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# Transmembrane protein 139 (TMEM139) interacts with human kidney isoform of anion exchanger 1 (kAE1)

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ABSTRACT

Human kidney anion exchanger 1 (kAE1) mediates $Cl^-/HCO_3^-$ exchanges at the basolateral membrane of the acid-secreting α-intercalated cells. Mutations in *SLC4A1* gene encoding kAE1 are associated with distal renal tubular acidosis (dRTA). Several studies have shown that impaired trafficking of the mutant kAE1 is an important molecular mechanism underlying the pathogenesis of dRTA. Proteins involved in kAE1 trafficking were identified but the mechanism resulting in dRTA remained unclear. Thus, this study attempted to search for additional proteins interacting with C-terminal of kAE1 (Ct-kAE1) and involved in kAE1 trafficking to cell membrane. Transmembrane protein 139 (TMEM139) was identified as a protein interacting with Ct-kAE1 by yeast two-hybrid screening. The interaction between kAE1 and TMEM139 was confirmed by affinity co-purification, co-immunoprecipitation (co-IP) and yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA). In addition, flow cytometry results showed that suppression of endogenous TMEM139 by small interfering RNA (siRNA) and over-expression of TMEM139 in HEK293T cells could reduce and increase membrane localization of kAE1, respectively. The presented data demonstrate that TMEM139 interacts with kAE1 and promotes its intracellular trafficking.


## 1. Introduction

Human anion exchanger 1 (AE1) is a chloride and bicarbonate exchanger ($Cl^-/HCO_3^-$) which is involved in maintaining acid-base homeostasis [1]. It is encoded by *solute carrier family 4, anion exchanger, member 1 (SLC4A1)* gene and two AE1 isoforms, erythroid AE1 (eAE1) and kidney AE1 (kAE1), have been characterized [2]. The eAE1 contains 911 amino acids which organize into three structural and functionally distinct domains including a cytoskeleton-associated amino-terminal domain (360 amino acids), a central anion-transporting transmembrane domain (522 amino acids), and a short cytoplasmic carboxyl-terminal domain (29 amino acids) [3]. It is a major protein on red cell membrane, functioning in both electroneutral anion ($Cl^-/HCO_3^-$) exchange and cytoskeleton anchorage. The kAE1 lacks the first 65 amino acids present at the amino-terminus of human eAE1 and is expressed at the basolateral membrane of acid-secreting α-intercalated cells of kidney and mediates $Cl^-/HCO_3^-$ transport across the basolateral membrane to balance $H^+$ secretion across the apical surface into urine [4].

Mutations of *SLC4A1* gene can cause distal renal tubular acidosis (dRTA), which is characterized by an inability to acidify urine leading to systematic metabolic acidosis and several clinical manifestations. The *SLC4A1* mutations generate mutant kAE1 that maintains functions of anion exchanger activity but exhibits a basolateral trafficking defect or mis-targeting the mutants to the apical membrane instead of the correct basolateral membrane of the α-intercalated cells. Our group has been interested in identifying the protein that interacts with kAE1 and plays a role in its basolateral trafficking. We have reported several proteins that interact with kAE1 such as integrin-linked kinase (ILK), an actin binding protein, which acts as an interacting partner with the N-terminal of kAE1 [5]. In addition, adaptor-related protein complex 1 μ1A (AP-1 mu1A) [6], adaptor-related protein complex 3 μ1 (AP-3 mu1), adaptor-related protein complex 4 μ1 (AP-4 mu1), clathrin

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[7] and kinesin family member 3B (KIF3B) [8] were recently identified as partner proteins interacting with Ct-kAE1 and may play a role in kAE1 sorting and trafficking.

Since the molecular mechanism of kAE1 trafficking in kidney cells has not thoroughly been elucidated, this study was performed to identify additional kAE1 interacting proteins by yeast two-hybrid screening. Transmembrane protein 139 (TMEM139) was identified as a protein that interacts with Ct-kAE1. The interaction between TMEM139 and kAE1 was confirmed by affinity co-purification, co-immunoprecipitation, and yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA). Endogenous TMEM139 suppression by interference RNA and TMEM139 over-expression were also demonstrated to have effects on kAE1 expression at the cell surface of HEK293T cells.

## 2. Materials and methods

### 2.1. Plasmid constructions

Two plasmids namely pGBKT7-Ct-kAE1 and pcDNA3.1/Zeo-YFP [1]-kAE1 were constructed and reported in our previous study [6]. pcDNA3.1/Zeo-YFP[1] and pcDNA3.1/Zeo-YFP[2] were the gifts from Professor Stephen W. Michnick, University of Montreal, Canada. pGBKT7-Ct-kAE1 used in yeast two-hybrid screening contains DNA fragments encoding for the Ct-kAE1 expressed a fusion protein with GAL4-DNA binding domain. pcDNA3.1/Zeo-YFP[1]-kAE1 and pcDNA3.1/Zeo-YFP[2] encoded for two separate fragments of yellow fluorescent protein (YFP) inserted between the *Nhe*I/*Hin*dIII sites of the pcDNA3.1/Zeo vectors (Invitrogen, San Diego, CA, USA). pcDNA3.1/Zeo-YFP[1]-kAE1 encodes for a fusion protein of YFP[1] and full-length kAE1 (911 amino acids). PCR-based amplification using a cDNA library derived from human kidney tissue (Invitrogen, Carlsbad, CA, USA) as a template was performed to generate a full-length cDNA encoding TMEM139 (670 bp) flanked by *Bam*HI and *Xho*I sites. The amplified fragments were inserted in pcDNA3.1/HisB and pcDNA3.1/Zeo-YFP[2] to generate pcTMEM139-His and pcDNA3.1/Zeo-YFP[2]-TMEM139, respectively. The inserted sequences in all constructs and their in-frame junctions were confirmed by DNA sequencing.

### 2.2. Yeast two-hybrid screening

Yeast AH109 strain transformed with pGBKT7-Ct-kAE1 was used as the bait strain. The bait construct was previously tested [6] for correct protein expression prior to use for library screening. The prey strain, Y187, was pre-transformed with the prey plasmid, pACT2, which carried the GAL4-activation domain fused to a fragment from a human kidney cDNA library. The yeast two-hybrid screening was performed by following the manufacturer's protocol (Clontech). Mated diploids whose cDNA-encoded products interacted with the bait protein were selected by growth on SD/-Trp/-Leu/-His/-Ade plates supplemented with X-α-gal. The colonies with blue color when grown on the dropout plates were considered as positive clones. The prey plasmids were rescued from the positive colonies by transformation into *E. coli*. The isolated plasmids were used as a template to amplify the cDNA inserted fragments. The *Alu*I restriction patterns of the PCR products were generated and the representatives from different restriction patterns were chosen for the specificity tests. Specific interactions between the bait protein and the encoded products of isolated preys were tested by re-transforming the bait plasmid, the prey plasmid, empty vector, and two other plasmids containing unrelated genes (p53 and lamin C) into the swabbing yeast strains. The cDNA fragments of the positive clones from the specificity tests were sequenced and aligned with sequences from the NCBI database.

### 2.3. Cell culture and transfection

HEK293T cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% $CO_2$. Two days before transfection, the HEK293T cells were collected and plated in 6-well plates. The cultured cells were transfected with pcDNA3.1 vector or its derivative constructs according to the designed experiments by Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were collected for further analyses at 48 h post-transfection.

### 2.4. Affinity co-purification

The HEK293T cells were transfected with two plasmids, pckAE1 tagged with Myc (pckAE1-Myc) or pcDNA3-kAE1 containing full-length cDNA of kAE1, which was a kind gift from Professor Reinhart Reithmeier, University of Toronto, Canada, and pcTMEM139-His (plasmids containing coding sequence of the TMEM139 tagged with poly-histidine), and then cultured for 48 h. The transfected cells were collected and lysed in lysis solution. The mixture was added to $Co^{2+}$-chelate resin (BD Biosciences, Franklin Lakes, NJ, USA). The samples were subjected to SDS-PAGE and immunoblotting. The presences of kAE1 and candidate proteins were detected by anti-Myc and anti-His antibody, respectively.

### 2.5. Co-immunoprecipitation

Co-immunoprecipitation (co-IP) assay was also performed. HEK293T cells were co-transfected with pckAE1-Myc and plasmids containing coding sequence of the candidate proteins. Two days after transfection, the transfected cells were detached and collected by centrifugation. Cells were lysed and aliquots of the cell lysates were kept, the remaining cell lysate incubated with the anti-Myc antibody followed by precipitation with Protein G-Sepharose (Thermo Scientific, Waltham, MA, USA). The bound kAE1 protein and the candidate partners were eluted and detected by SDS-PAGE and immunoblotting using anti-Myc and anti-His antibody, respectively.

### 2.6. Yellow fluorescent protein (YFP) – based protein fragment complementation assay (PCA)

Two days before transfection, the HEK293T cells were collected and plated in 6-well plates. The cultured cells were individually transfected with 1 μg each of the YFP construct or co-transfected with its pairs of the constructs. Two days after transfection, the cells were fixed in 3.6% formaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 1% BSA. The coverslips were washed and mounted with Fluorosave. Cell fluorescence images were observed by using a fluorescent microscope with a green fluorescent filter at a wavelength of 395 nm.

### 2.7. RNA interference of TMEM139 and over-expression of TMEM139

Small interfering RNA (siRNA) derived from the mRNA sequence (5′CAGACCUCGUUCAUUUGACUAACAU3′) and directed against TMEM139, was purchased from Invitrogen (Waltham, MA, USA). The efficiency of TMEM139 knock down was examined by


**Fig. 1. Specificity test of kAE1 and TMEM139 interaction by growth of yeast diploids on synthetic dropout (SD)/-Ade-His-Leu-Trp/X-α-Gal agar plates**. +ve represents mated diploids between Y187 harboring pGBKT7-p53 and AH109 containing pTD1-SV40-T Ag. The -ve represents mated diploids between Y187 harboring pGBKT7-lamin C and AH109 containing pTD1-SV40-T Ag. TM, V, p53, and lamin C are mated diploids between Y187 containing pGBKT7-Ct-kAE1 (bait plasmid), and AH109 harboring prey plasmid which contained TMEM139 sequence (column TM), pGBKT (empty vector; column V), pGBKT7-p53 (unrelated gene, column p53), or pGBKT7-lamin C (unrelated gene; column lamin C), respectively. Positive interaction between Ct-kAE1 and TMEM139 was confirmed (TM) by growth and turning blue on SD/-Ade-His-Leu-Trp/X-α-Gal plates.

real-time PCR. Transfection of siTMEM139 was performed twice with a 24-h interval, using Lipofectamine 2000 (Invitrogen). HEK293T cells were plated into a 6-well plate, 24 h before the first knock down. pckAE1-Myc and/or pcTMEM139-His were transfected parallel with the second transfection of siTMEM139. The cells were harvested 48 h after the second transfection for further analysis.

### 2.8. Measurement of cell surface kAE1 expression by flow cytometry

The cells that were prepared as described in section 2.7 were collected and resuspended in chilled Buffer A solution (2% fetal bovine serum DMEM, 1% BSA and 10 mM $NaN_3$). The mixture was incubated with rabbit anti-Myc antibody in Buffer A at 4 °C for 3 h. They were then washed three times with chilled Buffer A and incubated in donkey anti-rabbit IgG conjugated with Alexa 488 fluorescein at 4 °C for 30 min. The cells were washed three times again and then fixed in chilled 1% formaldehyde before analysis using Beckman–Coulter EPICS Elite (BD Biosciences). The percentage of fluorescence-stained cells was determined to quantify the level of the cell surface expression.

## 3. Results

### 3.1. Identification of TMEM139 as a Ct-kAE1-binding protein by yeast two-hybrid screening

To search for candidate proteins that could interact with kAE1, a yeast two-hybrid screening was performed using the C-terminal

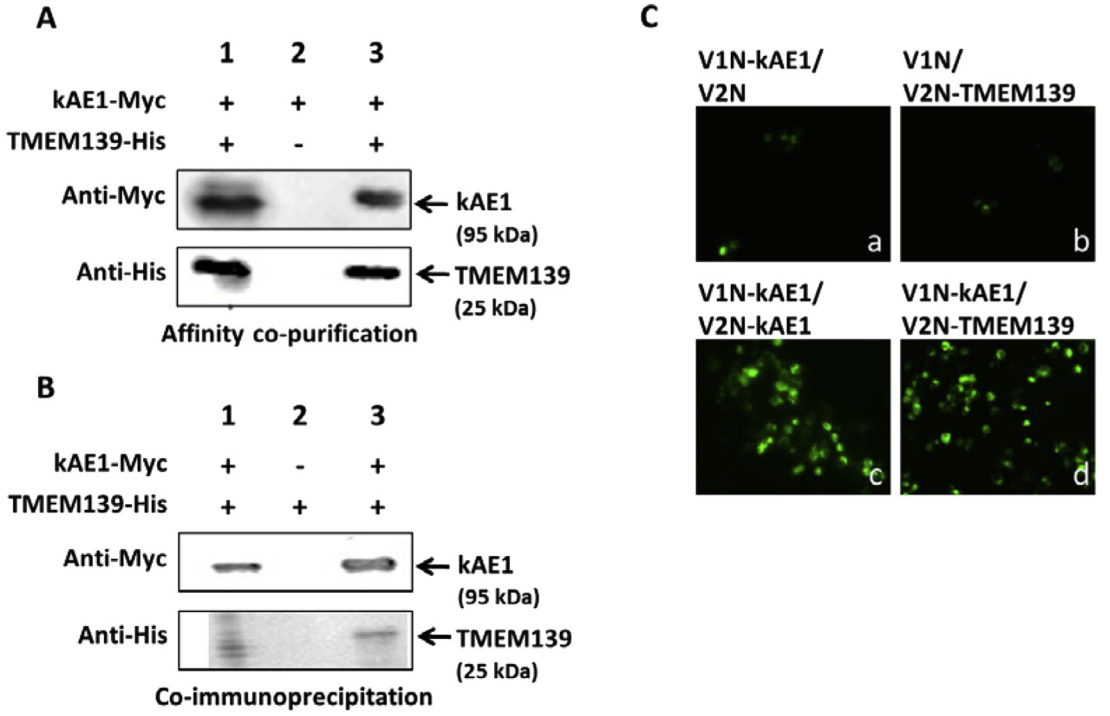


**Fig. 2. Verification of kAE1 and TMEM139 interaction by affinity co-purification, co-immunoprecipitation assays and YFP-PCA**. (A) Affinity co-purification of kAE1 and TMEM139 expressed in HEK293T cells. Lysate from individually transfected or co-transfected HEK293T cells were incubated with $Co^{2+}$ beads. Input and affinity-purified samples were resolved on SDS-PAGE and analyzed by western blot method. Lane 1 is cell lysate inputs. Lanes 2 and 3 are purified samples. Lane 3 illustrated co-purified kAE1-Myc with TMEM139-His. The co-purified complex was detected by both anti-Myc and anti-His antibodies. (B) Co-immunoprecipitation of kAE1 and TMEM139 expressed in HEK293T cells. Lysate from individually transfected or co-transfected HEK293T cells were incubated with anti-Myc antibody to immunoprecipitate kAE1-Myc and associated complexes. Input and immunoprecipitated samples were resolved on SDS-PAGE and analyzed by western blot method. Lane 1 is cell lysate inputs. Lanes 2 and 3 are immunoprecipitated samples. Lane 3 illustrated the co-immunoprecipitation of TMEM139-His with kAE1-Myc. The co-immunoprecipitated complex was detected by both anti-Myc and anti-His antibodies. (C) The cells were co-transfected to express (a) V1N-kAE1 and V2N or (b) V1N and V2N-TMEM139 as negative control, showing no fluorescent signal. (c) The cells were co-transfected to express V1N-kAE1 and V2N-kAE1 as positive control; dimerization of kAE1 resulted in V1N and V2N complementation generating intracellular green fluorescent signals. (d) The cells were co-transfected to express V1N-kAE1 and V2N-TMEM139, showing intracellular green fluorescent signals indicating their interaction.

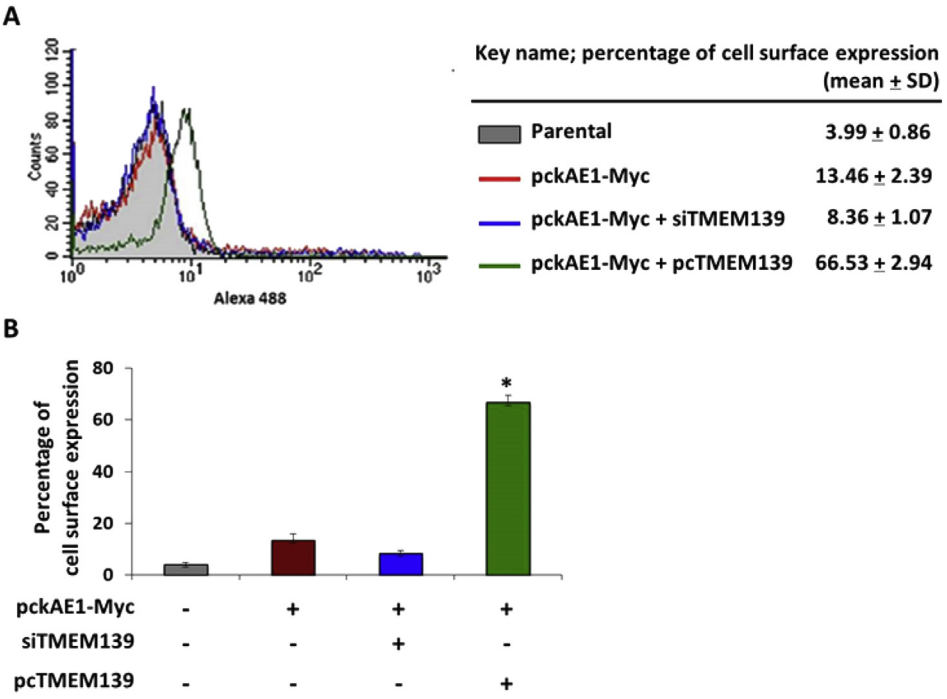


**Fig. 3. Cell-surface expression of kAE1-Myc in transfected HEK293T cells measured by flow cytometry**. (A) The transfected cells were incubated with mouse anti-Myc antibody followed by anti-mouse IgG antibody conjugated with Alexa 488. Fluorescence intensities were detected by flow cytometry with kAE1-positive cells showing heterogeneous expression above background levels for parental HEK293T cells (gray box), kAE1-Myc expressed cells (red line), co-transfection of kAE1-Myc with siTMEM139 (blue line) and co-transfection of kAE1-Myc with pcTMEM139 (green line). Percentages of cell surface expression of kAE1-Myc (mean ± SD) in different conditions are also indicated. (B) The bar graph showing percentages of cell-surface expression of kAE1-Myc. Colors of histograms are corresponding to the colors of flow–cytometry profiles in (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

part of kAE1 (Ct-kAE1, amino acids 876–911) as a bait. The bait construct, pGBKT7-Ct-kAE1, was used to screen a human kidney cDNA library. One positive clone containing a cDNA sequence encoding for TMEM139 protein (amino acid 1–670) was selected. The prey plasmids containing complete TMEM139 cDNA sequence isolated from the initial screen were re-transformed for re-mating. Mated diploid cells containing Ct-kAE1 and TMEM139 cDNA sequence could grow on the selective medium and showed a blue colony indicating the activation of reporter genes in these cells (Fig. 1).

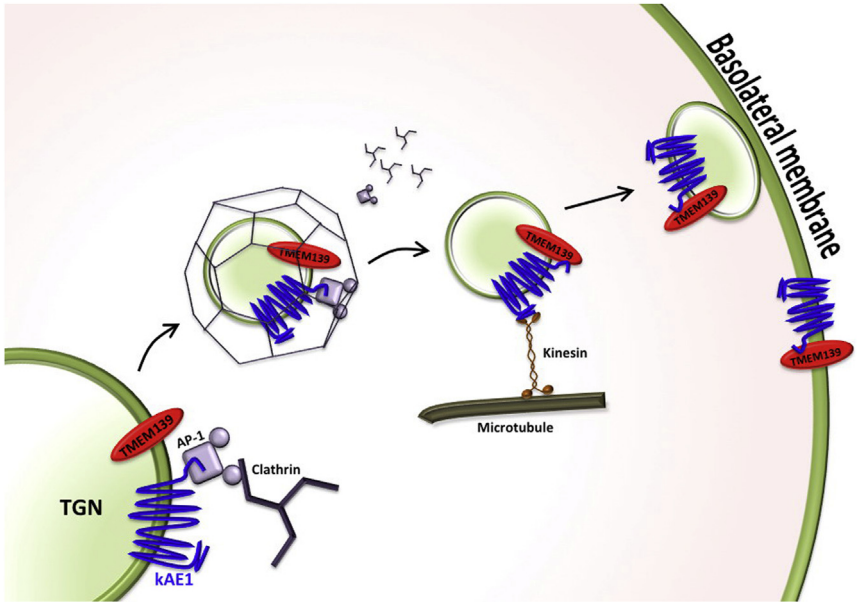


**Fig. 4. Proposed model for kAE1 trafficking and kAE1-TMEM139 complex**. We previously reported that kAE1 interacts with AP1 mu1A and clathrin. Kinesin, a motor protein, interacts with kAE1 in the kAE1-TMEM139 complex to transport this complex along microtubule to express at the basolateral membrane of the acid secreting alpha-intercalated cells in the distal tubule of nephron in the kidney.

### 3.2. kAE1 interacted with TMEM139 in HEK293T cells as examined by affinity co-purification, co-immunoprecipitation (co-IP) and yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA)

HEK293T cells were co-transfected with expression plasmids which produce TMEM139-His and kAE1-Myc. Affinity co-purification was performed using Cobalt ($Co^{2+}$) resins, which bound to His-tagged proteins. The results showed that kAE1-Myc was co-purified with TMEM139-His as detected by anti-Myc antibody (Fig. 2A, lane 3). Co-IP of kAE1-Myc using anti-Myc antibody could detect TMEM139-His as analyzed by using anti-His antibody in western blot analysis (Fig. 2B, lane 3).

YFP-PCA was used to investigate the interaction between kAE1 and TMEM139 within HEK293T cells by detection of intracellular green fluorescent signals. The HEK293T cells were co-transfected to express V1N-kAE1 and V2N-kAE1 as a positive control, with the dimers of kAE1 resulted in V1N and V2N complementation (Fig. 2C, picture c) showing intracellular green fluorescent signals. Similarly, when the cells were co-transfected to express V1N-kAE1 and V2N-TMEM139, intracellular green fluorescent signals were observed (Fig. 2C, picture d).

### 3.3. Suppression of the endogenous TMEM139 by siRNA and over-expression of TMEM139 reduced and increased membrane protein kAE1, respectively

HEK293T cells were co-transfected with either pckAE1-Myc and siTMEM139 or pckAE1-Myc and pcTMEM139-His to examine the effect of suppression and over-expression of TMEM139 protein. The efficiency of siRNA was determined by real-time PCR and western blot analysis (data not shown). Flow cytometry was used to detect the kAE1 expression at the cell surface and it was found that parental HEK293T cells and the cells expressing kAE1-Myc had mean fluorescence intensities of 3.99 ± 0.86% and 13.46 ± 2.39%, respectively (Fig. 3). HEK293T cells with suppression of TMEM139 showed lower level of kAE1-Myc on the cell surface (8.36 ± 1.07%) than those of control cells, but they showed a higher level of kAE1-Myc (66.53 ± 2.94%) in the cells with over-expression of TMEM139.

## 4. Discussion

Yeast two-hybrid screening by using Ct-kAE1 as bait was used to isolate proteins interacting with kAE1. cDNA encoding for TMEM139 was obtained and chosen for this study, as TMEM139 has previously been shown to interact with a protein namely SMURF2 [9]. This protein is an E3 ubiquitin-protein ligase which functions in protein trafficking by controlling interaction between WW-domain of the ubiquitin and PY-domain of protein target or adaptor protein [10]. It was proposed here that TMEM139 may play a role as an adaptor protein facilitating trafficking of kAE1. However, biological function of TMEM139 in human has not been reported.

TMEM139, a member of the type I transmembrane protein [11], is the protein spanning the membrane once with its N-terminus on the extracellular side of the membrane. Interaction between kAE1 and TMEM139 *in vitro* was observed by affinity co-purification and co-IP. The interaction in human kidney (HEK293T) cells was also shown by YFP-PCA. The endogenous TMEM139 was suppressed by small interfering RNA (siRNA) in HEK293T cells as detected by real-time PCR and western blot analysis (data not shown). This result is consistent with the significant reduction in kAE1 expression at the cell surface as analyzed by flow cytometry. In contrast, over-expression of TMEM139 in HEK293T cells resulted in higher level of kAE1 expression (Fig. 3). TMEM139 may function in intracellular kAE1 trafficking by unknown mechanisms. Since both kAE1 and TMEM139 are transmembrane proteins that interact with each other, they may co-traffic to cell membrane; thus, TMEM139 may act as a chaperone protein of kAE1, in a similar manner as glycophorin A (GPA) which is a chaperone protein of eAE1 [12].

We previously reported the interaction between “kAE1 and AP-1 mu1A” [6] and “kAE1 and KIF3B” [8]. AP-1 mu1A knock down reduced kAE1 expression on cell surface and also increased the accumulation of kAE1 in ER. It was suggested that AP-1 mu1A is required for the transportation of a cargo protein from TGN to the plasma membrane. In addition, it was found that a dileucine motif at Ct-kAE1, which was reported to be involved in basolateral trafficking [4], interacts with KIF3B. KIF3B or kinesin-like protein KIF3B also interacts with Nt-kAE1 and acts as molecular motors that transport the intracellular cargo from TGN along the microtubules. Thus, kAE1-TMEM139 may be a molecular complex involved in kAE1 transportation to express at the basolateral membrane (Fig. 4).

Further studies will be directed toward the role of TMEM139 in mutant kAE1 trafficking at the basolateral membrane such as in the G701D and SAO mutations. Taken together, the results of our study provide a better understanding on the trafficking machinery, protein–protein interaction and subcellular localization of kAE1 both in normal and abnormal conditions.

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