium and blood) membranes of the epithelial cells lining this portion of the nephron facilitate this reabsorption process (Figure 1.3). At the basolateral membrane, the sodium-potassium ATPase (Na^+ / K^+ ATPases; ratio of Na^+ : K^+ is 3 : 2, respectively) creates a net low sodium concentration inside the cell, thus creating the negative potential difference inside the cell [4]. This electrochemical gradient then becomes the driving force for many sodium-driven

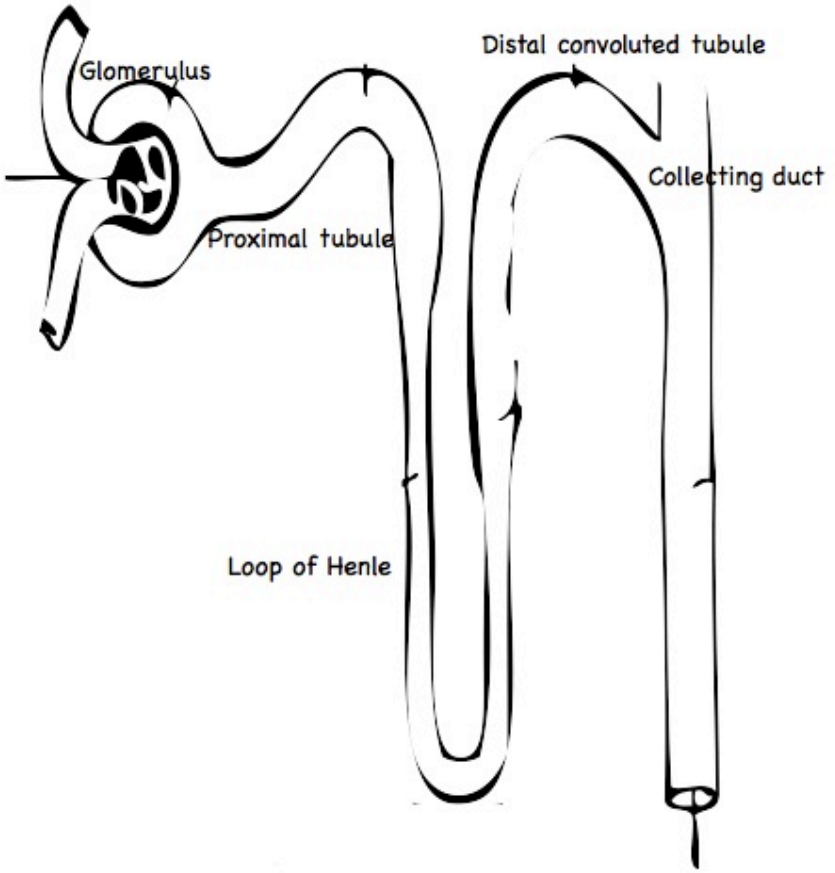

Figure 1.2 Schematic of the nephron.

Blood entering the nephron gets filtered at the glomerulus, then flows through to the proximal tubule, the thin descending limb of loop of Henle, the thin ascending limb of the Loop of Henle, the thick ascending limb of the loop of Henle, distal convoluted tubule, and finally the collecting duct to exit. The glomerulus, proximal tubule, and the distal convoluted tubule are found in the cortex of the kidney, while the loop of Henle and the collecting duct are found in the medulla.

transporters on the apical membrane of the proximal tubule. Sodium-proton exchanger 3 (NHE3) secretes protons to the luminal space, using this electrochemical gradient as driving force. The interplay between the apical NHEs and basolateral Na^+ / K^+ ATPases is crucial in driving these transport processes. Other than regulation of sodium and protons, a number of transporters are found along the apical membrane to facilitate reabsorption of filtered nutrients, such as phosphate, amino acids, glucose, water and other organic anions, usually coupled to sodium transport [4]. CA IV is found on the apical side of the proximal tubule epithelium along the brush border. It converts luminal bicarbonate and protons into water and CO_2. The CO_2 then diffuses into the epithelial cells, where CA II proteins mediate the conversion of CO_2 and water (which entered the cell via aquaporin-1 (AQP-1)) to bicarbonate and protons. An electrogenic sodium bicarbonate co-transporter on the basolateral membrane (NBCe1; transport ratio of sodium : bicarbonate is 1 : 2 [23] or 1 : 3 [24] respectively), and a less well characterized chloride-bicarbonate exchanger [25] complete the bicarbonate reabsorption process. Another important element of pH regulation that occurs in the proximal tubule is the secretion of ammonium. Filtered glutamine is converted to ammonium via glutaminase and phosphoenolpyruvate carboxykinases (Figure 1.1). The activity of these two enzymes is upregulated during chronic acidosis [26] to allow for buffering of acidic urine.

Patients with general dysfunction in the proximal tubule are diagnosed with Fanconi's syndrome [27]. The phenotypic defects could include the inappropriate excretion of

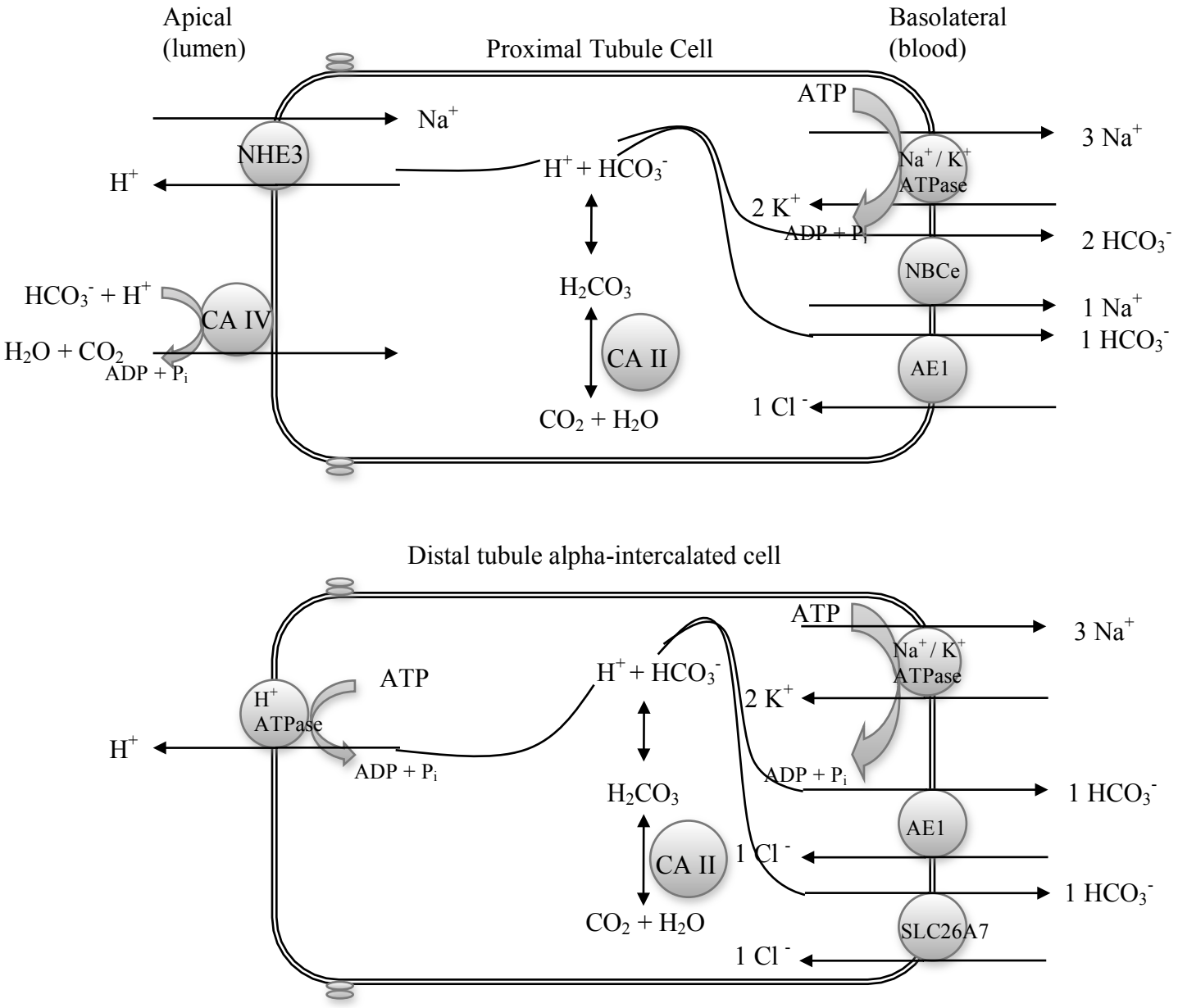

Figure 1.3 Proximal tubule epithelial cell and distal nephron alpha-intercalated cell (alpha-IC).

The major proteins involved in the process of bicarbonate reabsorption in these cells are depicted. The double ovals close to the apical membrane (left) by the cell edges represent tight junctions. CA IV is a peripheral protein on the brush border of proximal tubule cells, and not within the apical membrane itself.

amino acids (amino aciduria), calcium (calciuria leading to rickets), phosphates (phosphaturia), glucose (glucosuria), sodium (naturia), and small molecular weight proteins (proteinuria), as well as abnormally alkaline urine leading to proximal renal tubular acidosis (pRTA)(more details regarding pRTA are provided in Section 1.6.2).

1.4.3 Bicarbonate handling in the Loop of Henle

In the thick ascending limb of the loop of Henle, NBCn1's [28, 29], AE2's [30], and potassium chloride symporters [28, 31] all are on the basolateral membrane to facilitate bicarbonate reabsorption. The apical NHE3 and sodium potassium chloride co-transporter (NKCC2) help create the sodium gradient needed to drive many of the basolateral transporters to achieve bicarbonate reabsorption.

1.4.4 Bicarbonate handling in the distal nephron

The fine-tuning of ion reabsorption occurs in the distal regions of the nephron. This adaptive process is facilitated by the different proteins found in three diverse cell types of the distal convoluted tubule and collecting duct – principal cells, alpha-intercalated cells (alpha-IC's), and beta-intercalated cells (beta-IC's) [32]. Principal cells are important for their ability to alter the concentration of urine by changing the amount of water or sodium excreted, while IC's are important for achieving a balanced excretion of protons and bicarbonate in urine [3]. Since the focus of the thesis is on the importance of bicarbonate transport via the kAE1, only the intercalated cells will be detailed here (Figure 1.3).

IC's are thought to differentiate from the same parent cells, then acquire their respective functional properties [33]. The major role of the beta-IC's is to maintain a balanced plasma pH during metabolic alkalosis by secreting bicarbonate into the lumen via the apical Pendrin (Cl^- / HCO_3^- exchanger), and the basolateral H^+ ATPases to acidify the blood [33]. In contrast to beta-IC's, alpha IC's act oppositely, in that their main function is to reabsorb bicarbonate and pump protons to the luminal side of the nephron. Because of their opposing roles, alpha- and beta-IC's are upregulated during metabolic acidosis and alkalosis, respectively. Both alpha- and beta- IC's express high amounts of cytosolic CA II. [3], which helps facilitate a greater or lesser production of bicarbonate via conversion of CO_2 and water, depending on the rate at which bicarbonate is reabsorbed and protons are secreted via a feedback mechanism. In beta-IC's, the protons generated from CA II activity are shuttled to the blood via a H^+ ATPase on the basolateral membrane, while bicarbonate ions are excreted by the apical Cl^- / HCO_3^- exchanger [3]. In alpha-IC's, the protons generated from CA II activity are excreted into the lumen via a H^+ ATPase on the apical membrane, while the bicarbonate is reabsorbed via the basolateral Cl^- / HCO_3^- exchanger (kAE1) at a 1 : 1 ratio [3]. Intracellular Cl^- ions get shuttled out of the cell via chloride channels along the basolateral membrane of alpha-IC's. Na^+ / K^+ ATPases are also found on the basolateral membranes of alpha-IC's, facilitating reabsorption of sodium.

1.5 Anion exchanger 1

AE1 is encoded by the *SLC4A1* gene [34] and exists in 2 isoforms. In humans, two alleles encode AE1 on chromosome 17 [35] with a different promoter for the kidney and erythroid isoforms. The full length, 911 amino acid long AE1 [36, 37] is found in erythrocytes (RBCs; eAE1), whereas the product of an alternatively spliced, truncated isoform missing the first 65 amino acids is found in the kidneys (kAE1) [38, 39]. These isoforms share a common primary role – facilitating chloride-bicarbonate exchange – and similar structure with the exception of the N-terminal truncation in kAE1. Each e / kAE1 monomer has 12 – 14 transmembrane (TM) domains, intracellular amino (N) and carboxyl (C) terminal domains, and a single N-glycosylation site at asparagine (Asn) 642 position relative to the erythroid isoform [40-42]. Reconstitution of the membrane domain demonstrated that functional eAE1 was found in dimers [43, 44] and oligomers [45].

1.5.1 Erythroid AE1

The functional properties of eAE1 other than bicarbonate chloride exchange have been well studied. An important example is the role of eAE1 in respiration. The majority of CO_2 is carried throughout our body as bicarbonate. Since bicarbonate cannot freely diffuse into and out of RBCs, eAE1 is crucial for that process [46]. eAE1 also plays a great role in maintaining the structural integrity of RBCs.

Tetramerized eAE1 is capable of interacting with cytoskeletal components of RBCs including ankyrin, protein 4.1, 4.2, glycolytic enzymes G3P-dehydrogenaase, aldolase,

phosphofructokinase, tyrosine kinase $p72^{syk}$, and deoxyhemoglobin [47-53]. Alterations of these interactions makes RBCs fragile [54]. There is a wealth of information on eAE1 structure, function, and associated diseases, but since the focus of this thesis is on kAE1, only the key features of eAE1 relevant to the understanding of kAE1 will be discussed.

Mild proteolytic cleavage of eAE1 reveals two main domains: (1) a 43 kDa N-terminal domain (from Met 1 to Lys 360), important for membrane anchorage and maintaining RBC membrane rigidity [47]; (2) a 52 kDa C-terminal transmembrane domain (from Gly 361 to Val 911) important for anion transport [41, 55]. Residues 1 – 379 of the C-terminal domain is important for dimerization of AE1 [48]. Three tyrosine phosphorylation sites have been identified in eAE1 at amino acids 8, 359, and 904, and are suggested to stabilize its conformation and interactions with other proteins such as GAPDH [56, 57], while the transmembrane domain (residues 361 to 882) is responsible for Cl^- / HCO_3^- exchange [58]. Stilbene inhibitors, such as 4,4′-dinitro-2,2′-stilbene disulphonate (DNDS) and 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonic acid (H_2DIDS), compete directly for the active site of AE1 at lysine 539 and 851 [59, 60]. Because stilbene inhibitors bind to the active site of eAE1, their binding can be used to test if proper folding of e / kAE1 is maintained in mutants. However, it is important to keep in mind that this assay only looks at the folding of the active site, and not the entire protein. Therefore, it is more accurate to describe the assay as an estimation of the severity of misfolding of mutant proteins. The C-terminal tail of eAE1 (33 amino acids) contains acidic residues and short motifs that act as binding sites for chaperones and other

proteins. The L_{886} DADD motif of the C-terminus of eAE1 interacts with a basic histidine motif on the N-terminus of CA II [61, 62]. CA II tethers to the C-terminus of eAE1 forming a metabolon that effectively increases the rate of eAE1 anion exchange by directly providing the necessary substrate via conversion of water and CO_2 to bicarbonate and protons [63-65]. It is important to note that there has yet to be a good crystal structure resolved for eAE1, though crystals on various domains of eAE1 have been obtained and analyzed to generate the information above [43, 44, 66, 67].

1.5.2 Kidney AE1

kAE1 is an alternatively spliced [38, 39], truncated isoform of eAE1, missing the first 65 amino acids of the N-terminus [10, 39]. Like for eAE1, the cytosolic domain kAE1 is involved in the formation of stable dimers in solution [68]. Co-immunoprecipitation of differentially tagged kAE1 proteins in HEK 293 cells demonstrated that interaction of two kAE1 monomers form dimers [69, 70]. Also like for eAE1, localization and function of kAE1 is regulated by many different factors, detailed later. Unlike eAE1, kAE1 does not bind ankyrin or glycolytic enzymes as these interacting sites from eAE1 are deleted from kAE1 due to its truncated nature. However, the role of kAE1 in other interactions and functions in addition to anion exchange should not be ignored, as this is an area that has not been explored extensively yet. Thus far, studies have reported on the interaction of kAE1 with integrin-linked kinase [71], (ILK; a serine/threonine kinase that also interacts with actin), kanadaptin [72], nephrin [22], and adaptor protein 1 [73-75].

Controversy in some of these interactions remains to be elucidated. These interactions are further described below.

(1) Targeting of kAE1 is largely dependent on the phosphorylation state of the tyrosine residues at positions 359 in the N terminal domain and 904 in the C-terminal domain [73]. Phosphorylation of one or both of these residues allows for the regulation of the amount of kAE1 at the cell surface by stimulating internalization [73]. High concentrations of sodium bicarbonate in the extracellular medium of Madin Darby Canine Kidney (MDCK) cells expressing kAE1 caused an increased phosphorylation of these tyrosines [73] but failed to result in increased internalization of kAE1. This may be due to secondary effects of hypertonicity in inhibiting formation of clathrin coated pits, which was thought to facilitate endocytosis of proteins [76]. However, when the extracellular fluid of the outer medulla in mice became hypertonic due to induced alkalosis, there was a reduced level of kAE1 detected [77]. Altogether, these studies provide evidence that kAE1 internalization is regulated by tyrosine phosphorylation at two sites in the C-terminal tail when the extracellular fluid is alkaline due to metabolic alkalosis, and vice versa.

(2) GAPDH interacts with the N-terminal domain of eAE1 at D^{6}DYED and E^{19}EYED [47, 78, 79]. Recently, GAPDH was found to also interact with the D^{902}EYDE motif found on the C-terminus of kAE1 [57], forming a complex with kAE1 starting at the endoplasmic reticulum (ER). Correct localization of kAE1 to

the plasma membrane is dependent on this interaction, as knockdown of GAPDH resulted in intracellularly retained kAE1, though no changes in the total amount of kAE1 was observed. However, there remains yet another possibility, that GAPDH may negatively interfere with biogenesis and trafficking of other proteins that are vital for kAE1 trafficking to the membrane.

(3) The N-terminus of kAE1 interacts with the C-terminus of ILK, a serine, threonine kinase which enhances, but is not essential for, kAE1 trafficking to the plasma membrane in HEK 293 cells [71, 73]. This interaction allows kAE1 to indirectly interact with the actin cytoskeleton through the actin-binding property of ILK.

(4) kAE1 is expressed in the glomerulus at low levels [80] and helps maintain the structural integrity required for glomerular filtration [81]. Nephrin, an integral component of the podocyte slit diaphragm [81], has recently been found to complex kAE1, ILK and other proteins that are important for maintaining the structure of the glomerulus to prevent conditions such as albuminuria [82-84]. Co-expression of nephrin in *Xenopus* oocytes did not enhance chloride uptake by kAE1 [22], highlighting that in this part of the nephron, the role of kAE1 likely relies more on its structure than on its ion exchange activity.

(5) The kidney anion exchanger adaptor protein (kanadaptin) is a ubiquitous protein first identified to interact with mouse kAE1 from a yeast two hybrid assay

[72]. This study showed interaction of mouse kAE1 and kanadaptin in alpha-IC's, co-localizing at the intracellular vesicles and suspected to be a chaperone-like protein that helps with kAE1 trafficking to the cell surface. However, this interaction was not observed by others [85-87]. In fact, kanadaptin was found to have several nuclear localizing signals, localized to the nucleus, and no interaction or co-localization with kAE1 was observed [86, 87]. Furthermore, mouse kanadaptin cannot be found in the collecting duct in immunofluorescence experiments [86]. The role of kanadaptin as a chaperone for kAE1 trafficking remains controversial.

(6) Adaptor proteins mediate trafficking of membrane proteins to the correct location. Specifically, adaptor proteins 1A and B are found to interact with kAE1 to target its trafficking to the basolateral membrane [73-75]. They interact with the Y_{904}DEV motif at the C terminus of kAE1 [74] and lack of this interaction results in mistargetting of kAE1. Other interacting motifs between adaptor proteins and kAE1 are under investigation.

1.6 AE1 associated pathologies

Hereditary diseases caused by naturally occurring mutations of human AE1 arise from defects in both kidney and erythroid isoforms. Mutations at the same amino acid can lead to different consequences in RBCs and the kidney. This is due to presence of different

chaperones in the different cell type environments, and different roles of AE1 in addition to anion exchange.

For both isoforms, mutations can occur in one or both alleles independently, and can be inherited separately by descendants [88]. An individual with a different mutation in each allele is described as a compound heterozygote, while individuals with only one mutated copy of the allele are heterozygotes. Individuals with the same mutation in both alleles are homozygotes.

As both kidney and erythroid isoforms of AE1 are dimeric proteins, mutations can be categorized as dominantly and recessively inherited forms [88]. Dominant mutants negatively alter normal trafficking of WT e / kAE1 during co-expression, while recessive mutants are generally rescued partially in trafficking when co-expressed with WT e / kAE1 [88]. An individual with a single recessive mutation and a WT e / kAE1 typically display no disease symptoms, whereas individuals with a dominant mutation and a WT e / kAE1 display disease characteristics, although they are usually asymptomatic until adulthood. Individuals with two recessive e / kAE1 mutations display disease phenotype very early in life. The severity and type of the disease is dependent on the specific mutation an individual has.

1.6.1 eAE1 associated diseases

Mutations in eAE1 result in the diseases hereditary spherocytosis (HS) or Southeast Asian Ovalocytosis (SAO). HS is characterized by fragile RBCs leading to anemia while SAO is characterized by rigid and ovalocytic RBCs [89]. These two diseases are well characterized, but only the most relevant information pertaining to this thesis is discussed here. Patients with either of these conditions have RBCs that have a less rigid cytoskeleton, resulting in the RBC membrane collapsing onto intracellular organelles, and RBCs from these patients are recognized by the spleen and are degraded prematurely. These RBCs also have a net decrease in surface area, making them osmotically fragile. Interestingly, SAO, which is a deletion mutation in amino acids 400 to 408 of eAE1, results in a misfolded (no detectable binding DIDS or $[^3H]H_2$DIDS), nearly inactive (no detected sulphate transport activity), eAE1 protein that still reaches the cell surface [90-92]. This is one of the first examples in literature showing that misfolded proteins can still be targeted to the membrane.

1.6.2 kAE1 associated disease

Improper function of the nephron resulting in metabolic acidosis is called renal tubular acidosis (RTA) [93]. RTA caused by genetic mutations, also known as primary RTA, is the focus of this thesis. Earlier, the importance of two regions of the nephron for bicarbonate and proton regulation was elucidated in section 1.4.2 and 1.4.4 – the proximal tubule and distal nephron. As a consequence of their importance, defects in either region cause proximal (p) and distal (d) RTA, respectively.

The mode of inheritance and disease phenotypes are different for d / pRTA. While it is common for dRTA to be inherited as an autosomal dominant or recessive mutation, pRTA is usually autosomal recessive [93]. pRTA is mostly secondary to inability to reabsorb bicarbonate [94]. Patients suffering from pRTA have impaired urinary ammonium excretion after acid loading. Bicarbonate reabsorption is reduced, but the presence of distal bicarbonate regulation is usually sufficient in maintaining normal urine acidification in response to metabolic acidosis.

In contrast to pRTA, dRTA is characterized by the presence of metabolic acidosis, nephrocalcinosis, hypokalemia, and renal failure [95]. There is no further regulation of bicarbonate reabsorption or proton excretion at a site more distal than the distal convoluted tubule and collecting duct, and therefore malfunction in this region has more dire consequences than in the proximal tubule. Clinically, dRTA is diagnosed with the results of an ammonium chloride loading test [96]. Patients with dRTA cannot excrete the necessary levels of acid in response to high acid intake, and their urine cannot be acidified to pH $\leq$ 5.5 [93], whereas healthy individuals can reduce urine pH to as low as 4.2 in order to maintain a balanced metabolic pH [94]. These differences are also reflected when patients intake ingest large amounts of acids in their diet. dRTA is further classified into complete and incomplete dRTA. In incomplete dRTA, patients normally do not suffer from metabolic acidosis as they can maintain normal concentrations of bicarbonate in the blood. However, when challenged with a high load of acid, urine pH remains above 5.5. Patients with complete dRTA typically suffer from rickets or

osteomalacia in adulthood, as well as nephrocalcinosis and renal stones due to constitutive metabolic acidosis [93].

An interesting characteristic of some dRTA mutants is a mutation-associated defect causing these kAE1 to gain a non-selective cation transport capability [97, 98]. This was seen in R589H, G609R, S613F, S773P, delta850, A858D and G701D kAE1 mutants (refer to Figure 1.4 for the relative location of the mutations within the predicted secondary structure of kAE1) expressed in *Xenopus* oocytes. Of these, S773P, delta850, A858D, and G701D kAE1 had the greatest cation leak, which was still present upon co-expression with WT kAE1. It would be interesting to see if the morphology of the alpha-IC's change in the presence of these kAE1 mutants, as mutations with similar properties in eAE1 have also been described to cause a disease called hereditary stomatocytosis [99, 100], which is characterized by RBC swelling and lysis driven by an increased permeability and influx of cations [101].

Most cases of dRTA linked to kAE1 are the result of kAE1 trafficking mutants. Since kAE1 is not present at the cell surface, patients with dRTA cannot mediate buffering of acidic blood in the presence of systemic acidosis due to lack of bicarbonate reabsorption in the distal nephron. For example, the R589H kAE1 mutant was found to have functional activity in the erythroid isoform in RBCs [102], but the kidney isoform is retained in the ER and causes the kidney-associated disease dRTA in the patient (Figure 1.4 and Table 1.1). However, there are also mutations of kAE1 which retain normal trafficking, but have no functional activity. The S773P kAE1 mutant is correctly

localized to the basolateral membrane in polarized MDCK cells, but displays no functional activity, therefore this patient developed dRTA [103](Figure 1.4 and Table 1.1). The mode of inheritance for these mutations does not form a correlated pattern for localization and functional activity, as seen in Table 1.1. In fact, the number of dRTA mutation that result in mis-trafficking rather than misfolding is greater. Therefore, mis-trafficking mutants are the focus of this thesis.

1.7 Abolishment of AE1 function

Very few models of animals lacking AE1 have been developed because these animals do not have a high survival rate. Two murine animal models were described, one lacking eAe1 and the other lacking both eAe1 and kAe1 [104, 105] by using a nonsense mutation or alteration of the promoter site in or preceding the Ae1 gene respectively. The resulting mice displayed similar symptoms, as such, only one will be detailed here. Survival of homozygous knockouts was seen in only 39 of the 176 born, and 80 – 90 % of the 39 survive for only two weeks after birth. In addition, they were pale in appearance due to severe haemolytic anaemia, had retarded growth, splenomegaly, and spherocytosis. Proteins that usually form complexes with eAE1 in RBCs for structural support were absent or were decreased in levels compared to WT mice (25 % reduction of spectrin, 60 % reduction of ankyrin, and no detectable protein 4.2), but the relative abundance of these proteins were similar to WT mice, possibly the reason why homozygous eAe1 knockout mice still had normal membrane skeleton in RBCs. Heterozygous mice phenotype was undistinguishable from WT mice. Both models displayed similar phenotype in RBCs as described for the first study. In 2007, Wagner's group studied the

kidney related phenotype in the Ae1 null mice described previously. 12-week old surviving mice were analyzed. Typical symptoms of dRTA were observed (metabolic acidosis, hypercalciuria, hyperphosphaturia, hypocitraturia, inability to concentrate and acidify urine) [84].

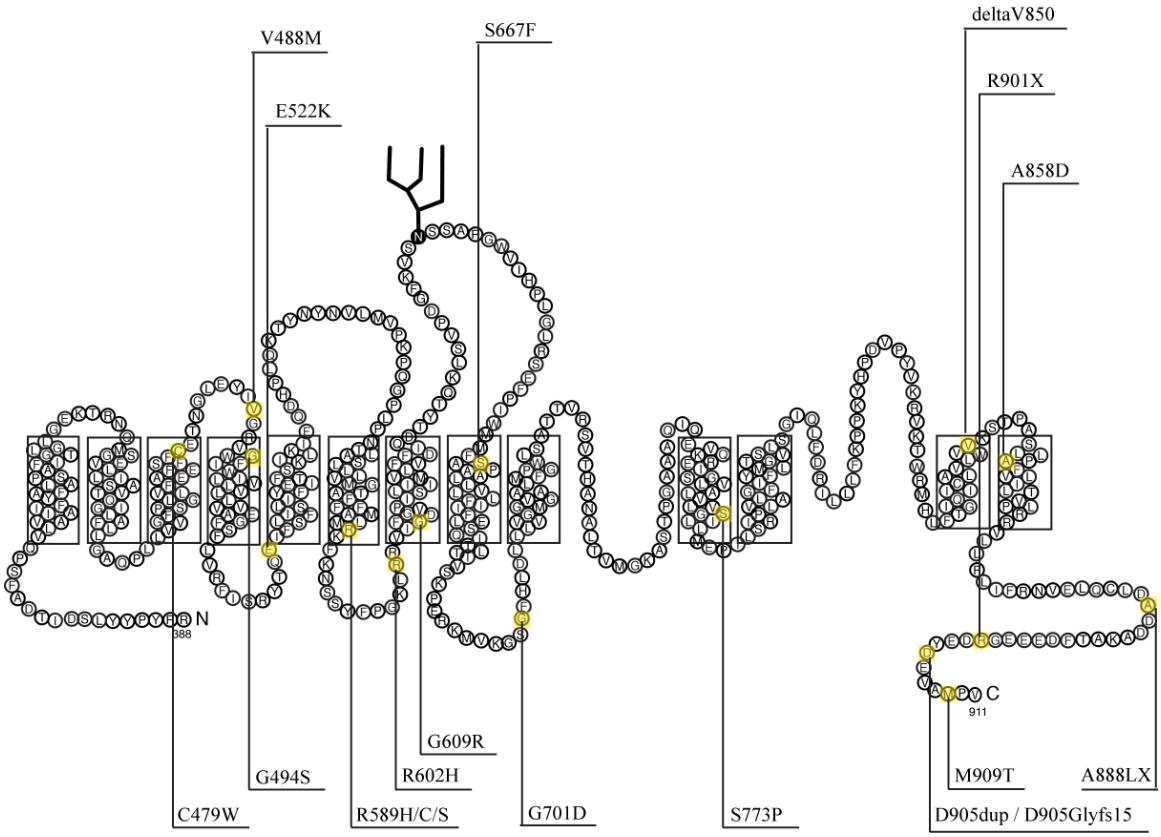

Figure 1.4 Topology model of kAE1 and mutations.[1]

Topology model with the relative positions of mutations causing dRTA. N-terminus is truncated and begins on amino acid 388. The N-glycosylation site is on asparagine 642 on the fourth extracellular loop.

[1] A modified figure from Bonar, P.T. and Casey, J.R. Plasma membrane Cl^- / HCO_3^- exchangers: structure, mechanism and physiology. 2008. Channels, **2**:51-59.

Table 1.1 Localization and modes of inheritance of characterized kAE1 mutations.

Mutation	Mode of inheritance for dRTA	Localization in MDCK cells[1]	References
R589H	Dominant	ER	[102]
R589C	Dominant	ER	[102]
R589S	Dominant	ER	[106]
G609R	Dominant	Apical and basolateral membranes	[107]
S613F	Dominant	ER	[102]
S667F	Dominant	ER	[108]
A858D	Dominant	Basolateral membrane	[92]
A888LX	Dominant	N/A	[109]
R901X	Dominant	Apical and basolateral membranes	[106]
D905Glyfs15	Dominant	N/A	[110]
D905dup	Dominant	N/A	[111]
M909T	Dominant	Apical and basolateral membranes	[112]
C479W	Recessive	ER	[113]
V488M	Recessive	ER	[114]
G494S	Recessive	N/A	[111]
E522K	Recessive	Basolateral membrane	[115]
R602H	Recessive	Basolateral membrane	[116]
G701D	Recessive	Golgi	[103]
S773P	Recessive	Basolateral membrane	[103]
deltaV850	Recessive	ER	[92]

[1] Detection method varies; please refer to original paper for full methods.

kAE1 at the basolateral membrane of alpha-IC's was confirmed to be downregulated compared to WT kAE1 using a functional assay monitored by 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) fluorescence (an intracellular pH reporter), in the presence or absence of DIDS. mRNA of kidney AE2, AE3, and AE4 were not upregulated, and the levels of pendrin were decreased. This was in agreement with these mice's inability to handle an acid load. AQP-2 was also mislocalized in these *slc4a*$^{-/-}$ mice, rendering inappropriate urine concentration and urine volumes that were two to three times higher than WT mice. Heterozygous *slc4a*$^{+/-}$ mice displayed WT phenotype, as shown previously.

Another model is in patients expressing the mutation V488M, or Band 3 Coimbra. V488M is located at the 4th transmembrane domain (Figure 1.3), which is a critical segment for proper insertion of the other kAE1 domains into the membrane [117]. Although this disruption suggests that kAE1 with this mutation is not properly inserted and therefore degraded, this is not the case as there is a detectable amount of V488M kAE1 in patients, shown below.

In 2000, a patient with homozygous V488M was characterized [118]. This patient was brought to the attention of Ribeiro *et al.*'s group from previous prenatal screenings of a Coimbra heterozygote mother. Her third pregnancy resulted in a female baby with edema, massive hepatosplenomegaly, RBC abnormal morphologies, and was immediately treated with blood transfusions, resuscitation, and ventilation. By age three, the patient had to be given oral sodium bicarbonate and monopotassium phosphate due to

hyperchloremic metabolic acidosis and nephrocalcinosis. mRNA analysis confirmed that these phenotypes were secondary to absence of eAE1, protein 4.2, reduced spectrin and GPA. The phenotypes observed in this patient is the best viable example of the resulting consequences in the absence of AE1 in humans. Indeed, mutations in this amino acid had dire effects, as seen in the phenotype of the heterozygous patient, which was characterized prior the homozygous patient in 1997 [114]. In the heterozygous state, these patients already suffered from mild HS and a 20 % decrease in eAE1 levels, and 26 %, 18 %, and 19 % decrease in protein 4.1, protein 4.2, and spectrin, respectively.

Knowing the consequences of downregulation and absence of AE1 function, it will be beneficial to find ways to restore this loss of function to improve patient conditions.

1.8 Thesis Objectives:

- To identify treatments that promote rescue of kAE1 trafficking mutants
- To identify degradation pathways involved in turnover of kAE1 trafficking mutants

2. Materials and Methods

2.1 Construction of AE1 mutants

Human kidney (kAE1) cDNA, containing the sequence encoding a hemagglutinin (HA) epitope in position 557 of the protein, the G701D kAE1 cDNA, and the R589H kAE1 cDNA cloned into the viral vector pFB-Neo (Stratagene) were previously described [119]. The addition of the HA tag was previously shown to have no effect on normal folding and function of kAE1 [119]. The kAE1-HA557 wild-type (WT) construct was submitted to site-directed mutagenesis using QuikChange site-directed mutagenesis kit (Stratagene). Using the QuikChange site-directed mutagenesis kit, we also introduced the C479W mutation in kAE1 devoid of HA epitope, subcloned in pcDNA3 vector (Invitrogen) [113, 119]. The presence of mutations was verified by automated DNA sequencing.

2.2 Transfections and viral infections

Viral infections were performed as previously described [120]. Briefly, HEK 293 cells were transfected with p-VPack-GP, p-VPack-VSV-G and pFB Neo kAE1-HA557 WT or mutant plasmids using FuGENE6™ (Roche Applied Science). Cell culture supernatant containing infectious viral particles were added to dividing MDCK cells complemented with 8 μg / ml hexadimethrine bromide (polybrene; Sigma). After 24 hours incubation, cells expressing AE1 were selected with 1 mg / ml geneticin. Despite the constant presence of geneticin in the cell culture medium, the level of kAE1 protein expression in MDCK cells progressively decreased within two to three weeks. Consequently, all our

experiments were performed within three weeks post-infection on a heterogeneous population of MDCK cells that have not been cloned.

2.3 SITS binding assay

MDCK cells expressing WT, G701D, C479W, and R589H kAE1 were grown to confluency in 6 well plates. Cells were lysed (see section 2.5) and normalized for total protein concentration using the BCA assay. A fraction of the total lysate was kept, labelled as the Total fraction, which provided a reference point of the total input. Another fraction of the lysate was incubated with 100 μM H_2DIDS for one hour at 4^{o}C in the absence of light. This fraction was then incubated with 50 μl of SITS beads in a 1x 228mM solution containing 0.1% triton for 20 minutes. Finally, a third fraction of the total lysate was incubated with SITS beads only. Samples were washed three times with PBS containing 0.1% triton and eluted with Laemmli's Sample Buffer and resolved on a 8% SDS-PAGE gel.

2.4 Treatments

For rescue treatment, confluent MDCK cells expressing the WT or mutant kAEI proteins were treated with the indicated glycerol, DMSO concentrations or incubated at 30^{0}C for 16 hours prior to lysate preparation, immunostaining and flow cytometry. Twenty-four hour incubations with 3μM of VX-809 (Selleck) at 37 ^{0}C were also used to treat cells expressing mutant kAE1. All treatments were supplemented to DMEM F12 containing 0.05 mg/ml penicillin/streptomycin, 10 % FBS and 1 mg/ml geneticin (Invitrogen). To

block protein synthesis, MDCK cells expressing WT or mutant kAE1 proteins were treated with 10μg/ml cycloheximide (CHX; Sigma) in DMEM F12 containing 0.05 mg/ml penicillin/streptomycin, 10 % FBS and 1 mg/ml geneticin. CHX treatment allowed us to study a specific pool of proteins inside the cell under various conditions. Incubation with 10 μM of epoxomicin (Cayman Chemical) was used for four hours to block proteasomal activity. 20 μM of leupeptin (Sigma) was used to inhibit lysosomal activity.

2.5 Lysate preparation and western blots

MDCK cells expressing kAEI WT or mutants were lysed with phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at pH 7.4) containing 1 % Triton X-100 and protease inhibitors leupeptin (Sigma), aprotinin (Sigma), pepstatin (Sigma), and PMSF (Sigma). Protein concentrations in cell lysates were quantified using BCA assay (using bovine serum albumin (BSA); Sigma). 5 – 15 mg (amount varies between experiments) of proteins were diluted in 2X Laemmli sample buffer (Bio-rad) and resolved on 8 % SDS-PAGE gels. Proteins were then transferred to nitrocellulose (Bio-rad) or PVDF (Bio-rad) membranes. The membranes were blocked with 3 % milk in TBST (5mM Tris base, 15mM NaCl, 0.1 % Tween-20), incubated in 1 % skim milk in TBST containing mouse anti-HA (Covance) primary antibody, followed by anti-mouse secondary antibody coupled to horseradish peroxidise (HRP). Probed proteins were detected with enhanced BM chemiluminescence Blotting Substrate (POD; Roche) or ECL Plus / Prime Western Blotting Detection System (Amersham, GE) on film (Kodak). Intensities of the bands from Western blots were quantified using ImageJ after

background was removed from quantification.

2.6 Immunocytochemistry

HEK 293 and MDCK cells expressing WT or mutant kAE1 were grown on glass coverslips or on semi-permeable Transwell polycarbonate filters (Corning Inc.), fixed with 4 % paraformaldehyde (Canemco Supplies) in PBS followed by incubation with 100 mM glycine in PBS (pH 8.5) to quench non-specific fluorescence. The kAE1 proteins located at the cell surface were detected with mouse (Covance) or rat (Roche) anti-HA primary antibody followed by either anti-mouse or anti-rat secondary antibody coupled to Cy3 fluorophore (Jackson Immunoresearch Laboratories). Cells were then permeabilized with 1 % Triton X-100 in PBS for ten minutes and incubated with mouse Bric155 antibody (IBGRL, Bristol, England) or a rabbit antibody detecting the last 15 residues of AE1 (gift from Dr. Reithmeier), followed by a mouse or rat anti-HA primary antibody followed by a secondary Alexa 488 antibody (Invitrogen Molecular Probes) to detect intracellular kAE1. 4'-6-Diamidino-2-phenylindole (DAPI) (Sigma) was used for staining the nucleus. Samples were visualized with a WaveFx Spinning Disk confocal microscope using the 60X oil immersion lens. Cell counts were done using multiple 20x frames taken from different experiments until approximately 200 cells were counted. Counts were solely based on presence of cell surface kAE1 in kAE1 expressing cells.

2.7 Immunoprecipitations

MDCK cells expressing kAE1-HA557 WT or mutants were lysed in PBS containing 1 % Triton X-100 and protease inhibitors. Aliquots of the cell lysates were saved as total fraction, the remaining cell lysates were incubated with rabbit anti-myc antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by Protein G-sepharose. The bound AE1 proteins were eluted with Laemmli's Sample Buffer before detection by western blot using a mouse anti-HA antibody (Covance).

2.8 Functional assay

MDCK cells expressing WT or mutant kAE1 were grown on 11 x 7.5 mm glass coverslips in 6 cm^2 dishes until 70 % confluent. Cells were treated with 1 % DMSO or 1 % glycerol at 37 ^{0}C or at 30 ^{0}C for 16 hours and VX-809 for 24 hours. The complete procedure has been described previously [121]. Briefly, the coverslips were rinsed three times with serum-free Optimem medium (Gibco), followed by incubation of 10 μM BCECF-AM (Sigma) for ten minutes at 37 ^{0}C or 30 ^{0}C for the temperature treatment. Coverslips were then placed in fluorescence cuvettes and cells were perfused with: Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM magnesium sulphate, 10 mM Hepes, 2.5 mM NaH_2PO_4 , 25 mM $NaHCO_3$) containing 140 mM chloride, then replaced with chloride free medium containing 140 mM gluconate to induce intracellular alkalization. BCECF intracellular fluorescence was calibrated with buffers at pH 6.5, 7.5, or 7.0 containing 100 μM nigericin sodium salt (Sigma). The Ringer's buffers are continuously bubbled with air : CO_2 (19:1). A Photon Technologies International (PTI) (London, Ontario, Canada) fluorimeter was used to read

the fluorescence fluctuations produced from the samples. Excitation wavelengths 440 and 490 nm and emission wavelength 510 nm were used (calibrated to the fluorimeter). Transport rates of the cells were determined by linear regression of the initial H^+ flux (first 30 seconds), normalized to pH calibration measurements. All measurements were done using the Felix software.

2.9 Amplex red

To quantify the amount of kAE1 at the cell surface, we used the reagent Amplex UltraRed (Invitrogen) [122-124] which binds to horseradish peroxidase (HRP) to give fluorescence or colorimetric signal that is proportional to the amount of HRP bound. For our purposes, we chose to detect via fluorescence. A convenient property of Amplex UltraRed is its impermeability to the membrane, which allows us to quantify the amount of kAE1 at the cell surface.

A number of modifications to the experimental procedure have been done, but to no avail. To start, cells were grown on 24 well plates to 60 %, 70 %, 80 % or 90 % confluency on the day of the experiment. 16 hours prior to the experiment, the cells were treated with 1 % DMSO, 1 % glycerol, 30^0C, or kept at control conditions. At the start of the experiment, cells were kept on ice (for the entire experiment) and fixed with 4 % paraformaldehyde, then cell surface kAE1 was probed with a primary antibody against the HA tag (Covance), followed by a secondary antibody conjugated to HRP. Antibody incubations were done in fetal bovine serum-containing-DMEM to block non-specific antibody binding, and have been optimized for concentrations that gave the highest signal

to noise ratio in WT expressing cells. A separate blocking step using fetal bovine serum-containing-DMEM did not give significant difference in results. Different blocking and antibody incubation solutions, such as 1 % BSA in PBS and goat serum, have also been tested, but the best results were obtained with fetal bovine serum-containing-DMEM. Between the primary and secondary antibodies, five washes with PBS containing 1mM MgCl and 0.1 mM CaCl (PBS^{++}) was done, and ten washes between secondary antibody and addition of Amplex UltraRed reagent was done, to prevent false amplication of signal due to reaction of Amplex UltraRed to unbound secondary HRP antibody. The fluorescence readouts for WT or mutant kAE1 were corrected for background noise by subtracting the value from non-transfected MDCK negative control cells and transfected MDCK cells incubated with secondary antibody only. Readings were made using the Synergy Mix Monochromato-based Multi-Mode Microplate reader (BioTek).

2.10 Flow cytometry

Cells expressing WT or mutant kAE1 were either treated with small molecules or incubated at low temperature for 16 hours before experiment. Cells were detached by trypsinization, fixed with 4 % paraformaldehyde, blocked and labeled with mouse anti-HA antibody for cell surface kAE1, followed by an Alexa 488 secondary antibody for fluorescence detection using FACsCalibur. WT or mutant kAE1 cells that displayed average fluorescence higher than untransfected cells for each treatment condition correspond to cells where kAE1 was rescued to the cell surface. In this pool of cells, an increase of average fluorescence of WT or mutant kAE1 cells after treatment compared

with untreated condition indicates that the amount of kAE1 at the surface of these positive cells was increased by treatment. Unpaired T-tests were conducted to measure statistical significance.

3. Functional rescue of a kidney anion exchanger 1 trafficking mutant in renal epithelial cells[1]

[1] This chapter is a modified version of a paper submitted for publication in PLOS One. All experiments shown in the figures of this chapter were conducted by Carmen Chu. Other authors of this paper are: Jennifer King, R who provided technical assistance in some experiments,. Todd Alexander for providing assistance for the functional assay and Emmanuelle Cordat who supervised the work and wrote the manuscript.

3.1 Introduction

Distal renal tubular acidosis (dRTA) is a dominantly or recessively inherited renal disease characterized by development of metabolic acidosis, inability to acidify urine, hypokalemia, nephrocalcinosis, and eventually renal failure if untreated [125]. Previous studies have identified several intracellularly retained but otherwise functional kAE1 mutants that cause dRTA [124, 126]. Up to 16 mutations in the *SLC4A1* gene have been identified to cause dRTA [115, 127]. Seven out of the eight eAE1 mutants isolated from dRTA patients' red blood cells displayed at least 50 % of WT levels of sulphate influx, supporting that the major hindrance of these mutants is trafficking, not misfolding [120]. Furthermore, these mutants are functional in *Xenopus* oocytes, but they are almost consistently retained intracellularly in renal epithelial cells.

In particular, this chapter focuses on the restoration of correct localization of two such mutants – G701D and R589H kAE1. A recently discovered, functionally inactive mutant, C479W kAE1 [113], serves as a negative control.

3.1.1 Rescue of trafficking mutants

Trafficking mutants causing severe diseases are not exclusive to the AE1 protein. Investigations on rescuing trafficking of several mutant membrane proteins are more mature than for kAE1, and therefore serve as an important source of inspiration for my studies in rescuing kAE1 trafficking mutants.

One of the most developed areas of study with regards to trafficking rescue is for the deltaF508 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) mutant. CFTR is a large glycoprotein, found on the apical membrane of epithelial cells in the lungs, pancreas, intestine, liver, sweat glands, and reproductive tract [128, 129]. It has 1480 amino acids, 12 transmembrane helices, and the whole protein can be separated into two repeated motifs linked by a cytoplasmic regulatory domain with phosphorylation sites [128]. It functions as a cyclic adenosine monophosphate (cAMP)-regulated chloride channel and is also permeable to bicarbonate ions (permeability reaching 25 % that of chloride ions) [70], suggesting that CFTR participates in a large fraction of bicarbonate secretion in the pancreas and intestine, two organs where bicarbonate secretion is key for normal physiology. Mutation of the gene encoding CFTR causes cystic fibrosis (CF), which is characterized by excessive absorption of sodium chloride as a result of faulty chloride secretion in the lungs, leading to a vulnerable epithelium that has a reduced mucosal layer, decreased mucosal clearance, an increased susceptibility to bacterial infections and subsequent inflammation and damage [130, 131]. Ninety percent of all CF patients have one or more deletions at the phenylalanine at position 508 of CFTR [126, 132].

Deletion of F508 in CFTR results in a partially functional protein that is retained in the ER [126]. Up to 100 % (the value varies depending on the cell line that deltaF508 CFTR is expressed in) of newly synthesized deltaF508 CFTR becomes degraded because the folding of the mutated protein is kinetically unfavorable for normal interactions with chaperones which assist CFTR maturation [133]. A study on rectal biopsies of 51 CF

patients showed that 21 patients had residual chloride secretion (measured by outward current changes in response to carbachol) that is about 30 times lower than non-CF patients; four of which were homozygous for deltaF508 [134, 135]. The presence of these partially functional deltaF508 proteins at the cell surface suggests that these proteins are not grossly misfolded but are normally prematurely degraded [136].

In studies of deltaF508 and WT CFTR expressed in model epithelial cell lines, incubations with chemical chaperones (such as glycerol, phenylbutyrate, or dimethyl sulfoxide) or at lower temperatures (30^{o}C or less) successfully rescued trafficking of deltaF508 CFTR [137-140]. This provides a proof of principle in that partly misfolded CFTR can be rescued to the membrane. In fact, deltaF508 CFTR rescued with many of these conditions have functional activity at the membrane [137, 141].

In addition to CFTR, chemical rescue of trafficking mutants in other proteins have also been well studied with promising results. A number of vasopressin receptor (V2P) mutants, such as V206D, S167T, L44P, and del62-64, are retained intracellularly in the ER. Since this receptor is crucial for binding of arginine-vasopressin (which induces the cAMP cascade to increase levels of AQP-2 at the membrane), mutations in this protein leads to nephrogenic diabetes insipidus (NDI) due to inability to concentrate urine. V206D V2R expressed in MDCK cells are normally ER retained, but expression at the cell surface increased in 70 % of the cells counted after treatment with 4 % glycerol or 1 % DMSO for 16 hours [142]. In another study, intracellularly retained mutants A98P V2R and L274P V2R are rescued to the cell surface when treated with 100 mM

trimethylamine-methyl-N-oxide (TMAO), 2 % DMSO or 26^{o}C for six hours [143]. Another example is AQP2 – an important water channel that is found in the principal cells of mammalian kidneys and in the epithelial cells along the inner medullary collecting duct. Mutations in AQP2 also lead to NDI [144-146]. Mutant T126M AQP2 is retained in the ER [147, 148], but was rescued to the membrane by 2 %DMSO and 0.75 %TMAO for four hours, which are relatively low concentrations compared to the 1M of glycerol that was needed to see the same rescue effect [147]. The effectiveness of these treatments lead to increases in transcription, translation, proper trafficking, and / or protein function, but not necessarily improvements in all these processes. For instance, Robben *et al*. found that 1 % TMAO for two hours increased synthesis of V206D V2R, but did not enhance its trafficking [142].

Based on the knowledge gathered from chemical treatment studies, another area of interest has evolved – the search for correctors, that is, small molecules which, when used as a treatment, will improve the trafficking and function of mislocalized mutants. For this thesis, small molecules are defined as small (<800 Daltons), organic compounds that potentially have rescuing effects for the target protein. For example, 4-phenylbutyrate, curcumin, and 4-cyclohexyloxy-2-{1-[4-(4-methoxybenzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline (VRT-325) all provide partial restoration of trafficking of deltaF508 CFTR [140, 149]. However, the extent of rescue and effectiveness of these drugs varies between different studies and different cell lines. For example, curcumin has been shown to correct trafficking defects of deltaF508 CFTR [149, 150] and functional activity of correctly targeted CFTR mutants by irreversibly increasing the open probability of the

channel via a covalent modification [49, 151], but others have shown evidence that counter the rescuing properties of curcumin for mutant proteins [152].

VX-809 is a drug that improves trafficking and function of deltaF508 CFTR, discovered recently from a 164 000 small molecule screen [153]. In Fischer Rat Thyroid (FRT) cells, VX-809 increased deltaF508 CFTR chloride transport by five times. In HEK 293 cells, VX-809 was able to increase ER exit of deltaF508 CFTR six times to reach 34 % of WT CFTR ER exit levels. Furthermore, 28 % of the rescued deltaF508 CFTR trapped in the ER by Brefeldin A was resistant to degradation. In human bronchial epithelial cells, VX-809 treatment increased deltaF508 CFTR maturation eight times and enhanced chloride transport four times (~14 % of WT levels). Even more encouragingly, in a recent clinical trial where CF patients with homozygous deltaF508 CFTR were given 200 mg of VX-809 per day for 28 days, increase in CFTR transport activity was seen [154]. Since several kAE1 mutants are also functional but trapped in intracellular compartments, the ability of this drug to enhance organelle exit of mutated proteins begs for an attempt to test the effects of this drug on kAE1 mutant rescue.

3.1.2 kAE1 mutants studied in this chapter

As detailed below, much is known about two of the mutants studied in this chapter – R589H and G701D kAE1, as these were the first characterized dominant and recessive kAE1 mutations, respectively. Table 3.1 summarizes the current knowledge for these mutations. The third mutant, C479W kAE1, was recently characterized [113] but is

functionally inactive and therefore serves as a negative control in the experiments described in this chapter.

3.1.2.1 R589H kAE1

A point mutation from R589 to H589 (R589H) was the first reported autosomal dominant kAE1 mutation [102, 106, 155] (Figure 3.1). This mutation is located on transmembrane domain six and this particular amino acid has been proposed to be a mutational hotspot because two other mutations were found at the same amino acid in different patients – R589C and R589S [116]. All three variants of R589 kAE1 mutations were dominantly inherited and all retained intracellularly when expressed in HEK 293 cells [69]. R589H kAE1 exhibited normal transport activity in the patients' RBCs [102, 155] and *Xenopus* oocytes [155, 156], and normal half-life and expression levels in HEK 293 cells [69], comparable to WT kAE1 levels. R589H kAE1 was retained in the ER when expressed in HEK 293 and MDCK cells [120], regardless of polarization of the cell. Co-expression with WT kAE1 led to retention of the WT kAE1 protein inside MDCK and HEK 293 cells and both R589H and WT kAE1 were trapped intracellularly. Interestingly, when the single glycosylation site of kAE1 was mutated from an asparagine to an aspartate (N642D), R589H kAE1 was capable of getting to the membrane, as did another ER-retained dominant mutant [157]. This suggests that interactions with the oligosaccharide chain of kAE1 may be another mechanism to prevent mutated kAE1 from reaching the cell surface and that perhaps other sites of intrinsic regulatory signals exist within kAE1 and remain to be discovered. This potential glycosylation-dependent retention regulation is likely specific to the kidney isoform of AE1 and unnecessary in eAE1, as R589H eAE1

Table 3.1 kAE1 mutations studied in this thesis.

kAE1	WT	G701D (TM 9)	R589H (TM 6)	C479W (TM 3)
Mode of inheritance for dRTA	N/A	Recessive [103]	Dominant [155]	Recessive [113]
Functional activity (% of WT) in:				
***Xenopus* oocytes**	Yes	Yes (with GPA) (100 %) [103]	Yes (50 – 80 %) [155]	No
RBCs	Yes	No (intracellular)	Yes (80 %) [155]	No
Localization in polarized MDCK cells	Basolateral membrane	Golgi	ER	ER
SITS binding	100%	63 % of WT [158]	75% of WT [69]	N/A
Mutants co-expressed in heterozygous patients		G701D [103] delta400-408 [92, 159] S773P [116] E522K [115] C479W [113] A858D [160] G494S [111]	WT [155]	G701D [113]

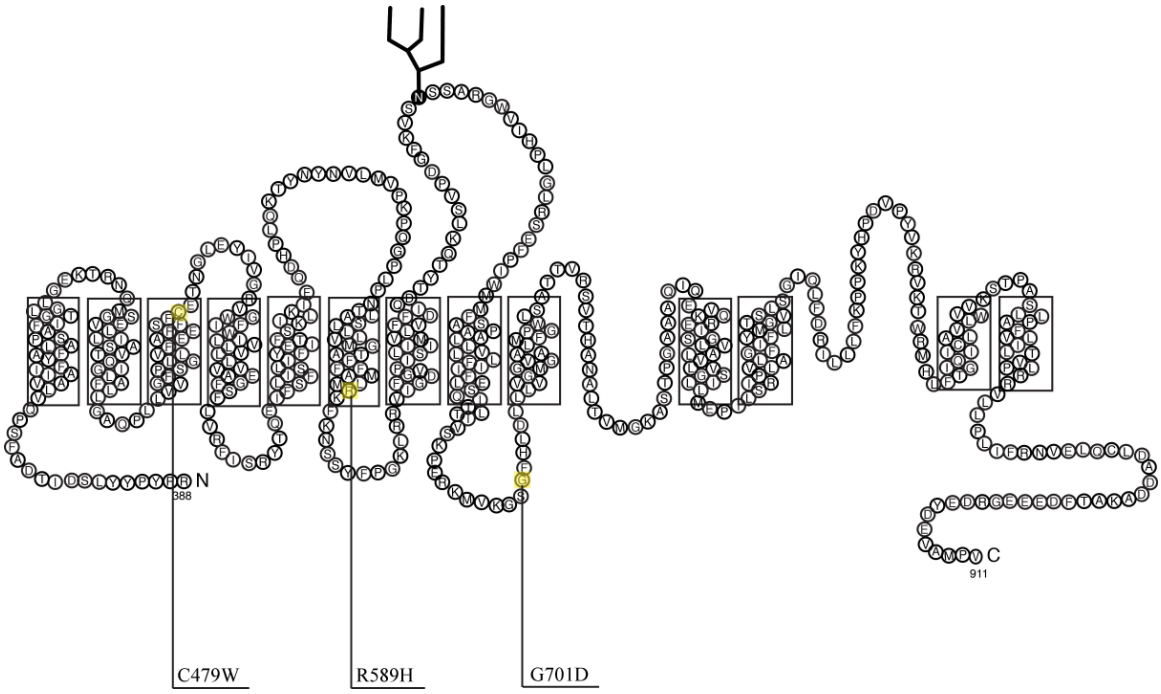

Figure 3.1 Topology model of kAE1 and the localization of mutants studied in this thesis[1]

[1] A modified figure from Bonar, P.T. and Casey, J.R. Plasma membrane Cl^- / HCO_3^- exchangers: structure, mechanism and physiology. 2008. Channels, **2**:51-59.

expressed in HEK 293 cells reached the cell surface [69] without any additional mutation at the glycosylation site.

3.1.2.2 G701D kAE1

Point mutation at amino acid 701 (G701D) of kAE1 was the first recessive mutation identified [103]. It was first characterized in a homozygous state [103], and subsequently found in several other patients, heterozygous with SAO kAE1 [92], S773P kAE1 [158], and E522K kAE1 [115]. The patient with both S773P and G701D mutations suffered from dRTA, rickets, and bilateral nephrocalcinosis. Both parents were asymptomatic – the mother has a single G701D mutation; the father carries a single S773P mutation. The patient with the double mutations of E522K and G701D suffered from dRTA and severe HS.

The G701D mutation is located at transmembrane domain nine [103] (Figure 3.1). It is retained intracellularly in the Golgi of MDCK cells and LLC-PK1 cells – a porcine kidney epithelial cell line [158]. However, this mutant is not grossly misfolded, as it retains 63 ± 11 % of WT kAE1's ability to bind to SITS beads, which targets the active site of kAE1 [158]. When co-expressed with GPA in *Xenopus* oocytes, G701D kAE1 reaches the cell surface [103]. GPA is found in RBCs and promotes trafficking of eAE1 to the membrane [49], but is not found in renal alpha-IC's. The capacity for this mutant to get further along in the biogenesis pathway than other mutated proteins (typically retained in the ER), together with its ability to carry out some level of chloride

bicarbonate exchange when localized at the membrane, makes it a particularly attractive target in trafficking rescue studies.

3.1.2.3 C479W kAE1

The C479W kAE1 mutation was discovered from a patient in Edmonton who was diagnosed with HS shortly after birth, and later with distal renal tubular acidosis at age 2.5 [113]. After a splenectomy and starting daily oral intake of sodium bicarbonate and potassium chloride supplements, her condition had improved. In 2008, we were introduced to the patient and successfully characterized the mutations responsible for her disease phenotype – C479 --> W in one allele, and G701 --> D in the other allele encoding the gene for AE1 (Figure 3.1). This was an exciting moment as she was the first patient to have the C479W mutation, thereafter named Band 3 Edmonton 1, and it was the first time the G701D mutation is found from a patient of non-Asian background [113].

The C479W mutation is a recessive mutation for dRTA (Table 3.1), as genotyping of her parents showed that the father was a heterozygote with C479W and WT kAE1. He suffers from a mild form of HS, but displays no renal symptoms. C479W was retained intracellularly at the ER in HEK cells, as well as in non-polarized and polarized MDCK cells. When injected into *Xenopus* oocytes, C479W kAE1 is detected at the cell surface, but has no functional activity. Co-immunoprecipitations show that C479W, G701D, and WT kAE1 can all interact with each other, but the ability of C479W kAE1 to reach the cell surface is not seen even when dimerized with WT kAE1. Conclusively, C479W

kAE1 mutant is mislocalized and functionally inactive, and serves as the negative control in these experiments.

3.1.3 Rescue of kAE1

Rescue of kAE1 is an area that has not been well explored. One study that focused on the effect of the glycosylation state on mutant trafficking [157] brushed upon a couple of treatment conditions which improved mutant kAE1 trafficking. They showed that MAL 3 (a small molecule modulator of Hsc 70), C3, and C4 rescued trafficking of R589H and R901X (missing the last 11 amino acids) kAE1 to the membrane, but not G701D kAE1 [157].

3.2 Results

3.2.1 Trafficking of G701D and R589H mutants can be rescued by chemical or low temperature incubations

In this study, we examined whether trafficking of one dominant (R589H) and two recessive (C479W and G701D) dRTA mutants of kAE1 could be restored to the plasma membrane by treating cells with DMSO, glycerol, low temperature or with the pharmacological chaperone VX-809.

We studied the trafficking of WT and mutant kAE1 by expressing them in MDCK cells. By quantifying the amount of kAE1 detectable after incubation with the protein synthesis inhibitor CHX, we have determined the half-lives of WT, G701D, R589H and C479W kAE1 to be twenty-three hours, four hours, one hour, and five hours respectively, in MDCK cells (Figure 3.2 and 3.3). These numbers are in good agreement with previously published half-lives measured for the same mutants in HEK 293 and MDCK cells [157]. We opted to incubate cells for 16 hours with each treatment or low temperature incubations as it encompasses the half-lives of all the mutants and preliminary results showed that treatment effects were evident at this timepoint.

Subsequent to treatment, WT and mutant kAE1 proteins were examined by immunofluorescence in the cells. Since the hemaglutinin (HA) epitope is located in the

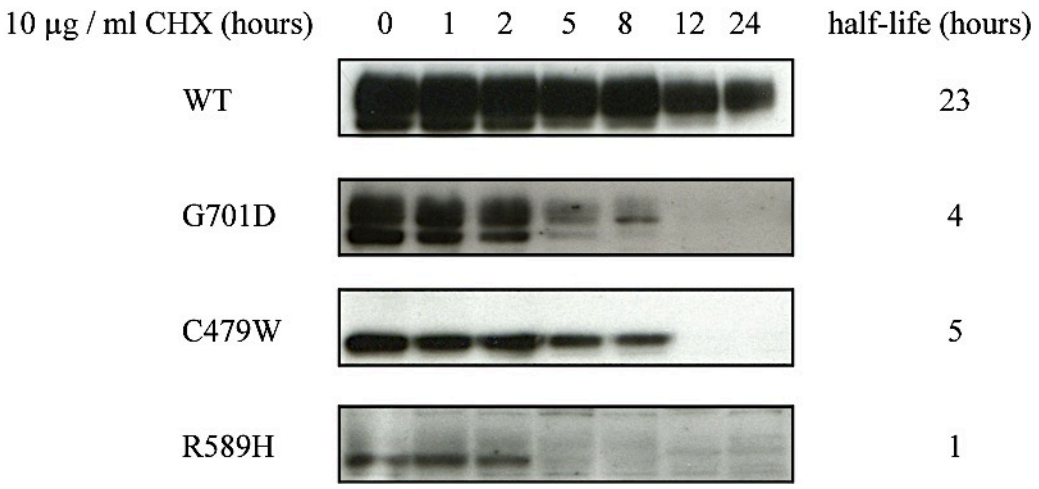

Figure 3.2 Half-lives of WT, G701D, C479W, and R589H kAE1.

Cells were treated with 10 µg / ml CHX for 0, 1, 2, 5, 8, 12 and 24 hours at 37^{o}C. Lysates were obtained from cells following these treatments and proteins were resolved on Western blots and half-lives were calculated based on degradation curves (Figure 3.3) produced from the densitometry measurements using ImageJ.

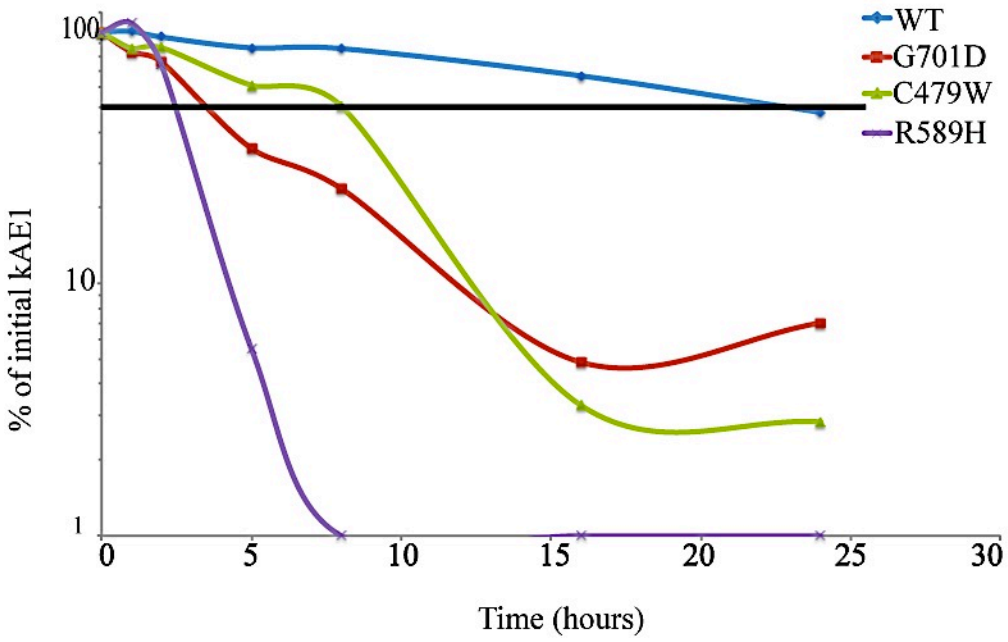

Figure 3.3 Degradation curves for WT, G701D, C470W, and R589H kAE1 mutants in MDCK cells.

Degradation curves were built based on densitometry measurements of each band at each time point of the cycloheximide experiment, measured using ImageJ. WT kAE1 followed a linear degradation pattern, while the mutants followed exponential decay. n = 1 experiment.

third extracellular loop for detection [119], we performed a two-step immunostaining prior and after permeabilization on polarized cells (see methods for details) (Figure 3.4). Red staining corresponds to kAE1 present at the plasma membrane while green staining shows total kAE1. Overlay images are shown for both the X-Y and X-Z planes. As observed on Figure 3.4 and 3.5, in comparison with WT kAE1, which was predominantly located at the plasma membrane in every condition, the G701D kAE1 mutant could be detected at the cell surface following incubations with either glycerol, DMSO or after low temperature incubation. Trafficking of the R589H kAE1 mutant was also minimally rescued by glycerol and low temperature incubation and function was corrected to some extent after low temperature or glycerol incubations, although not in a statistically significant way. No improvement in trafficking was seen for the misfolded C479W kAE1 proteins after any treatment. These findings indicate that chemical treatments or low temperature incubations can rescue trafficking of G701D or R589H kAE1 mutants to the basolateral membrane.

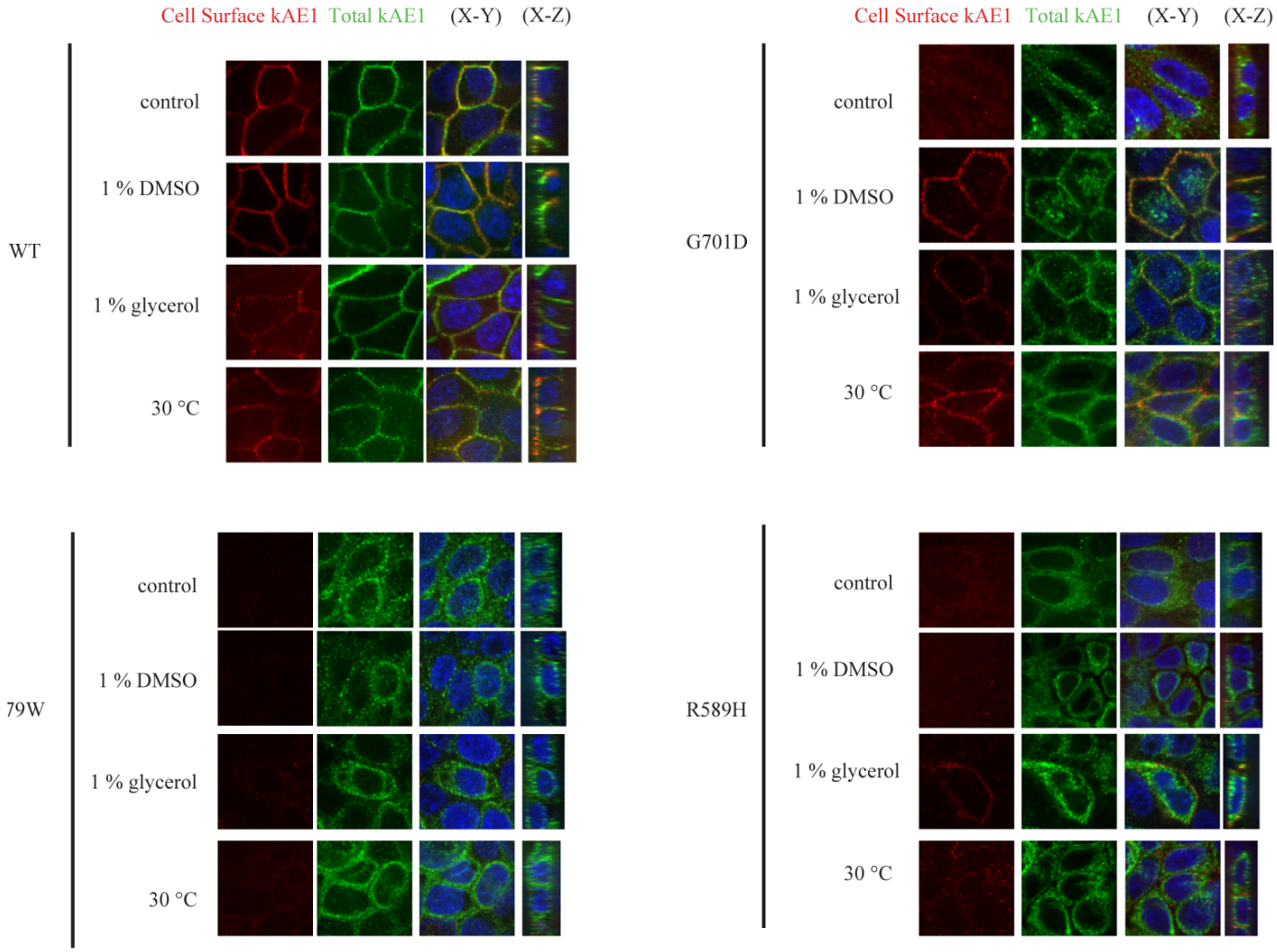

Figure 3.4 Low temperature incubations and chemical chaperones can partially rescue trafficking of mildly misfolded dRTA mutants.

Polarized MDCK cells expressing WT or mutant kAE1 treated in control conditions or with 1 % DMSO or 1 % glycerol for 16 hours were grown on glass coverslips, fixed, blocked and stained with a mouse anti-HA antibody followed by anti-mouse antibody coupled to Cy3 (red) fluorophore. Cells were then permeabilized, blocked and incubated with rat anti-HA followed by anti-rat antibody coupled to Alexa 488 fluorophore (green). Yellow staining corresponds to colocalization between green and red staining, seen in the overlay panels in X-Y and X-Z view. Bar represents 10 µm. The samples were examined with a spinning disk microscope using a 60 X oil immersion lens. Images are representative of 3 independent experiments.

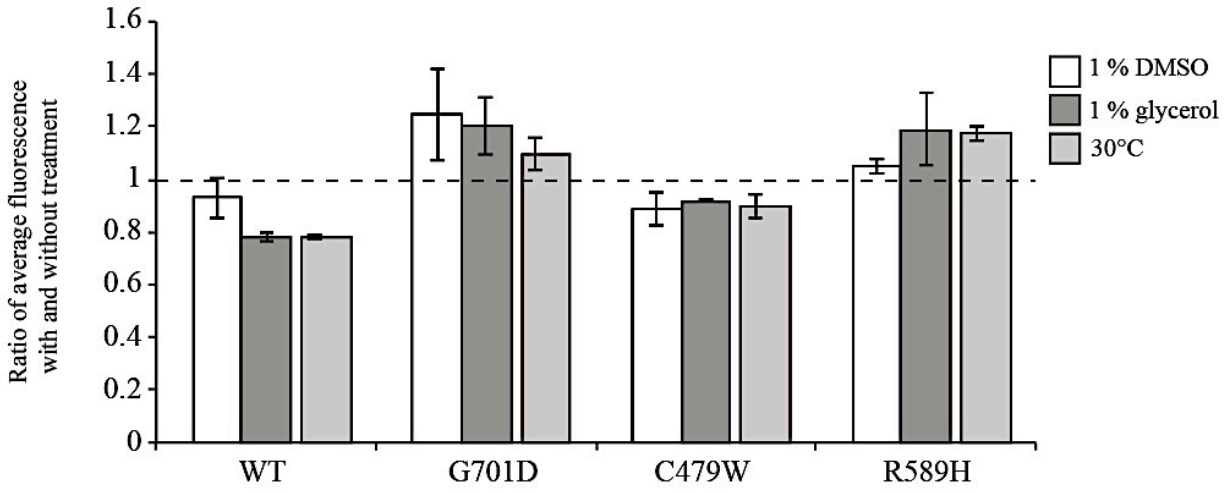

Figure 3.5 Low temperature incubations and chemical chaperones increase the level of mutant kAE1 expression at the membrane.

Cells expressing WT or mutants kAE1 treated for 16 hours in control conditions or with DMSO, glycerol or low temperature were trypsin digested, fixed, blocked and incubated with a primary mouse anti-HA antibody followed by a secondary antibody coupled to Alexa488. The cells were examined with a FACsCalibur flow cytometer. Values are mean ± SEM; n = 3 independent experiments.

To confirm our previous findings and to obtain quantitative measurements, we attempted to use cell surface biotinylation, however, the biotinylation reagent systematically leaked inside the cells despite reducing incubation time and temperature. Therefore, we instead used flow cytometry to detect surface expression of kAE1 on cells in control conditions, after treatment or low temperature incubations. We wondered if the increase in the number of cells with plasma membrane kAE1 was accompanied by an increase in the amount of kAE1 present within the plasma membrane relative to untreated cells. Treated or untreated cells expressing WT, G701D, C479W or R589H kAE1 mutants were fixed and stained using the HA antibody followed by a fluorescent secondary antibody. In parallel, we performed the same staining on permeabilized cells to confirm the total expression of the protein. If treatments resulted in an increased presence of kAE1 at the cell surface, we expected to detect a greater average fluorescence on non-permeabilized, treated cells relative to untreated cells. The relative increase of cell surface kAE1 was then calculated as the ratio of the average fluorescence in treated versus non-treated conditions for each mutant (Figure 3.4). We observed that the average fluorescence for G701D kAE1 mutant was increased by 24 ± 17 % (n = 3, ± SEM), 20 ± 11 % (n = 3, ± SEM)and 9 ± 6 % (n = 3, ± SEM), respectively, after DMSO, glycerol or low temperature incubation compared with G701D kAE1 in control conditions. Similarly, the average fluorescence of R589H kAE1 mutant increased by 5 ± 3 % (n = 3, ± SEM), 19 ± 14 % (n = 3, ± SEM)and 17 ± 3 % (n = 3, ± SEM), after DMSO, glycerol and low temperature incubations, respectively. None of the treatments or low temperature incubation resulted in changes of average fluorescence of the misfolded C479W kAE1 mutant. These data indicate that the amount of kAE1 present at the cell surface increases after chemical

treatment or low temperature incubation.

3.2.2 DMSO treatment partially rescues the functional activity of G701D kAE1 mutant

As the chemical treatments with DMSO and glycerol or low temperature incubations all rescued cell surface trafficking of the G701D kAE1 and R589H kAE1 mutants, we next asked whether the rescued mutants were functional at the cell surface. We carried out a functional assay using the ratiometric fluorescence–based pH sensitive dye BCECF-AM by following the initial rate of intracellular pH increase when cells pre-loaded with BCECF-AM in presence of chloride were perfused with a chloride free solution in presence of extracellular bicarbonate [121]. In these conditions, if kAE1 is functional and at the plasma membrane, entry of bicarbonate through kAE1 leads to an increased intracellular pH and subsequently augment the intracellular BCECF fluorescence. The bicarbonate-chloride transport rate in the initial 30 seconds of activity was measured and compared to non-treated conditions. Figure 3.6 shows that although all treatments improved G701D kAE1 trafficking, DMSO incubation was the only condition that showed a significant improvement in function at the plasma membrane. Low temperature incubation increased the average transport function of the R589H kAE1 mutant although not in a statistically significant way. To test whether the difference in functional rescue between G701D and R589H kAE1 was a result of changes in folding after DMSO treatment, we tested folding of the active site using SITS and H_2DIDS binding (Figure 3.7). Both mutants were able to bind to SITS beads to the same extent before and after

DMSO treatment. After pre-incubation with H_2DIDS, neither G701D nor R589H kAE1 were able to bind to SITS. These results indicate that rescued trafficking of the mutant

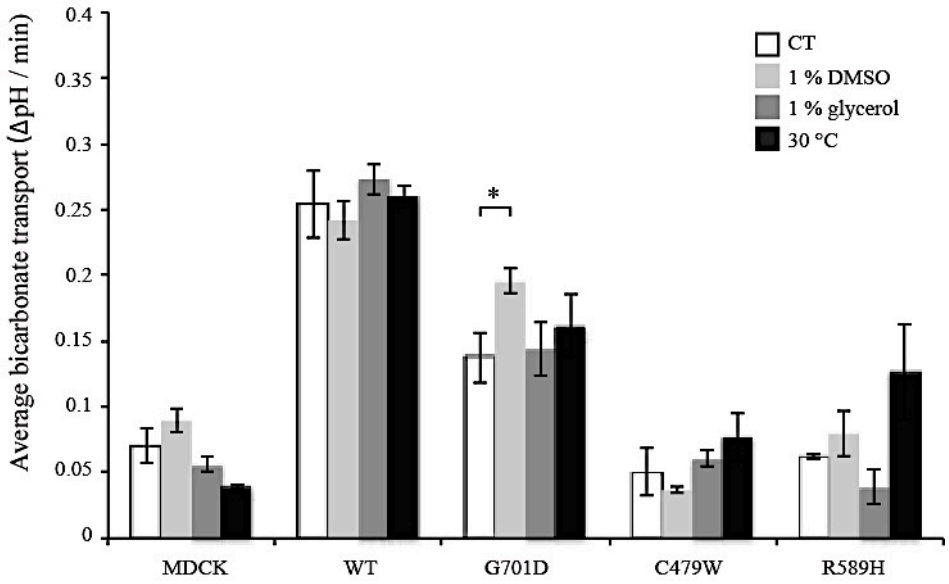

Figure 3.6 DMSO can functionally rescue G701D kAE1.

MDCK cells expressing WT or mutants kAE1 were grown on glass coverslips and kept in control conditions or treated for 16 hours with DMSO, glycerol or low temperature. After incubation with 10 μM of pH sensitive fluorescent probe BCECF-AM, coverslips were placed in a PTI cuvette and perfused with Ringer's solution containing sodium chloride. The buffer was then switched to a chloride-free Ringer's solution and the increase of BCECF fluorescence was recorded. After completion of the experiment, the BCECF fluorescence was calibrated using high potassium buffers containing nigericin at three different pHs. Nigericin allowed for equilibration of intracellular and extracellular proton concentration during this calibration step. Based on the standard curves built from the fluorescent levels of the pH buffers, the average change in pH units / minute in the first 30 seconds of activity was calculated for each mutant and WT with or without treatment. Values are mean ± SEM; n = 3 independent experiments. Asterisk indicates statistically significant difference with control condition ($P<0.05$).

proteins by several chemical treatments or low temperature incubation does not necessarily correlate to a detectable restoration of function at the plasma membrane. Thus, not all the treatments are equivalent in fully reverting the detrimental effect of the mutation.

3.2.3 The pharmacological corrector VX-809 also rescues G701D kAE1 mutant trafficking

The recently identified pharmacological corrector VX-809 (Figure 3.9) restored trafficking and function of the most common Cystic Fibrosis Transmembrane conductance Regulator (CFTR) mutant –deltaF508 – to levels where phenotypic improvement is seen in CF patients [153]. We wondered whether such corrector would, like DMSO, have a positive effect on kAE1 mutants' processing and folding. As previously reported, in MDCK cells, kAE1 is present as two main sub-populations on immunoblots: a high mannose-carrying sub-population present in the ER (low molecular band) and a complex glycosylated sub-population that has reached the medial Golgi (high molecular weight band) [161]. We determined that 3 μM is the smallest concentration of VX-809 that resulted in noticeable changes in protein amounts detected on Western blot. (Figure 3.9). Immunofluorescence experiments indicated that VX-809 rescued trafficking of the G701D kAE1 mutant to the basolateral membrane but had no effect on the C479W or R589H kAE1 mutants (Figure 3.9). Thus, VX-809 mimicked the rescuing effect of

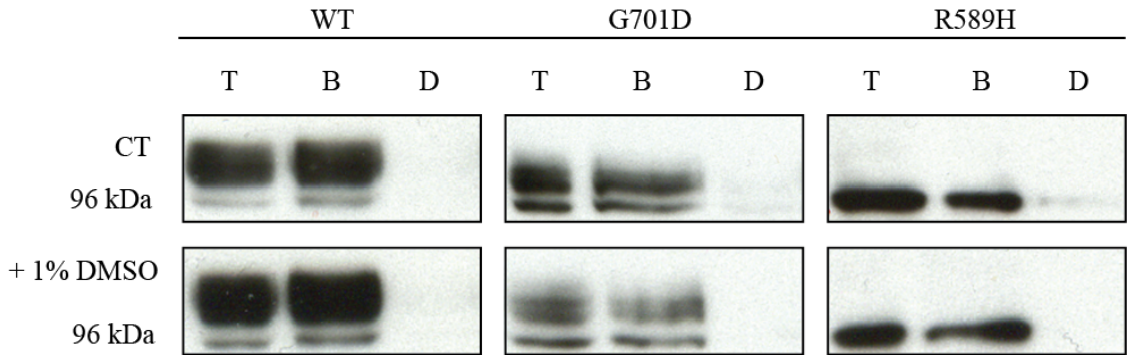

Figure 3.7 Folding of the active site for DMSO treated G701D and R589H kAE1 is not altered compared to control conditions.

Lysates containing WT, G701D, and R589H kAE1 were tested for their ability to bind to SITS beads in the presence (D lanes) or absence (B lanes) of the competitive inhibitor H_2DIDS. T lanes are the input total lysates. n = 1 experiment.

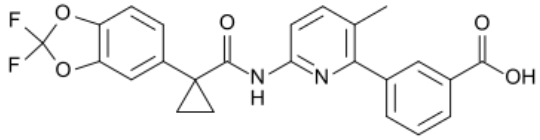

Figure 3.8 Structure of VX-809.
From Selleckchem.com products page. http://www.selleckchem.com/products/VX-809.html

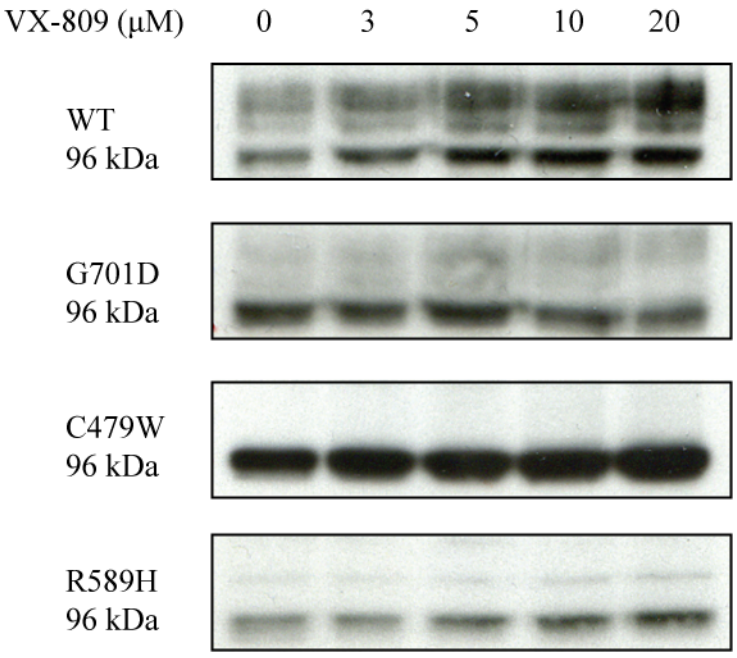

Figure 3.9 Dose optimization of VX-809 on WT and mutant kAE1.

MDCK cells expressing WT or mutant kAE1 were treated with increasing dose of VX-809 in the concentrations of 0, 3, 5, 10, and 20 μM over 16 hours. n = 4 independent experiments. Protein concentrations were normalized with actin.

low temperature incubations or chemical treatment on kAE1 processing to the cell surface. However, functional assays demonstrated that rather than improving the function of the rescued G701D kAE1 at the plasma membrane, VX-809 induced a decrease in activity of this mutant (Figure 3.11). Together, Figures 3.4, 3.5, 3.6, 3.7, 3.10 and 3.11 demonstrate that trafficking of mildly misfolded dRTA mutants can be rescued, however, not all the treatment have the ability to restore proper folding and function of the mutant.

3.2.4 DMSO incubation increases new synthesis of G701D and R589H kAE1 proteins

We next investigated the mechanism by which DMSO and VX-809 acted on kAE1 trafficking and cell surface processing. We performed an immunoblot using cell lysates collected from MDCK cells expressing WT or G701D kAE1 mutant that were either untreated or incubated with DMSO or VX-809 (Figure 3.12 A, lanes 1 - 2 & 5 - 6). When cells were treated for four hours with either DMSO or VX-809, density comparison of the immunoblot bands revealed that the overall amount of G701D kAE1 was increased by 41 and 38 %, respectively, while the amount of WT kAE1 was not greatly changed.

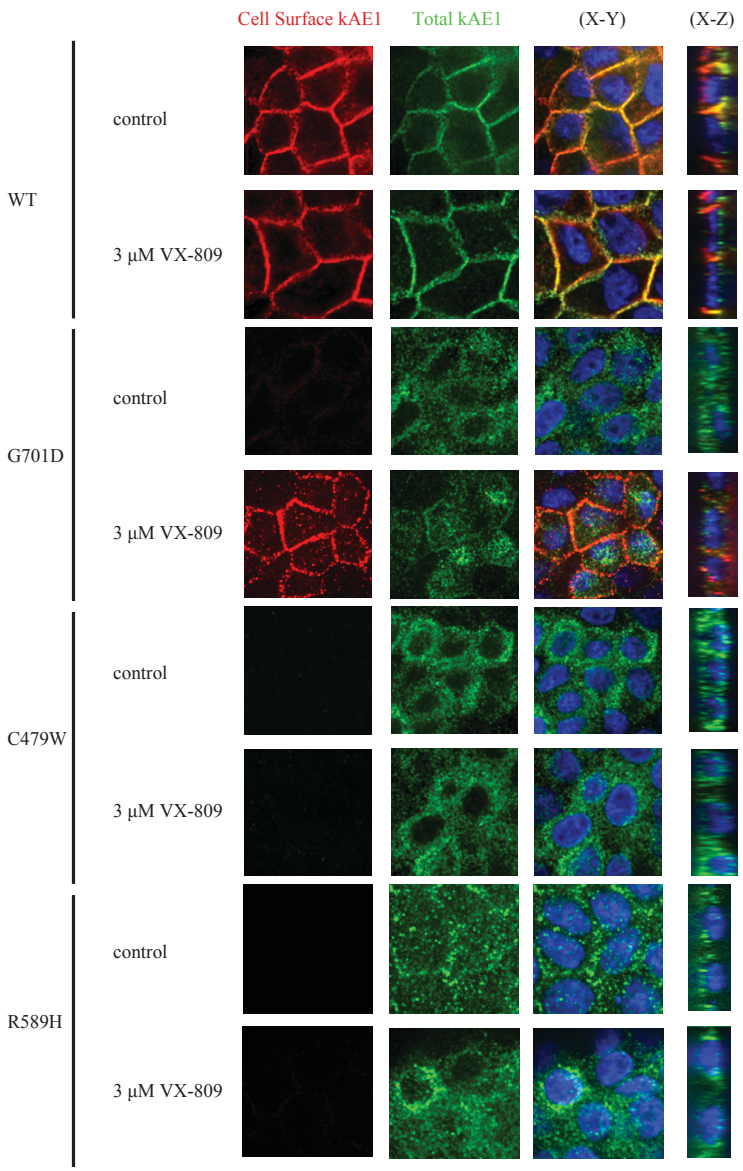

Figure 3.10 The molecular chaperone VX-809 rescues trafficking of G701D kAE1.

MDCK cells expressing WT or mutants kAE1 were either kept in control conditions or treated with 3 µM of the molecular chaperone VX-809 for 24 hours prior to fixation. We then performed a two-step immunostaining as follows: cells were blocked and stained with a mouse anti-HA antibody followed by staining with an Alexa 488 secondary antibody. Cells were then permeabilized and after blocking, stained again with a mouse anti-HA antibody followed by a Cy3 secondary antibody. The samples were examined with a WaveFx spinning disk using a 60 X oil immersion lens. Bar corresponds to 10 µm. Representative of 4 independent experiments.

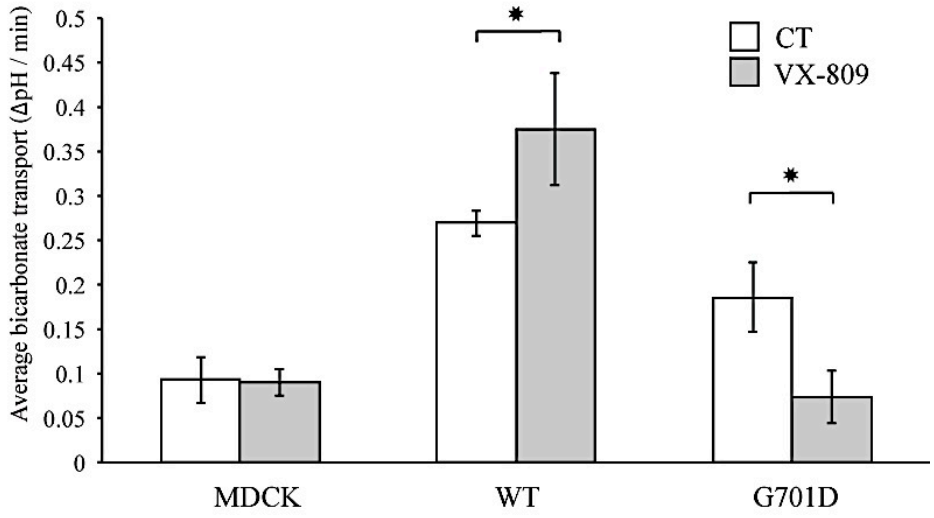

Figure 3.11 The molecular chaperone VX-809 does not improve functional activity of G701D kAE1.

MDCK cells expressing WT or G701D kAE1 were grown on glass coverslips and either kept in control conditions or treated for 16 hours with 3 μM VX-809. After incubation with 10 μM of pH sensitive fluorescent probe BCECF-AM, coverslips were placed in a PTI cuvette and flowed with a bubbled Ringer's solution containing sodium chloride. The buffer was then switched to a chloride-free Ringer's solution and the increase of BCECF fluorescence was recorded. After completion of the experiment, the BCECF fluorescence was calibrated using high potassium buffers containing nigericin at three different pHs. The variation of pH units per min was calculated for each mutant in control or treated conditions based on the intracellular pH values calculated using fluorescence intensities from the calibration buffers. Values are mean ± SEM; * = $p \leq 0.05$; n = 3 independent experiments.

Three possible mechanisms could result in increased kAE1 in treated cells: the treatment either increased protein synthesis, decreased protein degradation or acted on both. To discriminate between these hypotheses, we treated cells expressing WT or G701D kAE1 mutant either in control conditions, with CHX that inhibits protein synthesis [162], with CHX and DMSO or with DMSO only over four hours (Figure 3.12). We expected that if DMSO acts by increasing kAE1 synthesis, protein synthesis inhibition using CHX would cancel the rescuing effect of DMSO, and levels of kAE1 protein would be comparable to cells treated with CHX only. Therefore, the intensity of kAE1 bands in "DMSO + CHX" condition (lanes 3 and 7) should be similar to the one of kAE1 in "CHX" conditions (lanes 4 and 8). As seen in Figure 3.12, in lanes 7 and 8, after four hours incubation with DMSO and CHX, G701D kAE1 mutant was detected at a similar or lower amount than in cells treated with CHX only. This result indicates that chemical inhibition of protein synthesis completely reversed the effect of DMSO on kAE1 amount. Similar observations were made in cells treated with VX-809 (Figure 3.12, lower panels). These results suggest that both DMSO and VX-809 act by increasing the steady-state amount of kAE1 via promotion of kAE1 protein synthesis.

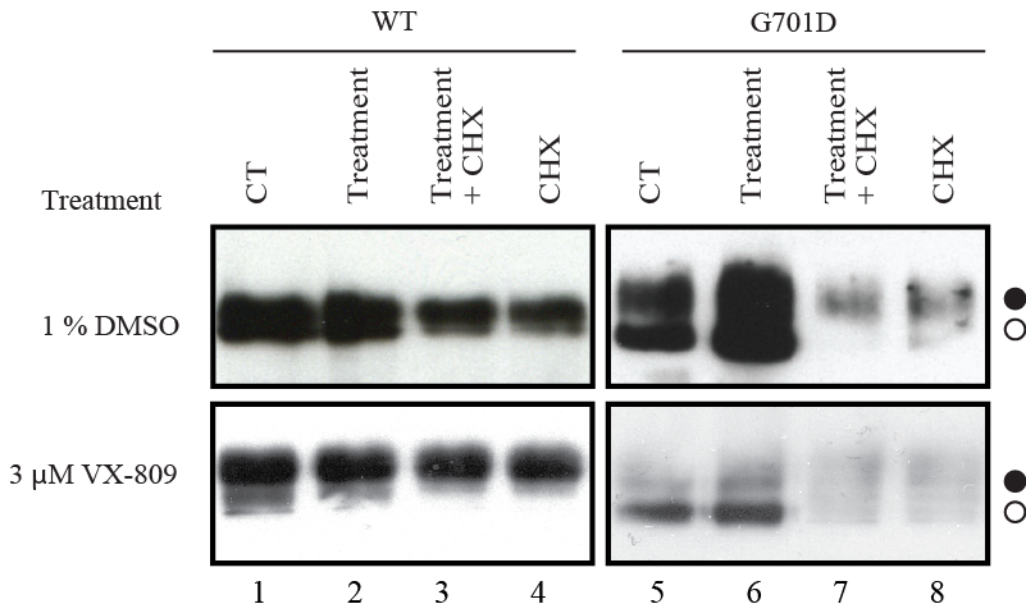

Figure 3.12 Both DMSO and VX-809 increase synthesis of the G701D kAE1.

MDCK cells expressing WT or mutants kAE1 were either kept in control conditions or treated for four hours with CHX (10 µg / ml), 1 % DMSO (top panels) or 3 µM VX-809 (bottom panels) in presence or absence of CHX prior to lysis. After protein concentration measurement, proteins (DMSO blots – 9.38 µg ; VX-809 blots – 6.48 µg) were resolved by western-blot and detected using a mouse anti-HA antibody followed by an anti-mouse antibody coupled to HRP. Filled circles correspond to kAE1 proteins carrying complex oligosaccharide, open circles indicate kAE1 proteins carrying high mannose oligosaccharide.

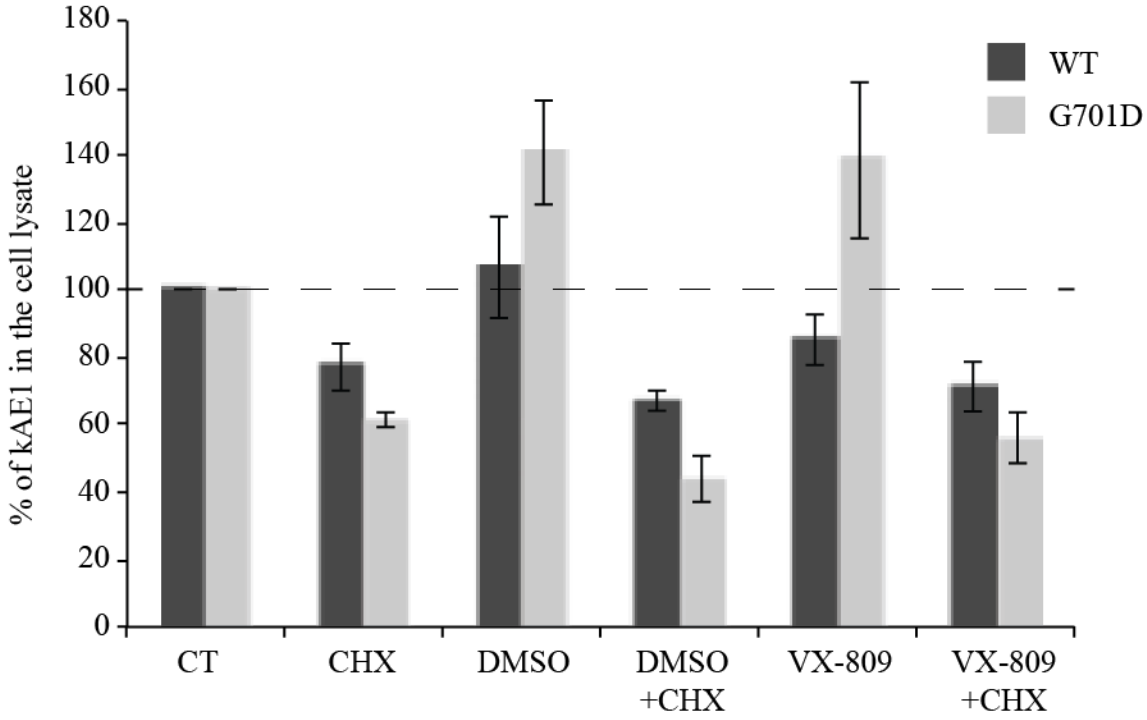

Figure 3.13 Histogram showing the effect of DMSO and VX-809 on G701D kAE1 synthesis.

Histogram showing the percentage of WT or G701D kAE1 proteins present in lysates from cells kept in control conditions or treated as indicated. Values are mean ± SEM; n = 3 independent experiments.

3.3 Discussion

In the present work, we determined whether trafficking to the cell surface and function of C479W, R589H or G701D dRTA kAE1 mutants could be rescued after treatment with chemical chaperones (DMSO or glycerol), one pharmacological agent (VX-809) or low temperature incubations. All the treatments partially rescued cell surface trafficking of the G701D kAE1 mutant, while a more modest rescue of the R589H kAE1 mutant was observed after low temperature incubation, DMSO or glycerol treatment. Our main finding is that DMSO treated cells expressing G701D kAE1 showed significant increase of functional activity after treatment. Despite trafficking rescue of the R589H dRTA mutant to the cell surface after glycerol, DMSO or low temperature treatment, there was no improvement in its functional activity. The inactive C479W dRTA mutant was not rescued by any of the treatments tested here. These findings indicate that (i) trafficking and function of the mildly misfolded G701D kAE1 dRTA mutant have the potential to be rescued; (ii) trafficking of the R589H mutant can be rescued but a corrector for function has yet to be identified, and (iii) DMSO, glycerol, VX-809, and low temperature treatments are not all equivalent in restoring functional trafficking of dRTA mutants to the cell surface.

The G701D kAE1 mutant is a recessively inherited dRTA mutant that is retained in the Golgi of renal epithelial cells [120]. Patients homozygous for this mutation display only dRTA symptoms independent of any RBC features: G701D eAE1 in the RBCs of patients display 98 % sulphate influx and 95 % of the inhibitor DIDS

binding sites compared with WT eAE1 [103]. When expressed in *Xenopus* oocytes, this mutant is inactive unless co-expressed with the erythroid restricted, chaperone-like protein glycophorin A. These previous findings suggested that this mutant is mildly misfolded and that its functional rescue may be possible. In the present study, we show that glycerol, DMSO, VX-809, and low temperature incubations rescued G701D kAE1 trafficking to the basolateral membrane of MDCK cells (Figures 3.4, 3.5, and 3.10). However, DMSO is the only treatment that also significantly restored function of the mutant at the cell surface (Figure 3.6). This finding suggests that DMSO incubation enabled G701D kAE1 dRTA mutant to reach a conformation close to WT kAE1 and to the one adopted by G701D kAE1 in presence of GPA. Interestingly, the G701D kAE1 mutant was rescued by all the treatments in this study, suggesting that this Golgi-retained mutant may have a greater potential to be rescued than ER retained mutants. Our experiments indicate that DMSO incubations dramatically increased the overall protein synthesis of the G701D kAE1 mutant (Figure 3.12 and 3.13), as did incubations with the VX-809 pharmacological chaperone. However, in contrast with VX-809 and other treatments described in this study, DMSO incubations may also promote better folding of this mutant, since G701D kAE1 was partially functional at the cell surface. We did not detect any improved ability of the G701D kAE1 mutant to bind the stilbene inhibitor SITS after DMSO treatment (Figure 3.7), but this assay does not exclude folding improvement at kAE1 transport regions different from the inhibitor binding site.

The functionally inactive C479W kAE1 mutant was not rescued by any chemicals that we have tried in these experiments, suggesting that the folding of the mutant cannot be

improved. The cellular quality control machinery likely detects this mutant as terminally misfolded, targeting it for degradation regardless of the treatment applied. The C479W mutation is located at the boundary between the third transmembrane domain and the second extracellular loop [113]. Interestingly, in the second extracellular loop, another recessive dRTA mutation, V488M, also impairs kAE1 proper folding and trafficking [120]. The dramatic effect that these two neighbour mutations C479W and V488M kAE1 have on the protein folding supports that this region of kAE1 is important for folding and that these mutants are likely terminally misfolded.

Trafficking of the R589H kAE1 mutant was slightly improved after chemical treatments or low temperature incubation. Although we observed a trend suggesting improved function after glycerol or low temperature incubation, the difference was not statistically significant ($p = 0.3$ between R589H control conditions and $30^{o}C$ treated conditions, which showed the greatest difference); indicating that restoration in trafficking does not automatically warrant functional improvement.

Our experiments indicated that treatments that partially rescued cell surface trafficking (DMSO, glycerol, VX-809 and low temperature incubations), with an apparent association with a marked increase in the steady-state amount of kAE1 within a time as short as four hours. This increase might be a general effect of the treatments on increasing cellular protein synthesis. Alternatively, this result may either reflect an improved ability of the cellular chaperones to fold proteins or a decreased cellular degradation. Glycerol and DMSO incubations protect misfolded membrane proteins from

thermal denaturation, aggregation and misfolding [141]. They may allow for tighter packing and stabilization of premature G701D kAE1, which encourages proper folding, increases stability, and promotes further trafficking to the plasma membrane. They however likely act with subtle differences, as DMSO but not glycerol rescued functional activity of G701D kAE1. Growth at low temperature did partially rescue cell surface trafficking of the G701D kAE1 mutant, however not functionally. As low temperature incubation inhibits 26S proteasome function [163], it suggests that reducing 26S proteasome-mediated degradation would not be enough to treat dRTA patients with the G701D mutation. These patients may therefore need a molecular chaperone that would not only improve trafficking but also folding of this mutant.

Although the precise mechanism of action of DMSO is not understood fully, various studies linking DMSO and other proteins' activities have revealed its potential role in increasing packing of protein folding intermediates [141], and its effects on various signaling pathways [164]. Indeed, DMSO is not by any means a broad spectrum therapeutic, but it has been used repeatedly in the past for various disease treatment applications. The earliest applications include treatment of rheumatologic diseases [165, 166], chronic prostatitis [167], pulmonary adeno carcinoma [168], and dermatologic diseases [169]. Its anti-inflammatory and free radical scavenging properties have also been long established [167, 170-173]. In fact, treatment of interstitial cystitis (IC) with DMSO in humans has been FDA approved in 1978 [174]. Drugs can be engineered based on the characteristics known about DMSO, to have similar functional properties and rescuing effects targeting specific mutant proteins. Of course, detailed studies remain

to be conducted on the effects of DMSO or DMSO-like molecules (when engineered) on other proteins found in the same cells, before attempting DMSO-like therapy for dRTA patients.

The VX-809 molecular corrector partially rescued the maturation and chloride transport activity of CFTR deltaF508 in human bronchial epithelial cells [153]. However, although it partially rescued G701D kAE1 dRTA mutant trafficking to the cell surface, there was no improved functional activity observed in our experiments (Figure 3.11). This indicates that the correcting effect of VX-809 is unique to CFTR deltaF508 and may not apply to other mutant membrane proteins that display trafficking defects. This idea is supported by the lack of rescuing effect on mis-trafficking mutants of a related membrane protein, P-glycoprotein (P-gp) [153].

These results highlight an important factor in the search for kAE1 mis-trafficking correctors: the specificity of the drug to each specific kAE1 mutant. If the treatment has an effect on more than one protein, the net negative effects on other proteins may mask the positive kAE1 rescuing effects. An example is seen in the correlation between rescuing conditions for mutant CFTR proteins and P-gp. P-gp is an ATP-dependent drug pump located at the membrane of epithelial cells [175]. It is expressed in the intestine, liver, and kidney to efflux hydrophobic drugs and prevents their absorption [176]. Since its localization is similar to that of CFTR, many rescuing drugs for CFTR are made less effective as they are also substrates of P-gp [177]. A case in point is a five-fold reduction in the correcting effect seen with the drug KM11060 (a sildenafil analogue) on mutant

CFTR when P-gp is co-expressed in HEK-293 cells [177]. Although there is no evidence for the presence of efflux pumps in alpha-intercalated cells where kAE1 is found, their potential presence in this nephron segment should not be overlooked when evaluating the efficacy of a kAE1 corrector. In the absence of a good animal model for kAE1 mutants, co-expression rescue studies of kAE1 and P-gp may be worthwhile.

Taken together, our experiments have shown the potential for one dRTA mutant, G701D kAE1 to be functionally rescued at the plasma membrane of renal epithelial cells. Additionally, the other mildly misfolded mutant R589H could partially traffic to the plasma membrane after chemical or low temperature treatment, although we did not find a statistically significant difference of functional activity with control conditions. These encouraging findings suggest that extending the investigation to a larger number of molecular chaperones may identify chemicals that could potentially rescue functional trafficking of these mutants. Our study highlights that prospective alternative treatments for patients suffering from dRTA as a result of mutated kAE1 can be developed to target the source of the disease, rather than the resulting consequences. Our findings raise the hope that identification of kAE1 trafficking and functional potentiators may open new therapeutical opportunities for dRTA patients.

4. Identification of degradation pathways involved in mutant kAE1 turnover

4.1 Introduction

In the previous chapter, DMSO was shown to rescue the trafficking and subsequently the function of the mutant G701D kAE1 protein. A major obstacle remains in that the turnover of the rescued mutant was faster compared to the WT kAE1 protein. This may be a contributing factor for the absence of a potentially more pronounced functional activity measured at the membrane, as evidenced below. Because of this limitation, it is important to understand not only how these trafficking mutants can be rescued, but also the underlying mechanisms by which they are degraded.

The two major pathways by which proteins are degraded in a cell are the proteasomal and lysosomal pathway. This chapter describes the preliminary work conducted to understand the role of different quality control components on WT, G701D, C479W, and R589H kAE1 degradation.

4.1.1 Proteasomal pathway

Quality control pathways in the cell are present to eliminate improperly folded proteins in a timely manner. This is crucial for cell survival as accumulation of misfolded proteins is often cytotoxic, leading to apoptosis of the cell [178]. Degradation and quality control mechanisms are set in place to avoid cell stress. The proteasomal pathway is responsible for early recognition and degradation of newly synthesized, mutated proteins before they exit the ER. The collection of proteins that are involved in this process belong to the Endoplasmic Reticulum Associated Degradation (ERAD) family. Different ERAD

complexes are formed to target degradation of different types of mutated proteins [178]. A major classification split for ERAD substrates (synonymous to misfolded proteins targeted for ERAD) is based on the glycosylation state of the substrate. Glycosylated proteins are targeted to ERAD complexes formed by lectin-like proteins, whereas non-glycosylated substrates are targeted to ERAD complexes made up of other chaperones. However, certain chaperones, such as ER-degradation-enhancing α-mannosidase-like protein (EDEM)-binding ER resident protein (ERdj5), are capable of forming complexes with different combinations of chaperones based on the glycosylation state of the substrate [178-180].

4.1.1.1 Final attempts to salvage misfolded proteins in the ER

Upon *de novo* synthesis, proteins undergo oligosaccharide modifications in the ER. There are two types of glycosylations – N- and O-glycosylation. As kAE1 has a single glycosylation site which undergoes co-translational N-glycosylation only, we will focus on N-glycosylation. Asparagine residues are first decorated with the N-glycan $Glc_3Man_9GlcNAc_2$ by the oligosaccharide transferase complex [178]. Glucosidases 1 and 2 then successively cleaves off two of the glucoses, a process which is necessary for the newly synthesized protein to enter the "calnexin (CNX) cycle" [181]. CNX and calreticulin (CRT) complex with newly synthesized proteins to promote proper folding. This step is crucial for deciding the fate of the newly synthesized protein. If a protein is properly folded, the final glucose on the N-glycan is removed by glucosidase 2, leaving the protein with the oligosaccharide $Man_9GlcNAc_2$ which allows it to be released from

the CNX-CRT complex and be further targeted to the Golgi via vesicular transport. On the other hand, if the protein is misfolded forming globules or has exposed hydrophobic surfaces, the protein is recognized by UDP-glucose glycoprotein glucosyltransferase (UGGT), re-glucosylated on the N-glycan, and runs through the CNX circle again until classified as properly folded or terminally misfolded. There is some debate on whether the single re-glucosylation is a sufficient signal for ERAD targeting [182], but more commonly ER retained proteins are modified by mannosidases until its N-glycan only has eight or less mannoses remaining, which is a signal for ERAD recognition [183]. Mannosidases act slowly, therefore they require proteins to remain in the ER for a length of time (property of misfolded proteins) before the mannose can be cleaved off. EDEM 1, 2 and 3 are such mannosidases [178, 184, 185], and directly forms a complex with CNX to increases its accessibility and decreasing the time needed to find misfolded targets [186].

Prior to proteasomal degradation, labelling of these terminally misfolded proteins must occur. Several proteins take part in this process independently, acting as a security system to ensure that the appropriate proteins are sent to the proteasome. There are a number of soluble and membrane-bound proteins in the ER that recognizes ERAD substrates. The separation between these proteins is apparent in yeast, where defective proteins with mutations in the cytosolic domain, transmembrane domain, and ER luminal domains are differentially recognized by complexes formed by members of ERAD-C, ERAD-M, and ERAD-L, respectively [187]. Specifically, ERAD-L substrates OS-9 (ER lectin), XTP3-B (ER lectin), SEL1L (cargo receptor), and HRD1 (an E3) complexes to

recognize specifically soluble misfolded proteins [188], where the first three components are from the ER lumen and the latter is on the ER membrane.

4.1.1.2 Ubiquitination , retrotranslocation, and proteasomal degradation

Proteins that are targeted for degradation in the 26S proteasome are typically ubiquitinated [182]. Ubiquitin is a 76 amino acid cytosolic protein [189]. It binds to the amine groups of other proteins by forming covalent bonds. The process of ubiquitination is carried out by a subset of proteins that belong to the ubiquitin 26S proteasome system (UPS). Three major families of enzymes are involved: E1 ubiquitin activating enzyme, E2 ubiquitin elongation enzyme, and E3 ubiquitin ligase. There is only one E1 enzyme in mammals, and it is responsible for activation of ubiquitin and transfer of ubiquitin onto the ubiquitin elongase and ligase in preparation for protein loading in an ATP-dependent manner [190, 191]. A thioester bond links the C-terminal glycine of ubiquitin to the cysteine on E1 [192]. E2's then receive the ubiquitin from E1 on its cysteine residues. Next, E2 binds to E3 and transfers the now activated ubiquitin to the target protein, typically on the lysine residues via isopeptide bonds [192]. E2 and E3 remain complexed to the target protein until four or more ubiquitins have been attached. Specific E2's and E3's are used for different classes of proteins. – in yeast, ERAD-C uses the E3 Doa10, while ERAD-M and L use the E3 Hrd1. However, such clear distinctions have yet to be established components of human ERAD, as the number of each of these components is vastly greater in humans, and many components are thought to play overlapping roles.

Before elongation of the full ubiquitin chain occurs, retrotranslocation of the misfolded protein from the ER to the cytosol must occur to allow for cytosolic 26S proteasome driven degradation of the misfolded protein. In most cases, this process is conducted by the Derlin1/Derlin2/p97/Valosin-containing protein-interacting membrane protein complex in an ATP-dependent manner via recognition of the ubiquitin chain [178, 188]. E3's such as Doa10 and Hrd1 in yeast have been found within the retrotranslocation complexes, and similar compositions have been proposed for mammalian contexts, as the colocalization of E3's and the retrotranslocation complex would decrease the time spent on locating ubiquitinated, misfolded proteins that need to cross the ER membrane [182]. However, some misfolded proteins that had not gone through ubiquitination have been shown to be retrotranslocated by the 19S cap of the proteasome itself [188]. The retrotranslocation process is less well understood than most of the other steps involved in ERAD and remains to be explored.

This misfolded protein is now in the cytoplasm, with a modified oligosaccharide and recognizable by the proteasome and ready for degradation. The 19S caps of the 26S proteasome have ubiquitin receptors which allows attachment of the misfolded protein. The deubiquitylating enzymes on these caps either removes the ubiquitins on the misfolded protein one by one or removes the entire chain all at once, creating a ubiquitin-free substrate for the 20S proteasome core. The core contains subunits which have trypsin-like, chymotrypsin-like, and post-glutamyl peptide hydrolyzing activities to breakdown the misfolded protein [182]. The ubiquitin molecules which were cleaved off by deubiquitylases, can be recycled to mark other misfolded proteins for degradation.

4.1.1.3 Tools for studying the UPS-ERAD proteasomal degradation pathway used in this thesis

The earliest tools developed to study the proteasomal pathway are inhibitors of the proteasome. The most well known are MG-132 and its derivatives, which are permeable tripeptide aldehydes that reversibly inhibit mostly chymotrypsin-like activities [193]. However, there has been evidence that MG-132 also inhibits cysteine proteases found in lysosomes [193]. Lactocystins and their derivatives are more specific as they act by being a substrate for the proteasome, thereby blocking its protease activities directly and irreversibly [193]. Epoxomicin is another class of potent proteasome inhibitors which are membrane permeable and irreversible [194]. Like lactocystins, epoxomicin achieves its specificity by binding directly to the β-subunits of the proteasome-substrate binding site.

A number of temperature sensitive mutants for the enzymes of the UPS have been generated. Of particular interest are mutants of E1, as it universally regulates the first step of all mammalian ubiquitination events, and mutants of E3, as they finalize the ubiquitination process just prior ERAD degradation. We were lucky to have recently acquired cells that have a temperature sensitive knockdown of E1 (hereon TS20's) and cells with a short hairpin-RNA knockdown of the carboxyl terminus of Hsc70-interacting protein (hereon CHIP; an E3) (kind gifts from Dr. Gergely Lukacs).

TS20 cells were derived from E36 Chinese Hamster cells [195]. This cell line is a mutant for the G2 phase of the cell cycle and is temperature sensitive (TS) for ubiquitin

conjugation. Two hours at 40°C eliminated all ubiquitination; 42°C for ten minutes gave the same result. Ubiquitin conjugation was restorable in the presence of WT E36 cell extracts or purified E1's [189, 195], demonstrating that the defect in these mutants indeed were due to inactivity of E1 in non-permissive temperatures.

The other knockdown cell line is derived from HEK 293 cells, with a short-hairpin knockdown of CHIP, an E3 ubiquitin ligase. CHIP is a recently characterized co-chaperone of the Hsc70-Hsp90 complex, which is known for its chaperoning role in trying to refold misfolded proteins. Interestingly, CHIP is a negative regulator of the Hsc70-Hsp90 complex – binding of CHIP converts the complex to increase degradation of the bound, misfolded protein [196-198]. CHIP achieves this by decreasing the ATPase activity of this chaperoning complex, and instead uses its ubiquitin ligase activity to increase the ubiquitination of the bound misfolded protein. This targets the substrate, for example CFTR, for proteasomal degradation more quickly [199]. Recently, Okiyoneda *et al*. observed that internalisation of unfolded deltaF508 CFTR was decreased by 80% when CHIP was knocked down at 37°C [124]. Furthermore, normal recycling and delayed degradation of the deltaF508 CFTR was observed upon CHIP knockdown. Knockdown of Hsc70 or Hsp90, which are the interacting partners of CHIP, also produced similar results. Overall, knockdown of CHIP-associated ERAD complex components restored deltaF508 CFTR to levels of rescue under temperature permissive conditions at 26°C.

As most trafficking mutants of kAE1 are retained in the ER and have a shorter half-life than WT kAE1, the major pathway involved in their rapid turnover is likely the UPS-ERAD proteasomal degradation pathway. To test this hypothesis, the TS20 and CHIP cells serve as excellent models in demonstrating the importance of the components of UPS to the stability of mutant kAE1. Increased stability of mutant kAE1 in the absence of E1 or E3 CHIP would support the idea that the degradation of mutant kAE1 is UPS dependent. Hsc70, normally activated by binding of Hsp40, was recently shown to be involved in the degradation of kAE1; MAL3 inhibition of the Hsc70 ATPase activity prevented degradation of kAE1 [157]. CHIP is known to target the Hsc70-Hsp90 complex and inhibit its chaperoning activities, therefore this cell line is valuable to further demonstrate the dependence of mutant kAE1 degradation on balance of ERAD complexes. In addition, inhibitors of the 26S proteasome can be used to correlate the role of ERAD to the fate of mutant kAE1 proteins.

4.1.2 Lysosomal pathway

Ubiquitin dependent degradation of proteins is not exclusive to the proteasomal pathway. In fact, ubiquitination plays an important role for degradation of proteins at the plasma membrane via the lysosome. The ubiquitination process prior to lysosomal degradation shares the same E1, E2's, and E3's as the proteasomal system [200], thus the name UPS is somewhat misleading. The degradation signal on lysosome targeted proteins can be polyubiquitin chains, monoubiquitins attached to multiple sites, or attachment of a single ubiquitin molecule [200]. Because ubiquitin molecules themselves have seven different

lysines, the linkages of the polyubiquitinated chains can differ. The most common linkage of ubiquitin chains found on lysosomal targets is on lysine 63 (K63) of ubiquitin [201], but K48, K29, and K11 linkages have also been found [202]. These ubiquitins are recognized and isolated by ubiquitin-sorting receptors Eps 15 and Epsin [203] via their ubiquitin-interaction motifs (UIM's) to begin the internalization process. Endosomal sorting complex required for transport (ESCRT)'s, particularly ESCRT 0, then continue to actually pinch off the cargo-containing-vesicle formed at the membrane [202]. ESCRT 0 has multiple UIM's and vacuolar protein sorting gene 27/ hepatocyte growth factor regulated tyrosine kinase substrate (Hrs)/ signal transducting adaptor molecule (STAM) homology domains containing ubiquitin binding domains that allow association with ubiquitinated proteins [202]. Ubiquitinated proteins targeted for lysosomal degradation bud into intraluminal vesicles (ILVs) inside early endosomes assisted by other members of the ESCRT family (I – III) [200], Lysosome targets within ILVs are well segregated from proteins that can be recycled to the membrane, which accumulate in budding tubules of the early endosome [204]. Accumulation of ILVs as the endosome matures results in multivesicular bodies (MVBs), also known as late endosomes. MVBs then fuse with lysosomes where target proteins are degraded [204, 205].

4.1.2.1 Tools for studying the lysosomal pathway used in this thesis

As with the proteasomal pathway, initial studies for the involvement of the lysosomal pathway in degradation of a protein involved lysosomal inhibitors. The most commonly used are chloroquine, leupeptin, and ammonium chloride. Chloroquine works by

accumulating in acidic compartments of the cell, such as endosomes and lysosomes, and inhibits acidification of these compartments. As such, endosome-lysosome fusion events are prevented, and no degradation of the proteins in these compartments occur [206]. Ammonium chloride also acts similarly by inhibiting fusion of phagosome and lysosome [207]. Leupeptin is more specific; it reversibly inhibits the serine and cysteine proteases found in lysosomes [208].

Recently, we have received some new tools to study the involvement of the lysosome in degradation of kAE1. This is discussed in Section 4.3.

4.2 Results

4.2.1 Stability of WT and mutant kAE1 in MDCK cells

As observed in the previous chapter, the relative stability of WT kAE1 was much greater than mutant AE1 proteins in MDCK cells (Figure 3.2 and 3.3). WT kAE1 had longer half-life of 23 hours, while G701D, C479W, and R589H kAE1 had much shorter half-lives –four, five, and one hour respectively. SITS binding results from previous studies as well as one conducted to support these studies showed that G701D kAE1 had similar binding to SITS beads compared to R589H and WT kAE1, at 81% and 70% of WT kAE1 binding, respectively (Figure 3.7). C479W kAE1 was not tested as it was predicted to be terminally misfolded based on its lack of function even when expressed on cell surface of *Xenopus* oocytes and its inability to be rescued by co-expression of WT kAE1 [113]. The short half-life of G701D kAE1 suggests that there must be some factor besides gross misfolding that makes G701D kAE1 prone to premature degradation.

Understanding the degradation process of a protein is not trivial. To begin to understand the different mechanisms of degradation between WT and mutant kAE1 proteins, we studied the general question of which of the two major degradation pathways – the proteasomal or lysosomal pathway – is predominantly involved mutant kAE1 degradation.

4.2.2 Ubiquitination of kAE1 proteins

We first tested whether kAE1 WT and mutants are ubiquinated. The antibody for ubiquitin used here detects polyubiquinated species with K29, K46, and K63 linkages, monoubiquitinated proteins, but not unbound ubiquitins. Consistent with findings from Patterson and colleagues [157], we observed that upon inhibition of the 26S proteasome with epoxomicin, the overall amount of ubiquitination was increased for kAE1 WT and dRTA mutants (Figure 4.1). Ubiquitination is an important process to degradation. As described above, proteins are ubiquitinated prior to their degradation by the 26S proteasome, but ubiquitination can also occur to plasma membrane proteins before their internalization for either recycling or degradation by the lysosome [209]. The effects of lysosomal inhibition on ubiquitination of kAE1 remains to be investigated. These results therefore do not completely differentiate the pathway of degradation, but that degradation of kAE1 is at least partly ubiquitin dependent.

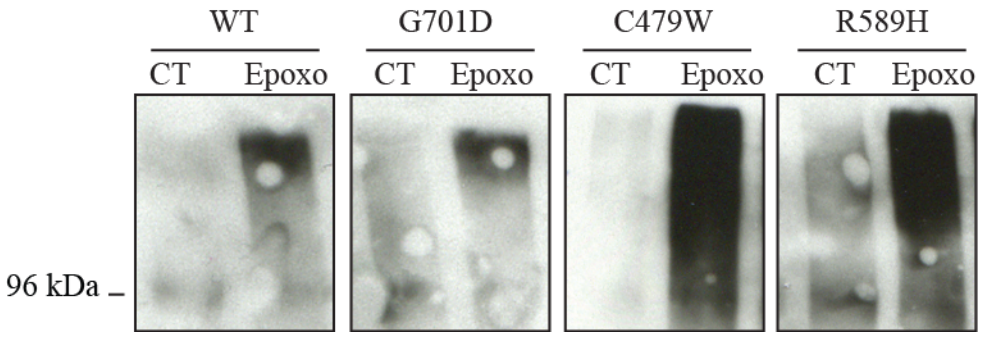

Figure 4.1 Ubiquitination of kAE1 proteins upon 26S proteasomal inhibition.
MDCK cells expressing WT, G701D, C479W, and R589H kAE1 were treated with 10 μM epoxomicin for four hours. Cells were harvested and immunoprecipitated with rat anti-HA antibody followed by protein G beads to pull down kAE1. Eluted samples were resolved on western blots and detected by mouse anti-ubiquitin; ubiquitinated protein fractions appear as a smear on western blot.

4.2.3 Involvement of the UPS in kAE1 degradation

To investigate further how ubiquitination is a hallmark of kAE1 mutants' degradation, we next looked at the effect of down-regulating components of the UPS system, which is critical for ubiquitination to occur. The process of ubiquitination is carried out by three major groups of enzymes as described above: E1 ubiquitin activating enzyme, E2 ubiquitin elongation enzymes, and E3 ubiquitin ligases. We were able to study the effect of E1 and one E3 on mutant kAE1 stabilization by using TS20 cells and HEK 293 cells that have a short-hairpin knockdown of CHIP. These cells were transiently transfected with WT or mutant kAE1 using the NEON electroporation system. Cells were grown for 24 hours after transfection before treatment with CHX and lysis. Complete knockdown of E1 in TS20 cells is achieved by incubation at 40^0C for two hours [195]. Consistent with previous findings, kAE1 migrates predominantly as the high mannose form in HEK cells [210].

Preliminary results show that absence of E1 and CHIP in cells expressing WT, G701D, and C479W kAE1 may lead to a stabilization of the mutant proteins at a steady state (Figure 4.2). These stabilizing effects are less obvious for the R589H kAE1 mutant and more experiments have to be done to confirm the effect. Inhibition of protein synthesis using CHX in the absence of E1 and CHIP also shows stabilization of mutant kAE1 proteins. This suggests that E1 and CHIP dependent ubiquitination and degradation of mutant kAE1 proteins is not exclusive to nascent kAE1 proteins, but also includes more mature forms of the proteins.

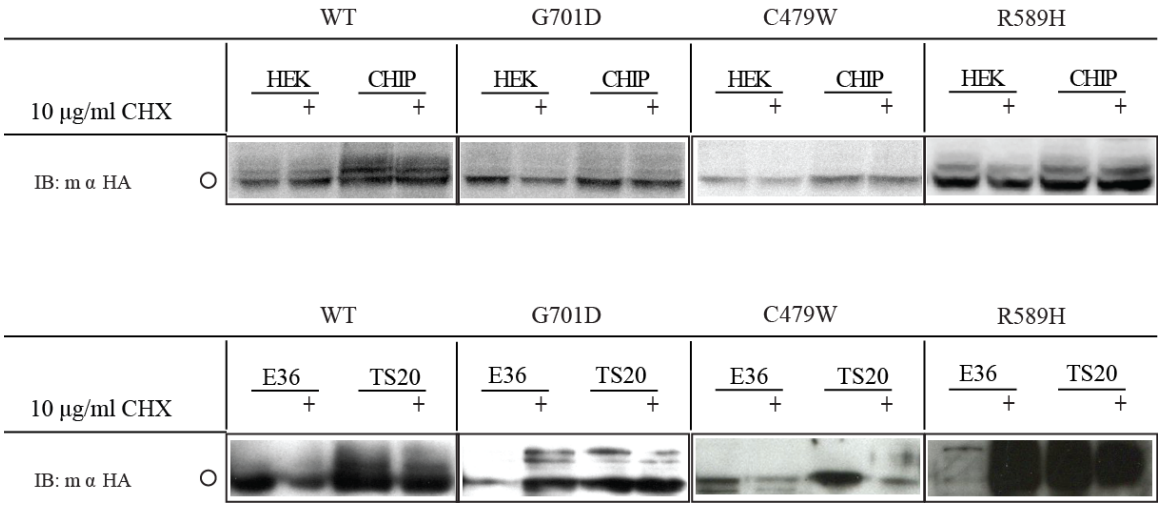

Figure 4.2 Knockdown of E1 and CHIP E3 stabilizes kAE1.

HEK 293, CHIP, E36, and TS20 were transiently transfected with WT, G701D, C479W, or R589H kAE1 24 hours before lysis. Cells were treated or not treated with CHX for three hours prior lysis. Lysates were resolved by western blots; kAE1 is detected by the HA epitope, and migrates as a single band at 96 kDa in these cells (empty circle) with very little complex band above.

4.2.4 Role of the proteasome and lysosome in degradation of kAE1

As mentioned above, evidence of ubiquitination of a protein is not enough to indicate how the protein is degraded. To test whether the 26S proteasome or lysosomes are involved in the steps subsequent to kAE1 ubiquitination, we first incubated cells expressing WT or mutant kAE1 with either epoxomicin, which inhibits the 26S proteasome irreversibly, or leupeptin to inhibit the lysosome, for four hours (Figure 4.3 and 4.4). The four hour time-point was chosen for these treatments because it was the first time-point where the treatment showed an effect on the levels of mutant kAE1 proteins present, and encompasses the shortest half-life of kAE1 proteins studied. Figure 4.3 shows that inhibition of the 26S proteasome in MDCK cells increased the amount of G701D kAE1 to 121 ± 20 % (SEM; n=3) of control conditions, suggesting the involvement of the 26S proteasome in degradation of G701D kAE1. Similarly, more C479W kAE1 (115 ± 18 % (SEM; n=3)) of control conditions) was detected in epoxomicin treated cells; R589H kAE1 also appeared to be slightly stabilized (105 ±19 % (SEM; n=3) of control). This suggests that all of these dRTA mutants are at least partially degraded by the 26S proteasome. Knowing the half-lives of G701D and C479W kAE1 to be four and five hours, if the degradation of these two mutants is solely dependent on the proteasome, then one would expect a 50 % increase in the amount of protein after blocking the proteasome for four hours. This is certainly not the case, suggesting involvement of other quality control mechanisms in turnover of mutant kAE1 or modulation of protein synthesis upon inhibition of degradation pathways. The amount of WT kAE1 remained unchanged after epoxomicin treatment (100 ± 2 % (SEM; n=3) of control conditions), similar to the case with R589H kAE1. This might be due to the

longer half-life of the WT protein, requiring longer times to see changes in protein. The little effect of proteasomal inhibition on the amount of dominant R589H kAE1 present suggests that this mutant is regulated differently compared to the recessive mutants studied in this thesis.

Interestingly, the G701D kAE1 was also greatly stabilized by the lysosomal inhibitor leupeptin (Figure 4.4). Treatment of the cells expressing G701D with 20 μM leupeptin for four hours resulted in the detection of 145 ± 15 % (SEM; n=3) compared to control conditions. There was no significant change for WT kAE1 after the four hour treatment with leupeptin with 102 ± 9 % (SEM; n=3) compared to control conditions. Altogether, these results indicate that both the 26S proteasome and the lysosome contribute to the G701D protein degradation, while C479W and R589H kAE1 are partly stabilized in the presence of proteasomal inhibitors.

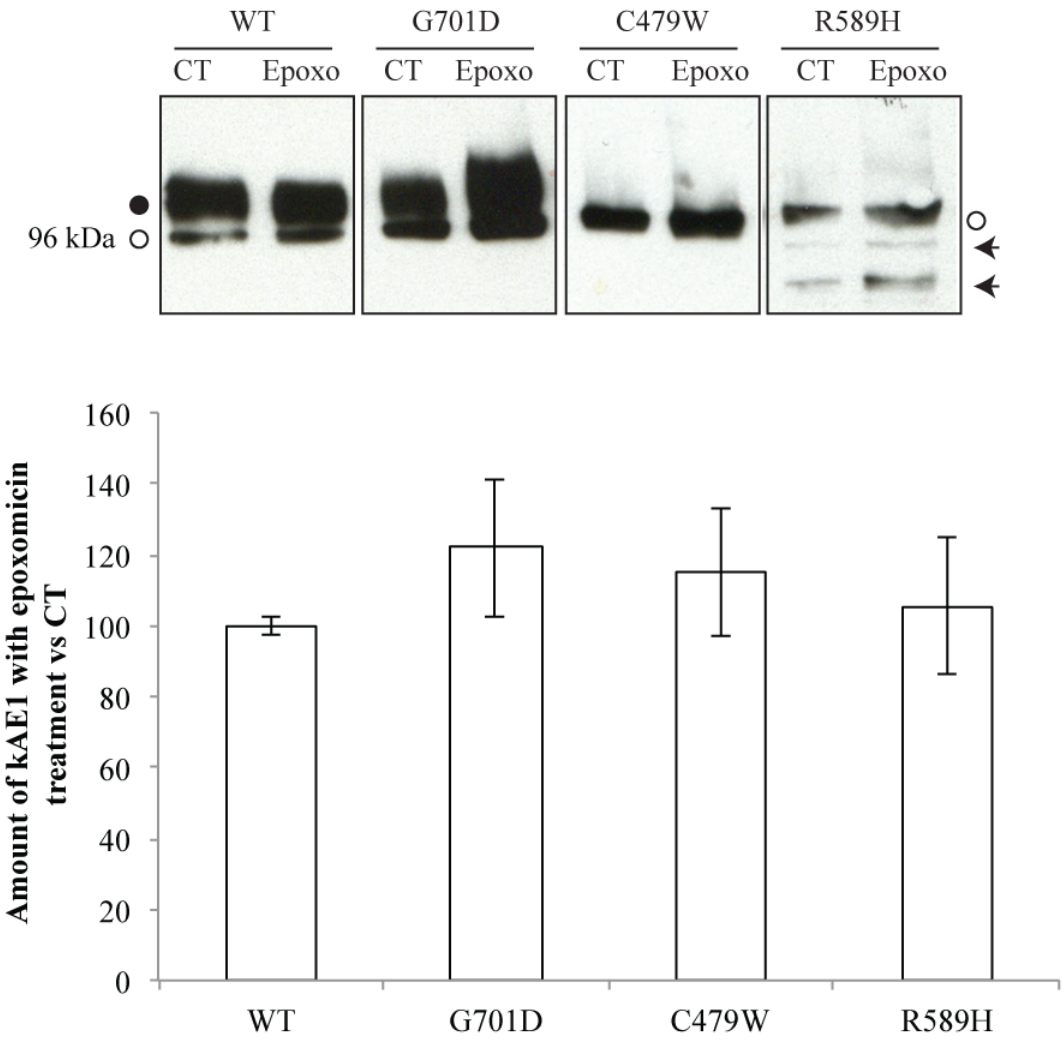

Figure 4.3 The proteasomal pathway is involved in degradation of mutant kAE1.
MDCK cells expressing WT, G701D, C479W, and R589H kAE1 were treated with 10 μM epoxomicin four hours. Cells were harvested and immunoprecipitated with rat anti-HA antibody followed by protein G beads to pull down kAE1. Eluted samples were resolved on western blots and detected mouse anti-HA antibody for kAE1. Migration of kAE1 at 96 kDa includes two species: kAE1 carrying complex oligosaccharide (filled circle) or high mannose (empty circle). Arrows denote degradative products. Error bars are SEM; n = 3 independent experiments.

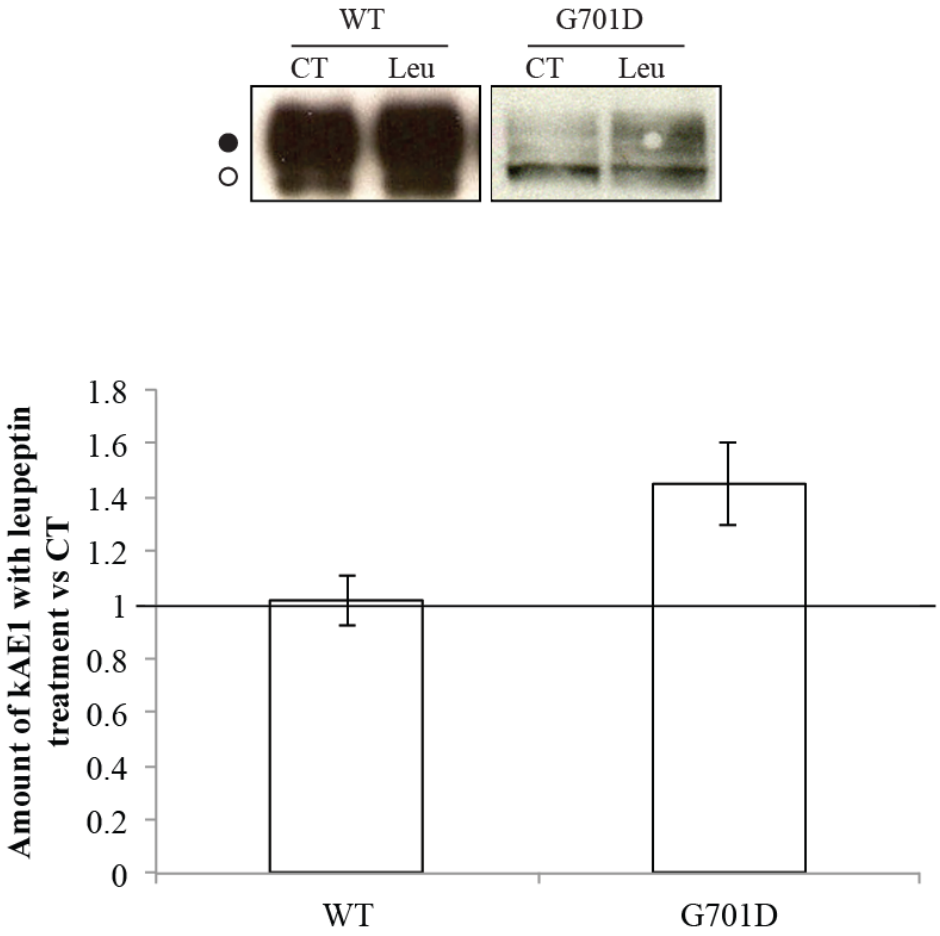

Figure 4.4 Lysosomal pathway is involved in degradation of kAE1 mutants.

MDCK cells expressing WT or G701D kAE1 HA were treated with 20 μM leupeptin for four hours. Cells were harvested and immunoprecipitated with rat anti-HA antibody followed by protein G beads to pull down kAE1. Eluted samples were resolved on western blots and detected by mouse anti-HA antibody for kAE1. kAE1 migrates at 96 kDa with a complex oligosaccharide band (filled circle) and a high mannose band (empty circle). Error bars are SEM, n = 3 independent experiments.

4.3 Discussion

Turnover of G701D, C479W, and R589H kAE1 mutants is quicker than WT kAE1 as seen with our half-life experiments (Figure 3.2). With lack of evidence for gross misfolding of G701D kAE1, characterization of the cellular machinery involved in dictating the fate of this particular mutant is especially interesting. We determined the relative contribution of the 26S proteasome and the lysosome in degradation of these dRTA mutants. Four hour treatments were used for these experiments because it was the first time-point that we saw an effect on the amount of mutant kAE1 proteins compared to CT conditions that also covers the shortest half-life of the mutants studied. This is a good time-point to use because it allows us to observe the early stabilization events by manipulation of the degradation pathways for these mutants, but a longer time-point would have allowed us to see the prolonged effects of these treatments on kAE1 proteins as well, although this may be cytotoxic. Extending the length of blocking the degradation pathways will be an interesting experiment to do in the future in addition to what is shown here.

Consistently with the work from Patterson and colleagues [157], we found that kAE1 WT and all the dRTA mutants are polyubiquitylated in epithelial cells, as demonstrated by the detection of polyubiquitin associated with immunoprecipitated kAE1. However, the dRTA mutants have a higher level of polyubiquitylation compared to kAE1 WT (as seen with the denser smears on the mutant blots in Figure 4.1), when the 26S proteasome was inhibited with the irreversible inhibitor epoxomicin. This reinforces the idea that kAE1 is degraded in a ubiquitin dependent manner, with partial involvement of the proteasomal

pathway.

Studies conducted in the E1 and CHIP knockdown cells further confirm the involvement of ubiquitination in the degradation of WT and mutant kAE1. Stabilization of WT and mutant kAE1 was evident after knockdown of E1 ubiquitin activating enzyme or cytosolic E3 co-chaperone CHIP, in the presence or absence of CHX (compare CHIP to HEK lanes in Figure 4.3). However, R589H seems to be regulated differently by the CHIP ligase. It is important to keep in mind the limitations of the time-point we chose – four hours was insufficient to see the prolonged effects of E1 and CHIP on kAE1 stability, therefore repetition of these experiments at longer time-points may be valuable. Also, since the E1 enzyme is only fully inactivated after a two hour incubation at 40^{o}C, the treatment time for these cells should have been extended to six hours to obtain an equivalent treatment time as the CHIP knockdown cells, such that the cells will be exposed to conditions without E1 for four full hours. Nevertheless, these observations add to previous evidence for kAE1 interaction with the Hsc-70-Hsp-90 complex [157], and binding of co-chaperone CHIP inhibits the complex's chaperoning ability by enhancing ubiquitination of mutant kAE1 substrate. It would be interesting to see if there are other co-chaperones that regulate CHIP activity, or if CHIP can be outcompeted by co-chaperones that favour refolding, rather than ubiquitylation for the Hsc-70-Hsp-90 complex. For example, HspBP1 has been shown to outcompete CHIP in regulation of CFTR turnover, allowing for maturation instead of degradation of CFTR proteins [199].

Inhibition of the 26S proteasome stabilized all the mutants (Figure 4.3), confirming the role of the proteasome in degradation of the kAE1 mutants. This is supported by the conventional role of ERAD and proteasomal degradation – to monitor and regulate the quality of newly synthesized proteins just as they exit the ER. In addition, both the G701D and kAE1 WT were stabilized by lysosomal inhibition. At steady state, G701D kAE1 is retained in the ER and the Golgi as detected by immunofluorescence experiments. It is partly stabilized in MDCK cells after inhibition of the lysosome, which suggests that, in addition to ERAD-proteasomal regulation, this mutant is also scrutinized by peripheral quality-control machinery at the Golgi, targeting it for lysosomal degradation. Proteins targeted for lysosomal degradation are typically endocytosed cell surface proteins [211]. The observation that G701D kAE1 is found in both the ER and Golgi at steady state, combined with the role of the lysosome in degradation of G701D kAE1, suggests that this mutant may be able to leave the Golgi and transiently reach the cell surface in the absence of any rescuing conditions. However, information from these lysosomal inhibitor studies alone are somewhat limiting as it remains unknown how kAE1 proteins are targeted to the lysosomes, but knowing the involvement of the lysosome in this mutant's degradation encourages more studies in this direction.

As pointed out earlier, both degradation pathways are ubiquitin dependent. One way to follow where ubiquitination occurs is by co-transfection of tagged ubiquitin, like ubiquitin-HA, and kAE1-myc (or any other tag to differentiate from the tag on ubiquitin) in MDCK cells. Using cell surface detection assays, kAE1 proteins ubiquitinated at the cell surface can be differentiated from those that are ubiquitinated immediately after ER

exit, targeted for proteasomal degradation. Under non-permeabilized conditions, these cells can be labelled with antibody specific for the myc tagged kAE1, lysed under conditions where no excess antibody is present, and pulled down with protein G beads. Protein complexes interacting with kAE1 target protein, including ubiquitin, should have been co-immunoprecipitated and can be detected using an antibody against the HA tag on western blot. Any interacting proteins detected during the immunoprecipitation is assumed to be a strong and specific interaction between the two proteins; weaker interactions may occur but transiently.

Recently, we received a few more tools to help us study the lysosomal pathway (courtesy of Dr. Gergely Lukacs). As mentioned in the introduction, lysosomal degradation of a membrane protein requires its endocytosis from the membrane. The proteins involved in containing, segregating, and budding off of the ubiquitinated targets at the cell surface are the proteins of the endosomal sorting complex required for transport (ESCRT) family [212]. There are three main complexes, ESCRT 0, 1, 2, and 3, each with their own subunits. The two knockdown cell lines we have obtained pertain to the Hrs and STAM subunits of the ESCRT 0. Recall that Hrs is important for targeting membrane proteins to early endosomes by binding to the ubiquitinated membrane proteins with its UIM [200], while STAM also has UIM's and helps in this vesicle formation initiation process and is later important for formation of the MVB. Previous studies showed that Hrs and STAM are important for CFTR turnover, as knockdowns stabilized the temperature sensitive mutant deltaF508 CFTR even at non-permissive temperature, 37^{o}C [124]. Using the knockdown cell lines of these two subunits, it will be interesting to see if lysosomal

degradation of kAE1 mutants (G701D kAE1) at the membrane still occur. If so, it would be interesting to tease out the other components that are involved in endosomal-lysosomal degradation of kAE1 proteins at the cell surface. Also, monitoring the activity of the proteasome in turnover of WT and mutant kAE1 in the absence of a properly functioning ESCRT-0 will be interesting. Already, based on the experiments done in this chapter, we suspect that there may possibly be a compensatory mechanism in place between the two major degradation pathways. Indeed, the resulting amount of G701D kAE1, for instance, was not equivalent to 100% stabilization when considering the turnout of inhibiting the proteasome and lysosome separately (21 % and 45 % increase of G701D, respectively). Studies in these cell lines will likely give us more insight into the interplay between the proteasomal and lysosomal degradation pathways for kAE1.

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5. Final Remark

Taken together, the experiments described in this thesis demonstrate the differences in trafficking, biosynthetic handling, and turnover of some kAE1 trafficking mutants causing dRTA. This disparity between kAE1 trafficking mutants is likely a result of the varying extent of misfolding due to these mutations. Investigation of kAE1 mutants G701D, R589H, and C479W has given us insight for better drug properties, targets and therapies that could rescue mutant kAE1 at a molecular level to improve dRTA patients' conditions. These rescue studies demonstrate the potential for kAE1 trafficking mutants to be re-localized and targeted to the basolateral membrane, and to have functional ability to carry out chloride bicarbonate exchange. Furthermore, the involvement of both proteasomal and lysosomal pathways in the degradation of WT and mutant kAE1 has been elucidated. Inhibition of either of these pathways has varying results for each kAE1 mutant. The results describing the fate of rescued kAE1 trafficking mutants are intriguing, and direct future studies towards the prevention of rapid degradation of rescued mutants relative to WT kAE1. Further elucidation of the many components of both degradation pathways and the role these play specifically on mutant kAE1 will provide better targets for restoring trafficking, function, and stability of mutant kAE1, allowing for improved therapies in this subset of dRTA Patients.

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