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Trafficking defect of mutant kidney anion exchanger 1 (kAE1) proteins associated with distal renal tubular acidosis and Southeast Asian ovalocytosis

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Abstract

Compound heterozygous *anion exchanger 1* (*AE1*) SAO/G701D mutations result in distal renal tubular acidosis with Southeast Asian ovalocytosis. Interaction, trafficking and localization of wild-type and mutant (SAO and G701D) kAE1 proteins fused with hemagglutinin, six-histidine, Myc, or green fluorescence protein (GFP) were examined in human embryonic kidney (HEK) 293 cells. When individually expressed, wild-type kAE1 was localized at cell surface while mutant kAE1 SAO and G701D were intracellularly retained. When co-expressed, wild-type kAE1 could form heterodimer with kAE1 SAO or kAE1 G701D and could rescue mutant kAE1 proteins to express on the cell surface. Co-expression of kAE1 SAO and kAE1 G701D also resulted in heterodimer formation but intracellular retention without cell surface expression, suggesting their trafficking defect and failure to rescue each other to the plasma membrane, most likely the molecular mechanism of the disease in the compound heterozygous condition.

Keywords: Distal renal tubular acidosis (dRTA); Kidney anion exchanger 1 (kAE1); Southeast Asian ovalocytosis (SAO); Compound heterozygous mutation; Trafficking defect; Epitope tag; Green fluorescent protein (GFP); Thai

Erythroid and kidney anion exchanger 1 (eAE1 and kAE1) are encoded by *AE1* or *SLC4A1* gene located on chromosome 17q21-q22 [1]. kAE1 is a truncated isoform of eAE1 with lacking of 65 amino acids at the N-terminus owing to the use of differential transcription and translation start sites [2,3]. eAE1 regulates red blood cell (RBC) morphology and chloride/bicarbonate (Cl^-/HCO^-_3) exchange in RBC whereas kAE1 mediates chloride/bicarbonate exchange at basolateral membrane of acid-secreting α-intercalated cells of distal nephron and collecting duct [3–7]. eAE1 defect results in morphological changes of RBC while kAE1 abnormality leads to distal renal tubular acidosis (dRTA)—a disease characterized by failure of kidney to appropriately acidify urine in the presence of systemic metabolic acidosis [8].

AE1 mutations have been found to be associated with both autosomal dominant (AD) and autosomal recessive (AR) dRTA [8,9]. The mutations associated with AD dRTA such as R589H, R589C, R589S, and R901X still maintain substantial anion transport activities in RBC and *Xenopus* oocytes [10–14], suggesting that the defect dose not simply associate with a reduction of anion

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transport function. Trafficking of mutant proteins to the cell surface of human embryonic kidney 293 (HEK 293) cells was impaired, causing their intracellular retention [15–17]. Moreover, kAE1 R901X and G609R exhibited mis-targeting to apical surface in polarized Madin-Darby canine kidney (MDCK) cells [18,19]. Co-expression of kAE1 R589H or R901X with wild-type kAE1 also caused intracellular retention of wild-type protein attributable to their hetero-oligomer formation and dominant negative effect [15,16].

The *AE1* mutation associated with AR dRTA was first reported in two Thai patients who carried a homozygous *AE1* G701D (*band 3 Bangkok I*) mutation and homozygous hemoglobin E (HBB E26K) and had severe hemolytic anemia and dRTA without abnormal RBC anion transport function [20]. eAE1 G701D and kAE1 G701D showed impaired chloride anion (Cl^-) transport and trafficking to the cell surface in *Xenopus* oocyte, which could be rescued by co-expression of glycophorin A (GPA)—an eAE1 chaperone [20]. The presence of dRTA phenotype without abnormal erythroid anion transport could be explained by the presence of GPA in RBC but absence in the kidney α-intercalated cells; thus, kAE1 G701D might fail to move to basolateral membrane in the kidney α-intercalated cells [21,22].

The combined dRTA and Southeast Asian ovalocytosis (SAO) condition was originally observed by our group in Thai patients who carried compound heterozygous SAO/G701D mutations [23,24], and were also reported later by other groups [25,26]. *AE1* SAO is caused by a deletion of 27 base-pairs in codons 400–408, resulting in an in-frame 9-amino-acid deletion at the boundary between cytoplasmic and membrane domains of AE1. The heterozygous *AE1* SAO mutation is insufficient to cause dRTA [23,25]. The transport function of eAE1 SAO is impaired in RBC and oocytes [25,27] although the protein could be inserted into plasma membrane [28]. The expression level and stability of eAE1 and kAE1 SAO proteins were significantly reduced and the mutant proteins were retained intracellularly in transfected HEK 293 and MDCK cells [29]. While homodimers of kAE1 and heterodimers of kAE1 and kAE1 SAO could traffic to the basolateral membrane of polarized cells, homodimers of kAE1 SAO were found to be retained in the endoplasmic reticulum and rapidly degraded.

To understand molecular mechanism of AR dRTA caused by compound heterozygous *AE1* SAO/G701D mutations, wild-type and mutant (SAO and G701D) kAE1 fused with different epitope tags or green fluorescent protein (GFP) were individually expressed and co-expressed in HEK 293 cells to examine protein expression, interaction, trafficking, and localization by several techniques. We found that trafficking defect of both kAE1 SAO and kAE1 G701D and their failure to rescue each other to the plasma membrane are most likely the molecular mechanism of the disease caused by this compound heterozygous condition.

Materials and methods

Plasmid construction. Wild-type (WT) *kAE1* cDNA was amplified from pcDNA3-kAE1 (a gift from Professor Reinhart A. Reithmeier) by PCR and inserted into *Not*I/*Xho*I-digested pcDNA3.1/HisB (Invitrogen, USA) to construct pcDNA3-kAE1 WT-His. pcDNA3-kAE1-HA expressing kAE1 with HA epitope fusion at its C-terminus was a gift from Miss Thitima Keskanokwong and Professor Joseph R. Casey. To generate pcDNA3.1-GFP, *GFP* gene was amplified by PCR from pDs-Green and cloned into *Eco*RV/*Not*I-cleaved pcDNA3.1 (+) vector (Invitrogen, USA). Then, *kAE1* was inserted into this plasmid at the site downstream to *GFP*, yielding pcDNA3.1-GFP-kAE1. The expressed fusion protein contained GFP linked to the N-terminus of kAE1. The plasmid constructs transformed in *Escherichia coli* were prepared by using QIAprep Spin Miniprep Kit (Qiagen, Germany) and examined for correct reading frames by DNA sequencing.

Site-directed mutagenesis. pcDNA3-*kAE1* WT-Myc, containing a sequence of Myc epitope inserted at the position 557 in the third extracellular loop of *kAE1*, was generated from pcDNA3-kAE1 by site-directed mutagenesis following the protocol of the QuickChange TM site-directed mutagenesis kit from Stratagene, USA. Eight plasmid constructs expressing kAE1 SAO or kAE1 G701D fused with either His, HA, Myc, or GFP (pcDNA3-kAE1 SAO-His, pcDNA3-kAE1 SAO-HA, pcDNA3-kAE1 SAO-Myc, pcDNA3.1 GFP-kAE1 SAO, pcDNA3-kAE1 G701D-His, pcDNA3-kAE1 G701D-HA, pcDNA3-kAE1 G701D-Myc or pcDNA3.1 GFP-kAE1 G701D) were created from the four plasmid constructs that were mentioned earlier by the site-directed mutagenesis method. Plasmid constructs propagated in *E. coli* were purified by QIAprep Spin Miniprep Kit (Qiagen, Germany). Mutations in these plasmid constructs were confirmed by DNA sequencing.

Cell culture and transfection. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PERBIO, UK), containing antibiotics, at 37 °C with 5% CO_2. The cultured cells at 40–60% confluent growth in 6-well plates were transfected with 2–5 μg of recombinant plasmid DNA per well either by using Lipofectin (Invitrogen, USA) according to the manufacturer's protocol or DEAE-dextran method [30]. The HEK 293 cells were individually transfected with each of the twelve plasmid constructs (four containing wild-type *kAE1* and eight consisting of mutant *kAE1*), and were also separately co-transfected with pcDNA-kAE1 WT-His and pcDNA-kAE1 SAO-HA (or pcDNA-kAE1 G701D-HA), pcDNA-kAE1 SAO-His and pcDNA-kAE1 G701D-HA, or pcDNA-kAE1 G701D-His and pcDNA-kAE1 SAO-HA, for expression or co-expression, affinity purification, co-immunoprecipitation, cellular localization, and cell-surface expression studies.

SDS–PAGE and Western blot analysis. Two days after transfection, the transfected HEK 293 cells were washed once with phosphate buffer saline (PBS) and lysed with lysis buffer on ice for 30 min. Proteins were collected after centrifugation and subjected to electrophoresis on 8% SDS–PAGE before transferring to nitrocellulose membranes. The membranes were blocked for 1 h with 5% skim milk in TBST (TBS with 0.1% Tween 20) and then incubated with either anti-His, anti-HA, anti-Ct AE1, or anti-Myc antibody for 2 h. After washing three times with TBST for 5 min each, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h. After washing another three times with TBST, the expressed proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) plus Western Blotting Detection System for 5 min according to manufacturer's instruction. The chemiluminescence signal was detected by exposing the membrane to an X-ray film in a cassette for 1 min and the film was developed by a film-developing machine.

Affinity co-purification. In this study, HEK 293 cells were individually transfected with the plasmid constructs or were co-transfected with four different pairs of the constructs described above. Two days after transfection, the cells were detached with PBS and collected by centrifugation at 3000*g* for 5 min. The cells were lysed with 500 μl of lysis buffer containing protease inhibitor cocktail on ice for 15 min. The insoluble fraction was removed by centrifugation at 4 °C. An aliquot of 400 μl of cell lysate