รายงานวิจัยฉบับสมบูรณ์

โครงการ

“การค้นหาและศึกษาคุณสมบัติของโปรตีนซึ่งจับกับโปรตีน anion exchanger 1 ที่พบในไตมนุษย์”

โดย

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บทคัดย่อ

ความผิดปกติของโปรตีนแอนไอออน เอ็กเช็งเจอร์-วัน ชนิดที่พบในไต (kidney anion exchanger 1 หรือ kAE1) อันเนื่องมาจากการกลายพันธุ์ของยีน *AE1* (หรือยีน *SLC4A1*) ทำให้เกิดโรคไตผิดปกติในการขับกรด (distal renal tubular acidosis) ผู้ป่วยโรคนี้มีสภาวะเป็นกรดในร่างกาย เนื่องจากไตไม่สามารถขับกรดออกทางปัสสาวะได้ จากผลการทดลองในเซลล์เพาะเลี้ยงชนิดที่ทำให้เกิดขั้ว (polarized cultured cells) พบว่า ในขณะที่โปรตีนปกติสามารถจะเคลื่อนย้ายไปอยู่ที่ส่วนฐานของเยื่อหุ้มของเซลล์ (basolateral membrane) โปรตีนที่ผิดปกติกลับค้างอยู่ภายในเซลล์ หรือถูกส่งผิดไปยังส่วนยอดของเยื่อหุ้มเซลล์ (apical membrane) อย่างไรก็ตาม ข้อมูลเกี่ยวกับการเคลื่อนย้ายของโปรตีน kAE1 หรือตัวบ่งชี้ว่าความล้มเหลวในการเคลื่อนย้ายของโปรตีนชนิดนี้ เกิดขึ้นได้อย่างไรยังไม่มีใครทราบ การเคลื่อนย้ายอย่างถูกต้องของโปรตีน kAE1 อาจจะจำเป็นต้องอาศัยส่วนทั้งที่อยู่ทางด้านปลายอะมิโน (amino terminus) และปลายคาร์บอกซี (carboxyl terminus) ของโปรตีน การเข้าใจกระบวนการเคลื่อนย้ายของโปรตีน kAE1 มีความจำเป็นจะต้องรู้ว่าโปรตีนอะไรบ้างที่มีปฏิสัมพันธ์กับโปรตีน kAE1 คณะผู้วิจัยจึงใช้วิธียีสต์ทู-ไฮบริด (yeast two-hybrid method) ในการตรวจกรองเพื่อค้นหาโปรตีนจากไตมนุษย์ที่จับกับโปรตีน kAE1 (kAE1-binding protein หรือ kAE1-BP) โดยใช้ส่วนของโปรตีน kAE1 ทางด้านปลายอะมิโน (amino terminus) และปลายคาร์บอกซี (carboxyl terminus) ซึ่งมีกรดอะมิโนจำนวน 338 และ 36 ตัว เป็นเหยื่อ (bait) เพื่อใช้จับกับโปรตีนที่ได้มาจากซี-ดีเอ็นเอ (cDNA) ซึ่งเตรียมมาจากไตมนุษย์ ลำดับนิวคลีโอไทด์ส่วนหนึ่งของซี-ดีเอ็นเอของโปรตีนที่ได้ ถูกนำมาใช้ในการในการค้นหาลำดับนิวคลีโอไทด์ของซี-ดีเอ็นเอที่สมบูรณ์และชนิดของโปรตีนในฐานข้อมูล NCBI GenBank และฐานข้อมูล EMBL โดยวิธี BLAST search จากการทดลองพบว่ามีโปรตีนหลายชนิดสามารถจับได้กับบริเวณปลายทั้งสองของโปรตีน kAE1 จากข้อพิจารณาด้านหน้าที่ของโปรตีนและข้อพิจารณาทางเทคนิค โปรตีน integrin-linked kinase (ILK) และ adaptor protein subunit mu1A (AP1mu1A) ได้ถูกคัดเลือกและนำมาศึกษาในเซลล์ชนิด human embryonic kidney (HEK 293) เพื่อยืนยันว่ามีปฏิสัมพันธ์กับโปรตีน kAE1 และหาความสำคัญทางชีววิทยาด้วยวิธีต่างๆ ได้แก่ co-immunoprecipitation, affinity co-purification, immunofluorescence staining และ cell surface biotinylation ผลการทดลองแสดงให้เห็นว่าโปรตีน kAE1 สามารถจะถูกตกตะกอนและถูกแยกร่วมกับ ILK และ AP1mu1 และพบว่าปรากฏอยู่ด้วยกันที่ผิวเซลล์ การศึกษาโดยวิธี flow cytometry พบว่าการแสดงออกของโปรตีน AP1mu1A ไม่มีผลต่อการปรากฏของโปรตีน kAE1 ที่ผิวเซลล์ ในขณะที่การศึกษาด้วยวิธี cell surface biotinylation แสดงให้เห็นว่า ILK ช่วยให้โปรตีน kAE1 ปรากฏบนผิวเซลล์ และ ILK ยังช่วยเพิ่มการแลกเปลี่ยนคลอไรด์และไบคาร์บอเนต (Cl^-/HCO_3^-) ของ kAE1 อีกด้วย ผลการศึกษาต่อมาแสดงให้เห็นว่าโปรตีน ILK ช่วยให้โปรตีน kAE1 ปรากฏที่ผิวเซลล์โดยการรวมตัวเป็นกลุ่ม (complex) กับโปรตีนชนิดอื่น ได้แก่ kAE1-paxillin-actopaxin ซึ่งช่วยเชื่อม kAE1 กับโปรตีนเอ็คตินโครงสร้างของเซลล์ (actin cytoskeleton) ข้อมูลที่ได้แสดงว่าโปรตีน ILK เป็นตัวเชื่อมโยงระหว่างโปรตีน kAE1 และโปรตีนเอ็คตินโครงสร้าง ซึ่งจะช่วยทำให้โปรตีน kAE1 อยู่สภาวะเสถียรและปรากฏเพิ่มขึ้นที่ผิวเซลล์

Abstract

Defect of human kidney anion (Cl^-/HCO_3^-) exchanger 1 (kAE1) protein caused by mutations of *AE1* (*SLC4A1*) gene results in a human kidney disease – distal renal tubular acidosis (dRTA), characterized by metabolic acidosis due to defective acid secretion in the distal nephron. While the wild-type kAE1 is normally located at the basolateral membrane of polarized cultured cells, the mutant proteins exhibited intracellular retention or apical mis-targeting. There is as yet no information as to the proteins involved in kAE1 trafficking process nor is there any indication as to how this process fails. The correct trafficking of kAE1 may require both of its amino- (N) and carboxy- (C) terminal regions. To understand the regulation of kAE1 trafficking process, it is necessary to identify the proteins that interact with it. Yeast two-hybrid (Y2H) system was thus employed to screen for kAE1-binding proteins (kAE1-BP) from human kidney cDNA library by using both N- and C-terminal regions of human kAE1, consisting of 338 and 36 amino acids, respectively, as baits. The partial sequences of prey-cDNAs obtained from the screening and sequencing were analyzed with homology BLAST search for full-length cDNA and proteins in the NCBI GenBank and EMBL databases. Many putative kAE1-BPs of both termini have been identified. From functional and technical consideration, integrin-linked kinase (ILK) and adaptor protein subunit mu1A (AP1mu1A) were selected for investigation in human embryonic kidney (HEK 293) cells to confirm their interaction with kAE1 and to understand their biological significance by using co-immunoprecipitation, affinity co-purification, immunofluorescence staining, and cell surface biotinylation methods. The results showed that kAE1 co-precipitated and co-purified with ILK or AP1mu1 as well as co-localized with either protein at the cell surface. The effect of AP1mu1A over expression on cell surface expression of kAE1 was examined by flow cytometry, showing that AP1mu1A did not affect to kAE1 expression at the cell surface, whereas cell surface biotinylation result showed that ILK promoted cell surface expression of kAE1 and it also enhanced Cl^-/HCO_3^- transport activity of kAE1. Further investigation found that ILK promoted kAE1 expression at the cell surface in HEK 293 cells by forming a kAE1-ILK-paxillin-actopaxin complex linking kAE1 to the actin cytoskeleton. These experimental data demonstrate that ILK provides a linkage between kAE1 and the underlying actin cytoskeleton to stabilize kAE1 at the plasma membrane, resulting in the higher level of cell surface expression.

Keyword: kindey anion exchanger 1 (kAE1), distal renal tubular acidosis (dRTA), yeast two-hybrid (Y2H), kAE1-binding proteins (kAE1-BP), integrin-linked kinase (ILK), adaptor protein subunit mu1A (AP-1 mu1A)

Executive Summary

Kidney anion exchanger 1 (kAE1) mediates Cl^-/HCO_3^- exchange at the basolateral membrane of kidney α-intercalated cells. It is an isoform of erythroid AE1 (eAE1) and lacks 65 amino acids at its amino terminus. This may cause some structural changes and altered protein-protein interaction in kAE1. Several mutations in the *AE1* gene have been found and associated with distal renal tubular acidosis (dRTA) such as *AE1* SAO, R589H, G701D, A858D, and R901X. dRTA is involved in defects of protein trafficking or mis-targeting to an appropriate site of the α-intercalated cells. R901X, resulting in a deletion of 11 amino acids at the C-terminus of kAE1 has been extensively studied, since mistargeting of kAE1 from basolateral to apical membrane is consistent with removal of basolateral localization signal at its C-terminus. The proteins interacting with motifs on both N- and C-termini may involve in kAE1 trafficking. The mechanism purposed is that an alteration of the mutant kAE1 interaction with chaperones or adaptor proteins preventing it to move to the cell surface or sending them to an inappropriate site. However, kAE1 adaptor proteins and mechanism of kAE1 trafficking in the kidney α–intercalated cells have not yet been identified. This study thus aims to identify proteins interacting with the N- and C-terminal kAE1 that may be involved in the kAE1 trafficking. The interacting proteins were screened from human kidney cDNA library by yeast two-hybrid. The selected interacting proteins are integrin-linked kinase (ILK) and AP1mu1 that use N- and C-terminal kAE1 for screening, respectively. Interaction between kAE1 with selected proteins was demonstrated in the co-expression experiments including co-immunoprecipitation, affinity co-purification, immunofluorescence staining, and cell surface biotinylation in human embryonic kidney (HEK 293) cells. The results demonstrated that kAE1 was co-precipitated and co-purified with AP1mu1 or ILK and two proteins (kAE1 with AP1mu1 or kAE1 with ILK) were co-localized in the plasma membrane. The effect of AP1mu1A over expression on cell surface expression of kAE1 was examined by flow cytometry, showing that AP1mu1A did not affect to kAE1 expression at cell surface. Interestingly, cell surface biotinylation result showed that ILK promoted cell surface expression of kAE1 and also enhanced Cl^-/HCO_3^- transport activity of kAE1. AP-1 complex is a heterotetramer containing mu1 and other subunits. Thus, over-expression of only AP1mu1A may not be

adequate to affect kAE1 trafficking to the cell surface and other subunits may also be required to demonstrate this effect. Mapping analysis revealed that kAE1 interacts with the C-terminus of ILK through the kAE1 calponin homology (CH) domain. In addition, the ankyrin repeats, PH domain and kinase activity of ILK were not required for binding ability to kAE1 and cell surface expression of kAE1. ILK also interacted with eAE1 suggesting that lack of the 65 amino acids of kAE1 would not alter the folded structure of CH domain in eAE1. Further investigation found that ILK promotes kAE1 expression at the cell surface in HEK 293 cells by forming kAE1-ILK-paxillin-actopaxin complex links kAE1 to the actin cytoskeleton. This study presents data demonstrating that ILK provides a linkage between kAE1 and the underlying actin cytoskeleton to stabilize kAE1 at the plasma membrane, resulting in the higher level of cell surface expression.

Objective

This project aims to isolate, identify and characterize human kidney proteins that interact with kidney anion exchanger 1 (kAE1); this is an initial step to study kAE1 trafficking in normal and abnormal conditions in human α-intercalated cells and molecular pathogenesis of a human kidney disease – distal renal tubular acidosis (dRTA).

Background and Rationale

Anion (Cl^-/HCO_3^-) exchanger 1 (AE1, band 3) is a membrane protein that involves in bicarbonate transport and maintaining acid-base homeostasis in human body. *AE1* gene (also known as *SLC4A1*) encodes both erythroid AE1 (eAE1) and kidney AE1 (kAE1). Transcription of the eAE1 in erythroid precursors is under the control of an erythroid-specific promoter upstream of exon 1 while the renal transcription arises from a distinct promoter within intron 3 of the *AE1* gene. kAE1 has 65 amino acids at the N-terminus shorter than eAE1. eAE1 serves the dual roles of Cl^-/HCO_3^- exchange and cytoskeletal anchorage of red cell membrane whereas kAE1 is the basolateral Cl^-/HCO_3^- exchanger of the acid-secreting type A intercalated cells of the kidney. The eAE1 is composed of 911 amino acids and its N-terminal cytoplasmic domain (~400 amino acid) is involved in interactions with ankyrin-1 and proteins 4.1 and 4.2, as well as glycolytic enzymes and hemoglobin. However, the shorter N-terminus of kAE1 does not bind to ankyrin, band 4.1, and aldolase, which may be due to the altered structure of cytoplasmic domain of kAE1. The identity of kAE1-binding proteins (kAE1-BPs) in the intercalated cells remains unknown, leading to the question: what are proteins that bind to the cytoplasmic domain of kAE1?

Since kAE1 is important in the process of bicarbonate reabsorption across the basolateral membrane of the type A intercalated cells of the renal collecting duct, the defect of kAE1 will lead to distal renal tubular acidosis (dRTA), the disease characterized by the incapability of the kidney to acidify urine in the presence of systemic acidosis, which is usually accompanied by hypocitraturia, hypercalciuria, hypokalemia, metabolic bone disease, nephrocalcinosis, and chronic renal insufficiency. Several mutations in *AE1* gene have been found to be associated with both autosomal dominant and autosomal recessive dRTA. The *AE1* mutations associated with autosomal dominant dRTA are missense mutations at the position 589 where arginine is substituted by histidine, serine or cysteine (R589H, R589S, R589C), at the position 613 where serine is substituted by phenyalanine (S613F), and a deletion of 11 amino acids at the carboxy terminus (AE1Δ11) while the *AE1* mutations linked to autosomal recessive dRTA are a change of glycine at the position 701 to aspartic acid (G701D), a deletion of valine at the position 850 (ΔV850) and a

substitution of alanine at the position 858 to aspartic acid (A858D). These mutations do not cause defect of Cl^-/HCO_3^- exchange but result in impaired trafficking or mistargeting of the mutant proteins. In cases of mutations associated with autosomal dominant dRTA, the trafficking of the wild-type is retarded by the mutant proteins, so called the dominant negative effect. However, it has yet been unknown how the protein trafficking fails or why mistargeting of kAE1 occurs. Therefore, it is necessary to identify kAE1-BPs to understand kAE1 trafficking and targeting in the normal and abnormal conditions.

The yeast-two hybrid system is a powerful method to demonstrate protein-protein interaction *in vivo*. It can be carried out to screen for novel proteins from cDNA library that specifically interact with a target protein of interest. The principle of this system is based on the modular structure of a eukaryotic transcription factor known as DNA-binding domain (DNA-BD) and activation domain (AD) without the necessity that they are covalently attached. Therefore, a bait gene is expressed as a fusion to the DNA-BD while another cDNA is expressed as a fusion to the AD. When the bait and library fusion protein interact in a yeast reporter strain, the DNA-BD and AD are brought into proximity and activate transcription of reporter genes. In this study, yeast-two hybrid screening was conducted to isolate proteins from cDNA library derived from human kidney cells that interact with the large N-terminal and C-terminal regions of kAE1. It has been known that the expression of full-length kAE1 is difficult in yeast because of the presence of 12-14 transmembrane domains which may be toxic to the yeast cells. The true interaction between kAE1 and kAE1-BPs would be confirmed and verified by co-immunoprecipitation, affinity co-purification, and immunofluorescence studies. If kAE1-BPs are identified, they will contribute to the understanding of normal and abnormal trafficking and targeting of human kAE1 and will provide a new insight into molecular mechanisms associated with the pathogenesis of dRTA.

Literature Review

Human anion exchanger 1 (AE1, band 3) is a prototype member of bicarbonate transporter superfamily involving in maintenance of acid-base homeostasis in human body. AE1 is encoded by *solute carrier family 4, anion exchanger, member 1* (*SLC4A1*) or *AE1* gene located on chromosome 17q21-22. The *AE1* gene comprises 20 exons. It encodes erythroid AE1 (eAE1), the major integral protein that serves the dual roles of Cl^-/HCO_3^- exchange and cytoskeletal anchorage of red cell membrane, and kidney AE1 (kAE1), which is the basolateral Cl^-/HCO_3^- exchanger of the acid-secreting type A intercalated cell of the kidney. Transcription of the eAE1 in erythroid precursors is under the control of an erythroid-specific promoter upstream of exon 1, while the renal transcription arises from a distinct promoter within intron 3 of the *AE1* gene. Thus, the resultant kidney transcripts encode kidney-specific kAE1 polypeptides lacking 65 amino acids at the N-terminus [1].

The eAE1 is composed of 911 amino acids and forms two structurally and functionally distinct domains. The ~500 amino acid C-terminus constitutes a highly conserved domain that is predicted to transverse the lipid bilayer up to 12-14 times [2]. This domain is responsible for anion transport and remains functional either when translated from cDNA clone encoding the isolated membrane domain or after proteolytic removal of a short cytoplasmic C-terminal tail contain a binding site of carbonic anhydrase (CA) II [3]. The ~400 amino acid N-terminal cytoplasmic domain is involved in a number of functions unrelated to anion transport such as anchoring the membrane protein to cytoskeleton via interactions with ankyrin-1 and proteins 4.1 and 4.2, as well as glycosolic enzymes and hemoglobin [4, 5]. Three-dimensional structure of N-terminal cytoplasmic domain eAE1 showed a globular structure, which is composed of 11 β-strands and 10 helical segments [6]. The first 54 residues at N-terminus contains 20 acidic residues, the strongly anionic region involved in most peripheral protein interactions [4]. Loss of this portion causing deletion of a central strand in the major β-sheet may alter the globular structure of eAE1 to a different form. Therefore, N-terminus of kAE1 does not bind to ankyrin, protein 4.1, and aldolase, which may be due to the altered structure of cytoplasmic domain of kAE1. However, the identity of kAE1-binding protein(s) in the acid-

secreting type A intercalated cells remains unknown. This leads to the question: what are proteins that bind to the cytoplasmic domain of kAE1?

The function of kAE1 is important in the process of bicarbonate reabsorption across the basolateral membrane of the type A intercalated cells of the renal collecting duct, which occurs in association with net acid excretion mediated by H^+-ATPase pump in the apical membrane of these cells. Failure of either acid excretion or bicarbonate reabsorption by these cells leads to distal renal tubular acidosis (dRTA), the disorder characterized by the incapability of the kidney to acidify urine in the presence of systemic acidosis [7]. The inadequate urinary acidification is usually accompanied by hypocitraturia, hypercalciuria, hypokalemia, metabolic bone disease, nephrocalcinosis, and ultimately to chronic renal insufficiency [8]. Familial dRTA can be inherited in both autosomal dominant and autosomal recessive patterns with a broad spectrum of clinical severity. Some patients with autosomal dominant dRTA remain asymptomatic until adolescence or adulthood, whereas others with autosomal recessive dRTA may be severely affected in children usually present with failure to thrive, growth retardation and rickets.

Defects in any one of several transporters and enzymes of the type A intercalated cells required for trans-epithelial acid excretion and bicarbonate reabsorption might cause heritable dRTA [8]. Among these components are H^+-ATPase, AE1, cytoplasmic carbonic anhydrase II (CAII), and at least in conditions of K^+ deprivation, H^+/K^+-ATPase. Although mutations in vacuolar H^+-ATPase have been found in autosomal recessive dRTA through whole-genome linkage mapping followed by candidate screening within the linked region [9], several mutations in *AE1* gene has been found to be associated with both autosomal dominant and autosomal recessive dRTA [10-12].

To date, the *AE1* mutations associated with autosomal dominant dRTA are missense mutations in codon 589 (R589H, R589S, R589C), S613F, and an 11-amino acid deletion at the carboxy terminus (AE1Δ11) while the *AE1* mutations linked to autosomal recessive dRTA are G701D, ΔV850 and A858D [13]. A current hypothesis is that these mutations caused dRTA either by preventing the movement of mutant and normal proteins to the cell surface (impaired trafficking) or sending the mutant proteins to inappropriate site (mis-targeting), which resulted in impairing bicarbonate movement across the basolateral membrane [14, 15]. Functional analysis

of the R589H, AE1Δ11 and G701D mutants were extensively studied by using either erythroid (eAE1) or kidney AE1 (kAE1) form expressed in *Xenopus* oocytes [16-19], human embryonic kidney (HEK-293) cells [14, 15], and Mardine-Darby canine kidney (MDCK) cells [20, 21]. The recessive G701D mutant showed inactive anion transport of both mutant eAE1 and kAE1 forms owing to impaired protein trafficking when expressed in *Xenopus* oocytes while patients' red cell content and AE1-mediated sulfate flux were normal [19]. Functional cell surface expression of the G701D mutant could be completely rescued by co-expression of a red cell membrane protein, glycophorin A (GPA). The co-existence of defective intercalated cell function with normal red cell function is explained by the presence of GPA in red cell and the absence in the kidney [19]. It was found that impaired trafficking of the G701D mutant in transfected HEK-293 cells may explain the loss of kAE1 at the basolateral membrane of the type A intercalated cells [22-24]. Unlike in erythroid cells, a functional equivalent of GPA in intercalated cells has not been identified. The mechanism by which *AE1* mutations cause autosomal dominant dRTA is more complicated. The two mutant AE1, R589H and AE1Δ11, exhibited mildly reduced function in *Xenopus* oocytes, without evidence of dominant negative effect behavior [17, 18, 25]. However, recent publications reported that *AE1* mutations associated with autosomal dominant dRTA could prevent mutant kAE1 accumulation at the cell surface in HEK-293 cells [14, 15] and MDCK cells [22]. Moreover, a more recent study has proposed that the presence of autosomal dominant dRTA in some patient may be due to mis-targeting of mutant protein and the explanation was due to the absence of tyrosine-based sorting motif which has been implicated as basolateral targeting signal in polarized epithelial cells [26]. In general, various membrane proteins are sorted to the basolateral membrane through the interaction of tyrosine-based targeting motifs in their cytoplasmic domains with adaptor protein complexes, one of which is AP-1B. However, the study cannot show the involvement of AP-1B in sorting mechanism of kAE1 [21]. So far, beyond observations that some mutations caused defects in trafficking to basolateral membrane of kAE1, it has yet been unknown how the protein trafficking fails and what checkpoint is present in normal trafficking of kAE1. Thus, to understand kAE1 transport, targeting, and regulation, it is necessary to identify kAE1 binding proteins (kAE1-BPs).

During the past few years, in an attempt to isolate the adaptor protein that binds to kAE1, a new protein called kanadaptin (kidney anion exchanger adaptor protein) has been identified as a protein binding to the N-terminal cytoplasmic domain of mouse kAE1 but not to eAE1 by yeast-two hybrid system [27]. Kanadaptin was expressed only in the collecting tubules and in the intercalated cells; it was co-localized with kAE1 in cytoplasmic vesicles but not when it had been delivered to the basolateral membrane [27]. These results raised the possibility that this protein is involved in the targeting of kAE1 vesicles to their final destination. However, other studies have reported that kanadaptin was able to accumulate in the nucleus and mitochodria of various epithelial and non-epithelial cultured cell types [28, 29]. Our group has demonstrated that kanadaptin did interact with kAE1 and may not play a role in targeting of kAE1 to basolateral membrane of intercalated cells [30, 31]. These finding supported the notion that neither AP-1B nor kanadaptin is involved in kAE1 impaired trafficking associated with dRTA. The possibility is that different binding protein(s) that interact with either C-terminal or N-terminal cytoplasmic domain of kAE1 may contribute kAE1 trafficking or targeting to basolateral membrane in human kidney.

The yeast-two hybrid system is among a few methods to demonstrate protein-protein interaction *in vivo* [32]. This can be carried out by a selection or screening for novel proteins from cDNA library that specifically interact with a target protein of interest. The principle of this system is based on transcription readout taking place within living yeast cells. This approach takes advantage of the modular structure of a eukaryotic transcription factor known as DNA-binding domain (DNA-BD) and activation domain (AD) without the necessity that they are covalently attached. Therefore, a bait gene is expressed as a fusion to the DNA-BD while another cDNA is expressed as a fusion to the AD. When the bait and library fusion protein interact in a yeast reporter strain, the DNA-BD and AD are brought into proximity and activate transcription of reporter genes. This technology can be used to identify novel protein interactions, confirm suspected interactions, and define interacting domains.

In this study, yeast-two hybrid screening would be conducted to isolate protein from cDNA library derived from human kidney cells that interacts with the large N-terminal and C-terminal region of kAE1. Subsequently, co-immunoprecipitation and affinity co-purification would be performed to demonstrate the specific interaction of the proteins. Further studies would be carried out in human kidney cell lines to

examine localization and distribution of interacting proteins by using immunofluorescence microscopy. If the binding proteins of human kAE1 are identified, they may contribute to the investigation of the normal and abnormal trafficking or targeting of human kAE1 and will provide a new insight into a molecular mechanism associated with the pathogenesis of dRTA.

Research Studies

Part I: Isolation and identification of protein interacting with kAE1 by yeast two-hybrid system

Yeast two-hybrid (GAL4-based) system was employed to screen for kAE1 interacting proteins from human kidney cDNA library by using both N- and C-terminal domains of kAE1 as baits. The yeast two-hybrid system is based on the principle that a protein of interest (bait) is expressed by fusion with DNA-binding domain (GAL4-BD) while interacting proteins (prey) are expressed from a cDNA library by fusion with activation domain (GAL4-AD). Yeast cells transformed with bait and prey plasmid constructs are combined to mate as diploid. Interaction between bait and prey will bring BD and AD to close proximity and activate reporter genes, which are selectable markers for positive yeast colonies containing required prey-cDNAs. Therefore, yeast two-hybrid screening is composed of construction of bait plasmids, examination of suitable bait protein properties, yeast two-hybrid screening of human kidney cDNA library, isolation and reduction of redundant clones from library screening, sequencing of cDNA inserts, searching for homology sequences from the databases and specificity test of protein-protein interaction in yeast.

The construct with a full-length kAE1 cDNA, cloned into a GAL4-BD vector – pGBKT7, failed to express in yeast cells; therefore, parts of cDNA encoding N- and C-terminal cytoplasmic domains of kAE1 were cloned and used for the screening, instead. The experimental procedures and results were summarized as follows:

1. Construction of bait plasmids

Human *AE1* cDNA sequences encoding kAE1 N-terminal domain (amino acids 66-403, NkAE1) were amplified by PCR using two primers: kAE001-NcoI (5'-CATGCCATGGACGAAAAGAACCAGG-3') and kAE403-SalI (5'-CGCGTCGACTTAGGGGCTGAATGCA-3'). In addition, the C-terminal sequences (amino acids 876-911, CkAE1) were amplified using two primers: Ct-kAE1F-EcoRI (5'-GGAATTCCTCATCTTCAGGAACGTGGAGC-3') and AE1R-SalI (5'-CGTGTCGACTTACACAGGCATGGCCACTTC-3').

The NkAE1 and CkAE1 were amplified to generate fragments with sizes of 1.02 kb and 121 bp, respectively. NkAE1 contains *Nco*I and *Sal*I and CkAE1 consists of *EcoR*I and *Sal*I restriction sites (underlined) for cloning into the GAL4-binding domain vector, pGBKT7. An aliquot of ligation mixtures was transformed into *E.coli* DH5α competent cells. The transformants were selected on LB plate containing 100 μg/ml of ampicillin. The putative positive clones were identified by using colony PCR screening and restriction enzymes analysis to confirm the presence of the inserts. The recombinant plasmids encoding NkAE1 and CkAE1 were named pNkAE1 and pCkAE1, respectively. To verify the presences of *kAE1* sequences, both the pNkAE1 and pCkAE1 plasmids were subjected to DNA sequencing. No mutation in cDNA fragment was detected in both pNkAE1 and pCkAE1 plasmids. Before using for mating and cDNA library screening, they were tested for fusion protein expression in yeast cells and auto-transcriptional activation of reporter genes, which were positive for the former but negative for the latter. They were then used for screening kAE1 binding proteins from human kidney cDNA library.

2. Examination of bait properties

To test properties of bait, pNkAE1 and pCkAE1 plasmids were transformed into the *MATα* yeast strain AH109 by using the standard lithium acetate method. The yeast transformants were selected on synthetic dropout medium lacking tryptophan (SD/-Trp) plate by incubation at 30 °C for 2-4 days. Autonomously transcriptional activation of baits was tested by culturing on SD/-Trp/X-α-Gal, SD/-His/-Trp/X-α-Gal and SD/-Ade/-Trp/X-α-Gal to examine whether the baits themselves activate transcription of *HIS3*, *ADE2* and *MEL1* (α-galactosidase) reporter genes. The results showed that yeasts transformed with either pNkAE1 or pCkAE1 appeared as very slightly blue colonies on SD/-Trp/X-α-Gal, which were not significantly different from yeast transformed with pGBKT7 vector (negative control). This background might be generated from the plasmid backbone itself. However, the yeast transformants did not grow on SD/-His/-Trp/X-α-Gal and SD/-Ade/-Trp/X-α-Gal plates, suggesting that these baits were unable to activate transcription of *HIS3*, *ADE2* and *MEL1* reporter genes.

Apart from transcriptional activation, expression of bait fusion proteins were also examined to ensure that the bait constructs were able to express fusion proteins,

GAL4-BD fused to NkAE1 (GAL4-BD-NkAE1) and to CkAE1 (GAL4-BD-CkAE1) in yeast cells. The GAL4-BD-NkAE1 and GAL4-BD-CkAE1 fusion proteins were extracted from the yeast strain AH109 transformed with either pNkAE1 or pCkAE1 while that transformed with pGBKT7 vector was used as a negative control. The protein samples were resolved on SDS-PAGE and the expressed proteins were detected by immunoblotting using mouse anti-GAL4-BD antibody as a primary antibody and followed by HRP-conjugated anti-mouse IgG antibody. The result showed that GAL4 DNA-BD antibody could detect expression of GAL4-BD at molecular weight of 22 kDa while the fusion proteins GAL4-BD-NkAE1 and GAL4-BD-CkAE1 were detected as expected proteins with molecular weights of 60 kDa and 26 kDa, respectively. Both pNkAE1 and pCkAE1 could thus be used as baits for screening kAE1 interacting proteins from human kidney cDNA library by the yeast two-hybrid system.

3. Yeast two-hybrid screening of human kidney cDNA library

The two-hybrid library screening was performed by yeast mating method. The transformant yeast (AH109 strain) with either pNkAE1 or pCkAE1 were grown in 50 ml of SD/-Trp liquid dropout medium at 30 °C for 16-24 h with shaking (~250 rpm). The cells were collected by centrifugation at 1,000 x g for 5 min and resuspended in the residual liquid. An aliquot (~1.0 ml) of yeast (Y187 strain) pre-transformed with kidney cDNA library, constructed in pACT2 to express the fusion protein containing GAL4-AD, was thawed out at a room temperature with gently vortex and combined with the bait strain in 2-L sterile flask containing 45 ml of 2x YPDA/kanamycin, then incubated at 30 °C for 20-24 h with gentle swirling (30-50 rpm) to allow mating. The mating mixture was transferred to a sterile centrifuge bottle and spun down by centrifugation at 1,000 x g for 10 min. The cell pellet was resuspended with 0.5x YPDA/kanamycin and spread on large (150-mm) plates containing SD/-Ade/-His/-Leu/-Trp dropout media. Plates were incubated at 30 °C until colonies appeared. The positive clones were further screened for α-galactosidase activity (*MEL1*) by growing the colonies on SD/-Ade/-His/-Leu/-Trp containing X-α-Gal (Clontech) indicator plate. The positive controls were performed using pGBKT7-53 (murine p53) and pTD1-1 (SV40 large T-antigen). Approximately $1x10^6$ independent clones in cDNA library were screened and interactions between the N-terminal kAE1 or the C-

terminal kAE1 and interacting proteins were examined by growth of diploid yeast cells on SD/-Trp/-Leu/-His/-Ade to verify the expression of HIS3 and ADE2 reporters.

From this initial screening, we found that 151 colonies could grow and show the blue colonies on the SD/-Ade/-His/-Leu/-Trp containing X-α-Gal plate when used the N-terminal kAE1 as bait. These positive clones were classified into three different groups, groups A, B and C, on the basis of the intensity of the blue colonies. Group A consisted of 107 colonies that gave strong blue colonies. Group B consisted of 26 colonies that gave moderate blue colonies while group C consisted of 18 faint blue colonies. The strong blue colonies indicated strong interactions between the N-terminal kAE1 and interacting proteins but the faint blue colonies might be false positives from the screening on SD/-Trp/-Leu/-His/-Ade plates due to a long incubation time at 30 °C. Finally, total 133 positive colonies including strong and moderate blue colonies were selected for further analysis.

For using the C-terminal kAE1 as bait, a total number of 1,250 colonies from the SD/-Trp,-Leu,-His,-Ade plate were obtained and they were transferred to SD/ Trp-Leu- master plates but a number of 584 from 1,250 colonies were re-screened on SD/-Trp,-Leu,-His,-Ade, and on SD/-Trp,-Leu,-His,-Ade/X-α-Gal plates (to observe the activation of MEL1 gene). Only the colonies showing strong blue on the plate containing X-α-Gal were selected for further analysis.

4. Isolation of cDNA positive clones and identification of redundant clones from library screening

To reduce the redundant clones from library screening, the plasmid DNA of positive clones were isolated and digested with restriction enzymes to create restriction digest patterns. First, the plasmid DNA from positive clones (133 clones from putative N-terminal kAE1-interacting proteins and 120 clones from putative C-terminal kAE1-interacting proteins) were isolated from yeast by standard lyticase method. Briefly, the positive colonies were grown in 5 ml of SD/-Leu to select the library plasmids. The cells were collected by centrifugation at 14,000 x g for 5 min and resuspended in lysis solution [5 units/μl of lyticase enzyme (Sigma) in TE buffer]. The cell suspension was incubated at 37 °C for 1 h with shaking and then 20% of SDS was added to the reaction and mixed vigorously. The tube containing

cell suspension was placed in liquid nitrogen and performed freeze-thaw cycles for 3 times and then mixed with vortex. The volume of cell suspension was brought up to 200 µl with sterile water and added with an equal volume of 1:1 of phenol and chloroform mixture solution, then centrifuged at 14,000 x g for 10 min. The aqueous upper phase was transferred to a fresh microcentrifuge tube. Ammonium acetate (10 M) was added to the aqueous solution followed by adding 2 volumes of absolute ethanol to precipitate DNA. The DNA pellet was collected by centrifugation at 14,000 x g for 10 min, then air-dried and resuspended in sterile distilled water.

These plasmids were used as DNA templates for PCR amplification of the inserted cDNAs in the library clones. PCR reaction was performed by using the primers flanking multiple cloning site of library plasmid named LibADf (5'-CTATTCGATGATGAAGATACCCCACCAAACCC-3') and LibADr (5'-CTATTCGATGATGAAGATACCCCACCAAACCC-3'). Since the size of cDNAs inserted in the library was varied from 0.5 kb to 4.0 kb, PCR amplification condition was optimized to amplify the cDNAs inserts of all positive clones. The PCR reaction was performed in a total volume of 25 µl containing 0.3 µg of plasmid DNA, 10 pmole of each primer, 2 mM dNTPs, 1x Pfu buffer (2 mM final concentration of Mg^{2+}) and the 0.9 unit *Pfu* DNA polymerase (Promega). The 40 PCR cycles were performed as follows: (i) denaturation at 95 °C for 2 min (ii) denaturation at 94 °C for 20 sec, (iii) annealing and extension with temperature and time depending on the size of inserts, followed by another 7 min of extension at 68 °C in the final step. An aliquot of PCR product was resolved by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. The results showed that cDNA inserts of all positive clones could be amplified. After that, the PCR products were purified by using QIAGEN PCR Purification Kit. The purified PCR products were subsequently digested with *Hae*III for the N-terminal positive clones and *AluI* for the C-terminal positive clones to eliminate duplicate clones from the library. The digested products were analyzed on 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. The digested fragment pattern results of PCR products were analyzed and compared among the positive clones. One of duplicate clones was used as a representative of the groups and subjected to DNA sequencing. After elimination of redundant clones, a total of 121 clones of putative N-terminal kAE1-interacting proteins and 102 clones of putative C-terminal kAE1-interacting proteins were obtained.

5. Sequencing of cDNA inserts and searching for homology sequences

(1) Clones of putative N-terminal kAE1-interacting proteins

To examine inserted cDNAs in the clones, partial DNA sequencing of the purified PCR products of all 121 clones of putative N-terminal kAE1-interacting proteins were performed by using a primer named LibInt-f (5'-CCAGATTACGCTAGCTTGGG-3') designed from the sequences which located upstream of cDNA insertion sites. The sequencing results showed 109 independent clones could be analyzed while those of the other 12 clones were not obtained since there were numerous repeat nucleotide (poly T) sequences in the inserted fragments. The obtained sequences were searched for homology in GenBank and protein databases by BLAST. The sequences of seventy-three clones were matched with known proteins while those of 36 clones were hypothetical proteins. The putative proteins that gave a high homology were further analyzed and classified into groups such as metabolism, transport and binding proteins, protein trafficking, apoptosis, membrane associated proteins, cytoskeleton associated proteins, transcription and translation factors, and hypothetical proteins (Table 1).

As mentioned, the normal trafficking mechanism of kAE1 to express on the cell membrane has been poorly understood. The criteria for selection of the independent clones for further study was therefore involved in the possibility of the proteins would be participating in kAE1 trafficking. Thus, 21 independent clones encoding known proteins namely aldolase B, GAPDH, giantin, pantophysin, vesicle-associated membrane protein, adaptor-related protein complex 1, ER translocon, Na^+/K^+ ATPaseβ3 subunit, Na^+/K^+ ATPase β1 subunit, cadherin EGF LAG G-type receptor 1, endothelial G-protein receptor 1, protocadherin LKC, laminin A, actin gamma 1, thymosin β4, WD repeat domain 1, dishevelle activator of morphogenesis 1, integrin-linked kinase, WD repeat and FYVE domain 3, nuclear factor, and randomly selected 14 clones encoding hypothetical proteins, totally 35 clones, were selected for specificity test of protein-protein interaction in yeast.

(2) **Clones of putative C-terminal kAE1-interacting proteins**

Different approach was performed for studies the 102 clones of putative C-terminal kAE1-interacting proteins. They were firstly confirmed for their interactions in a specificity test (see below). A number of 72 of 102 clones of putative C-terminal

kAE1-interacting proteins were tested and 28 clones could initiate the growth of yeast colonies on the SD media and produced strong blue colonies. The cDNA inserts of kAE1-interacting proteins in 28 prey plasmids were analyzed by DNA sequencing using LibRDf primer (5'-CTATTCGATGAAGATACCCCACCAAACCC-3') annealing to nucleotide sequence of pACT2 which was flanking the cDNA inserts. A total of 27 prey plasmids could successfully be analyzed while 1 clone was unsuccessful owning to unreadable sequencing profile. These 27 partial cDNA sequences were used for BLAST search in NCBI GenBank (http://www.ncbi.nlm.nih.gov) and EMBL (http://www.ebi.ac.uk) databases to obtain full-length cDNA sequences and identify their encoded proteins. Of 27 sequences of cDNA inserts analyzed, 17 encoding 14 known proteins, 7 encoding hypothetical proteins, and 2 encoding proteins known to be false positive (Table 2).

AP1mu1 and advillin from clone numbers 4 and 343, respectively, were selected based on our interest in protein trafficking. To determine the region of kAE1-BPs interacting with Ct-kAE1, the partial sequences of kAE1-BPs were aligned with full-length coding sequences of encoded proteins obtained from Human Genome Database. Moreover, the partial sequences were translated into amino acid sequences and aligned with the full-length proteins. The partial sequence of clone number 4 was identical to nucleotide sequence at the 3' region of AP1mu1 (positions 915-1,226), which encoded amino acid residues 306-408 of the full-length AP1mu1 (423 amino acids). In addition, the partial sequence of the clone number 343 was identical to nucleotide sequence positions 1,414-1,519 of advillin, which encoded amino acid residues 472-507 of full-length advillin protein (823 amino acids).

Table 1 BLAST searches of genes and proteins homologues to nucleotide sequences of human kidney cDNAs screened by yeast two-hybrid method using N-terminal kAE1.

Accession number	BLAST description	Proposed function	E-value
Protein involved in metabolism			
NM000282.1	Propionyl CoA carboxylase	Enzyme in catabolic pathway of amino acids	0.00
NM000035.1	Aldolase B*	Fructose metabolism	0.00
NM033055.1	Ortholog of mouse hippocampus	Carbohydrate transport and metabolism	0.00
BC014085.1	GAPDH*	Carbohydrate metabolism and membrane fusion	0.00
NM015423.2	Aminoadipate-semialdehyde dehydrogenase	Biosynthetic pathway of lysine	e -169
BC000306.1	L-3-hydroxyacyl CoA dehydrogenase	Mitochondrial beta-oxidation of fatty acids	0.00
AJ003100.1	Glycogen synthase 2	Glycogen biosynthesis	8e -42
AF069984.1	Nitrilase homolog 1	Nitrogen metabolism	6e -77
BT007538.1	Prosaposin	Lysosomal degradation of sphingolipid	1e -20
NM000016.2	Acyl CoA dehydrogenase	Mitochondrial beta-oxidation of fatty acids	0.00
AY195792.1	Mitochondrial protein (7 clones)	Electron-Transfer Proteins	0.00
Transport and binding proteins			0.00
AF030356.1	Peroxisome, pex1	Protein import into peroxisome matrix	0.00
AJ535113.1	MHC class I	Antigen presenting cells	3e -144
NM003361.1	Uromodulin	May regulate the circulating of cytokines	0.00
NM006288.2	Thy-1 cell surface antigen	Cell-ligand interaction	0.00
NM003107.2	Sex determining region Y box 4	Regulation of embryonic development	e-113
BC007034.1	Metallothioneine 2A (2 clones)	Bind to metal ions	0.00
BC007900.1	DAZ associated protein 2	Germ-cell specific binding protein	0.00
NM004048	Beta 2 microglobulin	Immunoglobulin	0.00
NM015423.2	Zn finger	Nucleic acid binding protein region	e-145
Protein trafficking			
NM004487.1	Giantin*	Cross-bridges of Golgi complex	0.00
BC020938	Pantophysin*	Vesicle trafficking	0.00
XM841900	Ubiquitin protein ligase	Protein degradation	0.00
BC020807	Proteosome	Protein degradation	0.00
NM003574.2	Vesicle-associated membrane protein*	Associated with SNARE proteins	e-118
NM178814.2	Adaptor-related protein complex 1, σ 3*	Role in protein sorting in trans-Golgi network	0.00
NM003145.2	ER translocon, TRAP β subunit*	Regulate the retention of ER resident proteins	0.00
NM001679.1	Na+/K+ ATPaseβ subunit*	Non catalytic domain, unknown function	0.00
NM001677.1	Na+/K+ ATPase β1 subunit* (2 clones)	Regulate assembly of α/β hetero domain	0.00
Protein involved in apoptosis			
NM014246.1	Cadherin EGF LAG G-type receptor 1*	Receptor in cell/cell signaling	e-135
NM030782.2	Cisplatin resistance related protein	Associated with apoptosis	2.7

Table 1 BLAST searches of genes and proteins homologues to nucleotide sequences of human kidney cDNAs screened by yeast two-hybrid method using N-terminal kAE1 (continued).

Accession number	BLAST description	Proposed function	E-value
Membrane associated proteins			
NM001081.2	Cubilin (2 clones)	Receptor for intrinsic factor B12 complexes	0.00
XM016625.5	Voltage-dependent anion channel (2 clones)	Channel through mitochondrial membrane	0.00
NM016121.2	K channel tetramerization domain	Protein forms a potassium-selective channel	0.00
NM0014002	Endothelial G-protein receptor 1*	Regulate differentiation of endothelial cells	0.00
NM0176575.2	Protocadherin LKC*	Cell-cell adhesion molecules	0.00
Cytoskeleton associated proteins			
BC000909.2	Laminin A*	Mediate attachment and organization of cells	0.00
BC053572.1	Actin gamma 1*	Cytoskeleton	0.00
NM021109.1	Thymosin β 4*	Organization of the cytoskeleton	0.00
NM005112.3	WD repeat Domain 1*	Induced disassembly of actin filaments	e-79
NM0149921	Dishevelle activator of morphogenesis 1*	Actin binding protein, cytokinesis	2e -84
BC001554.1	Integrin-linked kinase*	Mediating cell architecture	0.00
AF328728.1	Vimentin	Regulate intermediate filament protein	0.00
NM000146.2	Ferritin (4 clones)	Iron-storage Protein	0.00
NM001846.1	Collagen type IV	Constituent of the basement membranes	0.00
NM001101.2	β-actin	Cytoskeleton component	0.00
NM014991.3	WD repeat and FYVE domain 3*	Target cytosolic protein for degradation	0.00
Transcription and translation factors			
NM001030055	RhoGTPase activating protein	Termination step of protein translation	0.00
AF202445	dsRNA binding protein	Facilitating interaction with dsRNA	0.00
BC001633.1	TU translation elongation factor	Deliver aminoacylated tRNA to the ribosome	0.00
BC010046.1	Nuclear factor*	Signal transduction and cell communication	0.00
NM003110	Transcription factor SP1	Constitutive and induced expression of gene	0.00
NM001402.4	Translation EF1 α 1	Required for protein translation	0.00
AC009321.18	Poly T	Nuclear pore translocation	0.00
Protein involved in carcinoma and undefined function			
Z61349.1	CpG island	Located around the promoters of genes	0.00
BC004262.1	Similar to cactin	Interacts with the Drosophila IkappaB protein	0.00
BC040004.1	Similar to myeloid/lymphoid	Adenocarcinoma	0.00
BC040004.1	Similar to myeloid-linage	Carcinoma	0.00
BC002989.1	Human glutamine rich	ND	0.00
BC035531.6	Cas-Br-M retroviral transformed sequence	Lineage lymphoma b	0.00

Table 1 BLAST searches of genes and proteins homologues to nucleotide sequences of human kidney cDNAs screened by yeast two-hybrid method using N-terminal kAE1 (continued).

Accession number	BLAST description	Proposed function	E-value
Hypothetical proteins			
NM014749.1	KIAA0586*	Proline rich region	0.00
BC035980.1	Clone: 4133024*	Regulation of cytoskeleton	0.00
AC092038.3	Chromosome 3 clone RP11-89F18*	ND	0.00
AC112673.7	Chromosome 8 clone RP11-212F11*	ND	0.00
BC012018.1	Clone IMAGE: 4477559	ND	0.00
AC127515.9	Chromosome 17 clone RP13-6309	ND	0.00
AL136093.8	Chromosome 6 clone RP3-472A9*	ND	0.00
AL607077.4	Clone RP11-474I15	Reverse transcriptase	0.00
AE003589.3	Drosophila chromosome 26	ND	0.00
NM015936.1	Similar to tyrosyl-tRNA synthase	ND	0.00
NM025112.1	Similar to limonene synthase	Chloroplast precursor	0.00
NM015328.1	KIAA0828	Hydrolyse S-adenosyl-homocystein	e-160
NM004554.2	Similar to Nuclear factor activated T-cell	Signal transduction in atrial fibrillation	0.00
AC092755.4	Conserve TIG domain	Regulation of transcription	0.00
AP002961.2	Chromosome 11 clone RP11-2I22	ND	0.015
AC021744.14	Chromosome 8 clone RP11-284H18*	ND	0.00
NM032889.2	Clone MGC11308*	ND	0.00
AC005531.2	Chromosome 7 clone RP4-701016	ND	0.00
AC073578.17	Chromosome 12 RP11-897M7*	ND	0.00
AL022345.2	Chromosome 10 clone XX-Y738F9	ND	0.00
AL583783.6	Alu family	Polymorphisms in the human genome	0.00
XM037809.3	KIAA1671	Similar to tankyrase 1 binding protein	0.00
BC021670.1	Clone MGC: 22710	ND	0.00
AY274808.1	KIAA1749 (tropomyosin)*	Muscle protein inhibits contraction of actin	0.00
AP000769.5	Chromosome 11 CMB9-22P13	ND	0.00
BC010868.1	FLJ14600 *	Role in membrane sorting in mitochondria	0.00
XM037797.4	Clone MGC1842	ND	0.00
AC090868.4	Chromosome 15 clone RP11-795G3	ND	0.00
XM086408.4	KIAA1228 (conserve C2 domain)*	Ca_2^+-dependent membrane-targeting module	0.00
BC039469.1	Clone IMAGE: 4513453	ND	e-167
AC073848.4	Chromosome 4, clone RP11-669M16*	ND	0.00
AK122583.1	FLJ00294	Cytoskeleton associated protein	4e -94
AC005089.3	Chromosome 7 clone CTA-315H11	ND	0.00
AC139677.4	Chromosome 7 clone RP11-1070B7*	ND	0.00
NM033212.1	Clone LOC92922	ND	e-110
AK056173.1	FLJ31611*	ND	0.00

E-value = Expectation value; a low E-value score is a good hit to a model. Asterisks represent proteins selected for specificity test. ND = no data available.

Table 2 BLAST searches of genes and proteins homologues to nucleotide sequences of human kidney cDNAs screened by yeast two-hybrid method using C-terminal kAE1.

Accession Number	Clone No	Description	Functional classification
Protein involved in protein trafficking			
NM032493.2	4	Adaptor-related protein complex 1, mu 1 subunit	Protein transportation
NM004798.2	236/1	Kinesin family member 3B (KIF3B)	Protein and organelle transportation
Protein involved in signal transduction and electron transportation			
AY099353.1	157	Vav-1 interacting Kruppel-like protein (VIK)	Signal transduction and cell cycle regulation
NM004374.2	235	Cytochrome c oxidase subunit VIc (COX6C)	Electron transportation
Protein involved in organization of cytoskeleton			
BC066956.1	19	Vimentin	Cytosleleton
AY523969.1	342	Catenin (cadherin-associated protein), alpha-like 1 (CTNNAL1) gene	Cell-cell adhesion
AF041449.1	343	Advillin (p92)	Actin-binding protein
NM020384.2	350	Claudin 2	Cell-cell adhesion
Protein involved in metabolism pathway			
NM031208.1	1	Fumarylacetoacetate hydrolase	Metabolism of carbohydrate
BC045641.1	12, 13	Glutamine-fructose-6-phosphate transaminase 1 *	Metabolism of carbohydrate
BC083511.1	41	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism of carbohydrate
BC042142.1	73, 102	Aldehyde dehydrogenase 16 *	Metabolism of carbohydrate
BC018103.1	359	Aconitase 1	Metabolism of iron regulation
Cell cycle			
NM012311.2|	33, 70	KIN, antigenic determinant of recA protein *	DNA replication

Note: Asterisk (*) indicates the proteins that were identified twice.

Table 2 BLAST searches of genes and proteins homologues to nucleotide sequences of human kidney cDNAs screened by yeast two-hybrid method using C-terminal kAE1 (continued).

Accession Number	Clone No.	Description	Functional classification
Hypothetical proteins			
AC092815.2	28	Homo sapiens chromosome 1 clone RP5-1029K14	No data available
AL391644.12	40	Human DNA sequence from clone RP11-535F17 on chromosome 6	No data available
CQ731782.1	42	Sequence 17716 from Patent WO02068579	No data available
AC110997.4	106, 126	Homo sapiens chromosome 18, clone RP11-56J20	No data available
AC104452.2	153	Homo sapiens chromosome 3 clone RP11-949J7	No data available
BC045787.1	137	Homo sapiens chromosome 10 open reading frame 75 *	No data available
AC113395.2	236/2	Homo sapiens chromosome 5 clone RP11-418P19	No data available

Note: Asterisk (*) indicates the protein that was identified twice.

6. Specificity test of interactions between the N- or C-terminal kAE1 and positive clones in yeast

To verify interactions between the N- or C-terminal kAE1 and interacting proteins identified from the initial yeast two-hybrid screen, the second specificity test was performed by using pair-wise mating method. The isolated prey plasmids from *E.coli* were re-transformed into yeast strain Y187, whereas pNkAE1, pCkAE1, pGBKT7 vector, pGBKT7-53 or pGBKT7-Lamin C plasmid was transformed into the strain AH109. After that, the Y187 transformed with each candidate clones were mated with either bait (pNkAE1 or pCkAE1), pGBKT7 vector, pGBKT7-53 or pGBKT7-Lamin C, pre-transformed into the AH109. The true interaction between the N-or C- terminal kAE1 and interacting proteins were analyzed by growth of diploid yeast cells on SD/-Ade/-His/-Leu/-Trp containing X-α-Gal dropout plates. For the Nt-kAE1 interacting clones, 9 clones namely giantin (accession number NM004487.1), GAPDH (accession number BC014085.1), laminin A (accession number BC000909.2), pantophysin (accession number BC020938), ILK (accession number BC001554.1), thymosin β4 (accession number NM021109.1) and three unknown functional hypothetical proteins accession numbers: AC139677.4, AC073848.4 and AC073578.17 showed very strong blue colonies on SD/-Ade/-His/-Leu/-Trp containing X-α-Gal dropout plates when mating with pNkAE1. However, pantophysin (PPH), GAPDH, ILK were selected for further studies in cultured human kidney cells as they might play a role in normal kAE1 trafficking. For the Ct-kAE1 interacting clones, the specificity test was performed prior to DNA sequencing analysis. Twenty-eight clones showed strong interactions in the specificity test as mentioned above.

Part II: Interaction between kAE1 and interacting proteins in cultured human kidney cells

The first step of this part is the construction of human full-length kAE1 and kAE1 interacting proteins in eukaryotic expression vector. Human kidney cDNA was used as template for PCR-based amplification of cDNAs encoding kAE1 interacting proteins in order to clone them into a eukaryotic vector. Expression of full-length kAE1 and its interacting proteins were carried out in human embryonic kidney (HEK 293) cells. Specific interactions between kAE1 and interacting proteins were

demonstrated by co-immunoprecipitation, affinity co-purification, and immunofluorescence techniques. Cell surface biotinylation, fluorescence activated cell sorting (FACS) analysis, and transport activity assay were performed to examine the role of the interacting proteins on physiological function of kAE1.

1. Construction of recombinant plasmids encoding full-length human kAE1 and interacting proteins

To construct the recombinant plasmid encoding human full-length kAE1 containing hemagglutinin (HA) epitope tag at the C-terminus, pcDNA3/kAE1 plasmid was used as template in PCR amplification using specific primers, namely kAE1HindIII (5'-CCCAAGCTTATGGACGAAAAGAACCAGGAG-3') and kAE1XhoI (5'-CCGCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTAC ACAGGCATGGCCACTTC-3'). The sequence encoding for HA epitope tag was added into the latter primer. The PCR reaction in a total volume of 50 μl contained 17 ng of plasmid DNA, 10 pmole of each primers, 2 mM dNTPs, 1x Pfu buffer (2 mM final concentration of Mg^{2+}). After mixing the reaction and denaturing at 95 °C for 2 min, the 0.9 unit *Pfu* DNA polymerase was added (Promega). The PCR cycles were run on a thermal cycler GeneAmp®PCR system 2400 (Perkin Elmer Cetus, USA) for 40 cycles, each cycle consisting of (i) denaturation at 94 °C for 20 sec, (ii) annealing at 58 °C for 20 sec, (iii) extension at 68 °C for 3 min 30 sec, which was followed by extension at 68 °C for another 7 min in the final step. The expected amplified fragment of 2.5 kb corresponding to kAE1 was inserted to *Hind*III/*Xho*I digested pcDNA3.1(+) vector. The ligation mixture was transformed into *E. coli* DH5α and transformants were selected on LB plate containing 100 μg/ml of ampicillin. The *E. coli* colonies containing recombinant plasmids were analyzed by plasmid preparation and restriction endonuclease digestion to observe the presence of cDNA insert. As expected, the result showed two fragments of digested DNA corresponding to 5.4 kb of pcDNA3.1 and 2.5 kb of kAE1. Insert sequences were verified by automated sequencing. The results showed that the recombinant plasmid did not carry any mutation in the sequence of kAE1 which served as wild type kAE1 with HA-tagged at the C-terminus, namely pkAE1.

To clone the full-length of GAPDH, pantophysin, ILK, and AP1mu1, cDNAs derived from human kidney mRNAs were generated using oligo dT primer. The

cDNAs were then used as templates to generate the fragments corresponding to GAPDH, pantophysin, ILK, and AP1mu1 by using nested PCR. Pairs of primer for GAPDH amplification were 5'- CCGGAATTCGATGGGGAAGGTGAAGGTC-3' and 5'-CCGCTCGAGTTACTCCTTGGAGGCCATG-3', for pantophysin were 5'-CCGGAATTCGATGTCCGGCTTCCAGATC-3', and 5'-CTAGTCTAGATTATAT TCCGGTAGGAGGTG-3', for ILK were 5'-CCGGAATTCGATGGACGACATT TTCACTC-3' and 5'-CCGCTCGAGCTACTTGTCCTGCATCTTC-3', and for AP1mu1 were 5'-GGAATTCATGTCCGCCAGCGCCGTCTACG-3' and 5'-CGTCTAGATCACTGG GTCCGGAGCTGGTAATC-3'. The first round PCR was performed in 25 µl reaction volume containing 1x Pfu buffer, 2 mM dNTPs, 10 pmole of each primer, and 2 µl of human kidney cDNA. The PCR was started by prewarming cDNA at 95 °C for 5 min before adding 1 unit of *Pfu* DNA polymerase. The PCR was then performed for 35 cycles with the following profiles: 94 °C for 20 sec, 58 °C for 20 sec, and 68 °C for 3 min 30 sec. The final extension step was carried out at 68 °C for 7 min. The PCR products from the first round were used as the templates for nested PCR by the same condition as the first round PCR amplification. The amplified fragments of GAPDH and ILK containing *EcoR*I and *Xho*I sites and pantophysin and AP1mu1 with *EcoR*I and *Xba*I sites were digested with respective restriction enzymes and inserted into corresponding sites of pcDNA3.1/His B vector to generate poly-histidine tag (His-tag) at the N-terminus of the proteins. The recombinant plasmids were prepared and analyzed by restriction enzyme digestions. The results showed two fragments of DNA corresponding to pcDNA3.1/His B at 5.5 kb and the inserts of ILK at 1.35 kb, GAPDH at 1.00 kb, pantophysin at 0.72, and AP1mu1 at 1.28 kb. The inserted cDNAs were verified by automated sequencing and the recombinant plasmids were named pGAPDH, pILK, pPanto and pAP1mu1A. These plasmids were used to study protein expression in HEK 293 cells.

2. Human kAE1 and interacting proteins expressed in transfected HEK 293 cells

To examine the expression of kAE1 and interacting proteins (GAPDH, ILK, pantophysin, and AP1mu1), the recombinant plasmids (pkAE1, pGAPDH, pILK, pPanto, and pAP1mu1) were transiently transfected in HEK 293 cells by using standard calcium phosphate precipitation and DEAE methods. Two days post-transfection, lysates of the transfected HEK 293 cells were prepared by using 2x

sample loading buffer. The protein sample was set aside for loading to SDS-PAGE and immunoblotting to examine protein expression in HEK 239 cells. Immunoblots of kAE1 showed a strong protein expression at a molecular weight of 96 kDa as expected using anti-HA antibody against HA-tagged at the C-terminus of the protein. In addition, GAPDH, ILK, pantophysin or AP1mu1 were detected by using anti-His antibody against His-tagged at the N-terminus of these proteins. The results showed that proteins were highly expressed at molecular weights of 33, 51, 28 and 51 kDa for GAPDH, ILK, pantophysin, and AP1mu1, respectively (Figure 1). These results suggested that pkAE1, pGAPDH, pILK, pPanto and pAP1mu1 constructs could efficiently express the proteins of interest in the transfected HEK 293 cells.

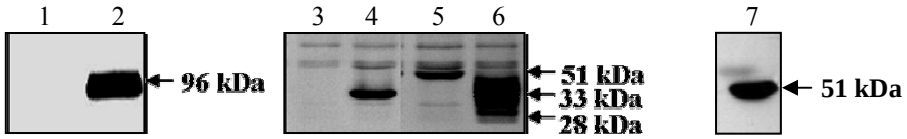

Figure 1 Immunoblots of kAE1 and interacting proteins expressed in human HEK 293 cells.

HEK 293 cells transfected with either pkAE1 (lane 2) or pGAPDH (lane 4), pILK (lane 5), pPanto (lane 6) and pAP1mu1 (lane 7) were lysed with 2x SDS-PAGE sample loading buffer and subjected to 10% SDS-PAGE and immunoblotting. Blots were probed with anti-HA and anti-His antibodies for detection of kAE1 and interacting proteins, respectively. Lanes 1 and 3 are untransfected cells probed with anti-HA and anti-His antibodies, respectively.

3. Co-immunoprecipitation of kAE1 and interacting proteins

In order to verify the interaction between kAE1 and its interacting proteins identified in the yeast two-hybrid screen, co-immunoprecipitation experiments were performed using HEK 293 cells transfected with pkAE1 alone or co-transfected either with pkAE1 and pILK, pkAE1 and pAP1mu1, pkAE1 and pGAPDH, or pkAE1 and pPanto. The mild lysis solution containing 0.5% Nonidet P-40 and 0.15 M NaCl was used to solubilize the HEK 293 cells for preserving interaction of kAE1 and its interacting proteins. Anti-His monoclonal antibody was used to precipitate 6xHis-tagged ILK (AP1mu1, GAPDH or pantophysin) from the lysates of co-transfected HEK 293 cells. The immune complexes were purified by using Protein A-Sepharose resin. Proteins were eluted with 2x SDS-PAGE sample loading buffer containing 2% (v/v) 2-mercaptoethanol and heated at 65 $^{\circ}$C for 5 min. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting. The presence of kAE1 in the immunoprecipitates was determined by immunoblotting using anti-HA antibody. The results showed that kAE1 precipitation was dependent on the expression of either AP1mu1, ILK, GAPDH or pantophysin (Figure 2). Thus, co-immunoprecipitation results indicated that AP1mu1, ILK, GAPDH and pantophysin associated with kAE1 in transfected HEK 293 cells.

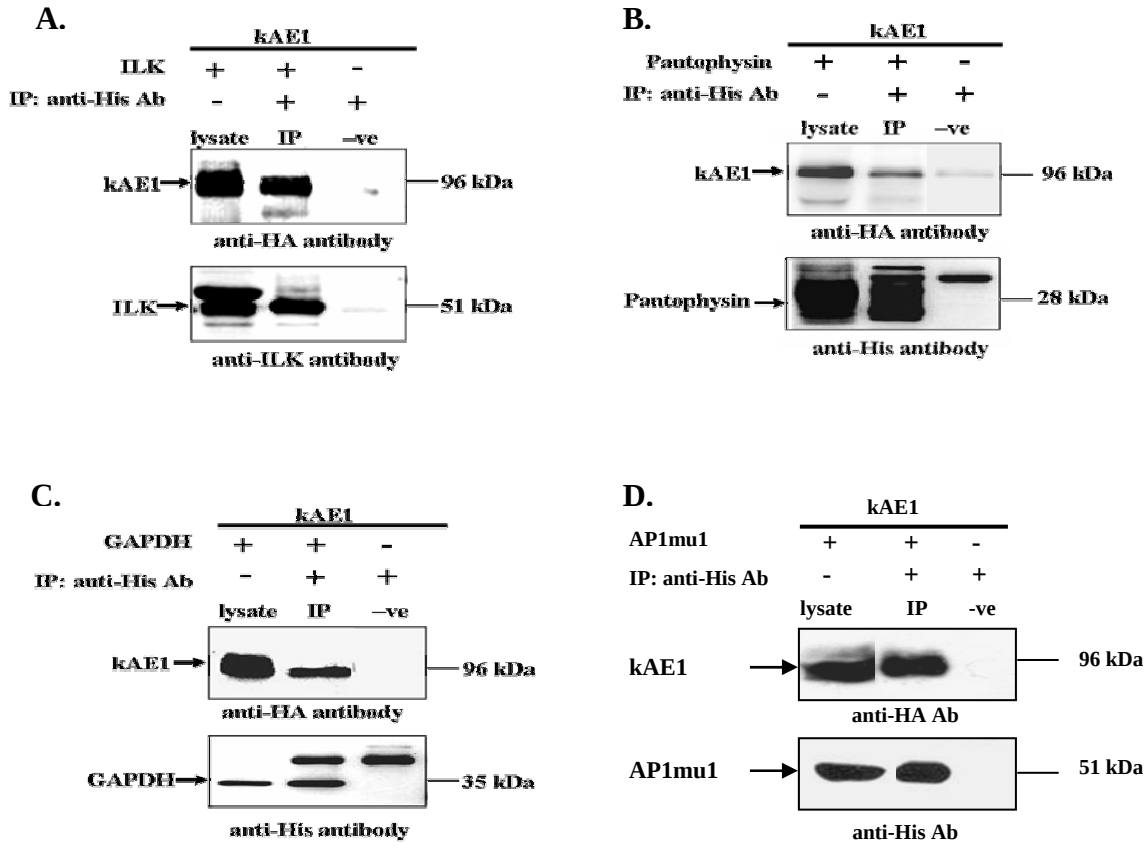

Figure 2 Co-immunoprecipitation of kAE1 and interacting proteins in HEK 293 cells. HEK 293 cells were individually transfected with pkAE1 or co-transfected with pkAE1 and pILK (A), pkAE1 and pPanto (B), pkAE1 and pGAPDH (C), or pkAE1 and pAP1mu1 (D). Cell lysates were either set aside for later analysis (lysate) or immunoprecipitated with anti-His antibody (IP). Cell lysates from cells expressing kAE1, but not interacting proteins, were also subjected to immunoprecipitation with anti-His antibody (-ve). Samples were subjected to SDS-PAGE and immunoblotting. Blots were probed for kAE1 using anti-HA antibody and for ILK with anti-ILK antibody while pantophysin, GAPDH, and AP1mu1 were detected by anti-6xHis antibody. The results (IP) showed that kAE1 was co-immunoprecipitated with ILK, patophysin, GAPDH, and AP1mu1.

4. Affinity co-purification of kAE1 and interacting proteins

To further verify the specificity of interaction between kAE1 and its interacting proteins, affinity co-purification using Co^{2+} resins was also carried out. N-terminal 6xHis-tagged ILK (GAPDH, pantophysin or AP1mu1) was co-expressed with kAE1 or kAE1 was expressed alone in HEK 293 cells. The complex of 6xHis-tagged ILK, GAPDH, pantophysin or AP1mu1 with kAE1 was purified by using Co^{2+} resins, which bind proteins containing 6xHis. The protein complex was then eluted from resins with high concentrations of imidazole-containing buffer. As expected, kAE1 was detected in the eluent using anti-HA antibody when co-expressed with 6xHis-tagged ILK, GAPDH, pantophysin or AP1mu1 (Figure 3). ILK, GAPDH, pantophysin and AP1mu1 were also detected in the bound fractions using anti-6xHis antibody. HA-tagged kAE1 did not bind to Co^{2+} resin. Thus, no band was detected by anti-HA antibody. Thus, ILK, GAPDH, pantophysin, and AP1mu1 interacted specifically with kAE1, as confirmed by both co-immunoprecipitation and co-purification.

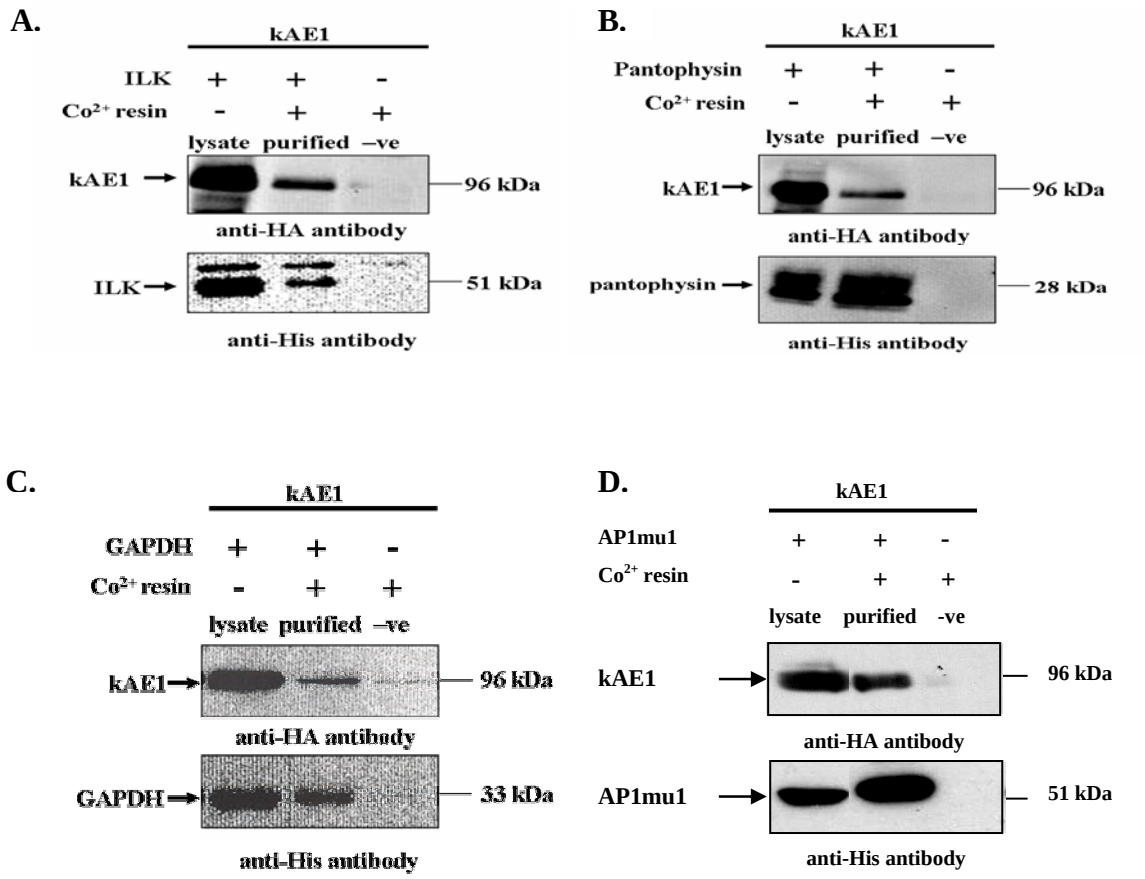

Figure 3 Affinity co-purification of kAE1 and interacting proteins.

HA-tagged kAE1 was expressed alone or co-expressed and co-purified with 6xHis-tagged ILK (A), pantophysin (B), GAPDH (C), or AP1mu1 (D) as indicated. Cell lysates were either set aside for later analysis (lysate), or incubated with Co^{2+} affinity resins at 4 ^{o}C (purified). Resins were washed thoroughly with washing buffer and subsequently eluted with 1.5 M imidazole containing buffer. Cell lysates from cells expressing kAE1 alone was also used in the process (-ve). Samples were subjected to SDS-PAGE and immunoblotting. Blots were probed with anti-HA and anti-6xHis antibodies to detect kAE1 and ILK (pantophysin, GAPDH, or AP1mu1), respectively. The results (purified) showed that kAE1 was co-purified with ILK, patophysin, GAPDH, and AP1mu1.

5. Localization of kAE1 and interacting proteins

Localization of kAE1 and its interacting proteins was investigated by immunofluorescence co-staining method. HEK 293 cells grown on coverslips in 6 well-plates were co-transfected either with pkAE1 and pAP1mu1, pkAE1 and pGAPDH, or pkAE1 and pPanto. LLC-PK1 cells were transfected with pkAE1 to observe the interaction between kAE1 and endogenous ILK. Two days after transfection, the cells were washed once with warm 1x PBS and fixed with 4% paraformaldehyde in 1x PBS at room temperature for 1 h. Cells were permeabilized in 0.2% Triton X-100 at room temperature for 15 min followed by washing twice with 1x PBS. Antibody against HA-tagged or anti-AE1 was used to detect kAE1 whereas anti-6xHis antibody was used to detect 6xHis-tagged AP1mu1, GAPDH, and pantophysin. Endogenous ILK in LLC-PK cells was detected with mouse anti-ILK antibody. After washing three times with 1x PBS for 5 min each, the cells were incubated in a mixtures of 1:8000 dilution of Cy3-conjugated donkey anti-rabbit IgG and 1:250 dilution of Alexa 488-conjugated goat anti-mouse IgG to visualize kAE1 and AP1mu1 (GAPDH, or pantophysin), respectively, while Cy3-conjugated goat anti-rabbit and FITC-conjugated rabbit anti-mouse antibodies were used to visualize kAE1 and ILK, respectively. Fluorescence images were captured by LSM510 confocal microscopy. Co-localization of kAE1 and pantophysin was predominantly in the endoplasmic reticulum and the *trans*-Golgi network but a small proportion of kAE1 was co-localized with pantophysin at the plasma membrane (data not shown). Pantophysin interaction with kAE1 might be involved in the regulation of kAE1 translocation between compartments in transfected HEK 293 cells. Interestingly, endogenous ILK in LLC-PK1 was stained mainly in intracellular and peri-nuclear regions, but was detected on the cell surface when the cells were transfected with kAE1. ILK co-localized with kAE1 particularly at the cell surface, suggested that ILK was possibly directed and attached to the cell surface via interacting with kAE1 (Figure 4). In addition, co-localization between AP1mu1 (or GAPDH) and kAE1 were observed at the cell surface (Figure 4).

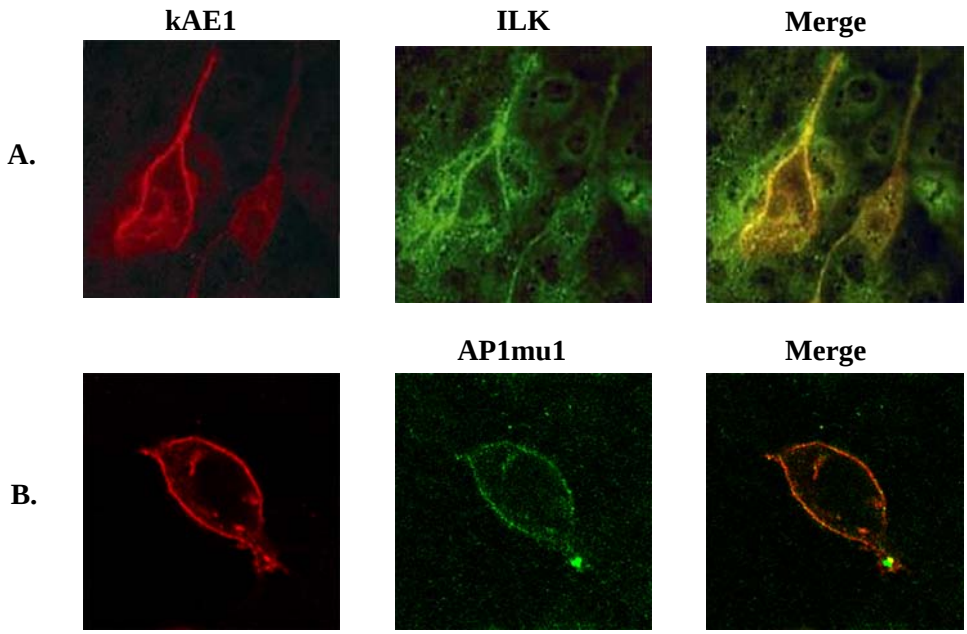

Figure 4 Co-localizations of kAE1 with ILK in LLC-PK1 cells and with AP1mu1 in HEK 293 cells.

Co-transfected cells were stained with anti-CkAE1 and anti-ILK antibodies or anti-HA and anti-His antibodies. Cy3- and FITC-conjugated antibodies were used to visualize kAE1 and ILK (A.), and Cy3- and Alexa 488-conjugated antibodies were used to visualize kAE1 and AP1mu1 (B.). Images were captured by Zeiss LSM510 confocol microscopy. kAE1 and kAE1-BPs (ILK and AP1mu1) were co-localized.

6. Effect of interacting proteins on cell surface expression of kAE1

Cell surface biotinylation was studied to examine whether the over-expression of ILK, GAPDH or pantophysin would affect the cell surface expression of kAE1 in HEK 293 cells or not (AP1mu1 was studied by FACS method – see below). HEK 293 cells transfected with pkAE1 alone or pkAE1 and pILK (pGAPDH or pPanto) were treated with a membrane impermeable biotinylating, NHS-SS biotin reagent. The biotinylated kAE1 proteins were bound to streptavidin resin and eluted from the resin by sample loading buffer containing 2% 2-mercaptoethanol to break disulfide bonds. Unfortunately, the amount of kAE1 in the biotinylated fraction could not be detected by anti-HA antibody because the biotinylated kAE1 eluted very poorly from streptavidin resin. The percentage of cell surface expression was therefore determined by comparing the amount of unbiotinylated kAE1 (lanes U) in the supernatant after incubation with streptavidin beads with the total expression of kAE1 (lanes T). It was found that a reduction in the amount of protein in the unbound fraction relative to the total fraction of kAE1 plus ILK was clearly evident compared with kAE1, while GAPDH and pantophysin had no effect on biotinylation of kAE1. By using the difference in the amount of kAE1 in the supernatant (lanes U) and total (lanes T) fractions, percentage of biotinylated kAE1 on cell surface was calculated with respect to comparable band densities by using (T-U/T) x 100%. HEK 293 cells expressing kAE1 alone was represented as 100% and compared to kAE1 co-expressed with interacting proteins. Thus, the percentage of cell surface expression was calculated to be 32 ± 7% kAE1, 31± 7% kAE1 plus pantophysin, 50 ± 7% kAE1 plus ILK, and 33 ± 3% kAE1 plus GAPDH (mean ± S.D, n = 4). It was shown that cell surface expression of kAE1 was increased by 50% in the presence of ILK transfected in HEK 293 cells. In control experiments, no biotinylation of cytoplasmic yellow fluorescent protein (YFP) was detected (0.009 ± 2%, mean ± S.D, n = 4), indicating that the labeling was restricted to the cell surface (Figure 5). Actin was used as an internal control to monitor protein expression. Expression of actin in each lane should be the same according to equal amount of protein loading. However, a reduction of actin was observed in unbound fraction of kAE1 when co-expressed with ILK but it did not change when co-expressed with GAPDH, pantophysin or when kAE1 was expressed alone (lanes U), implying that actin might be recruited to associate with kAE1 via interacting with ILK. These results indicated that cell surface expression of

kAE1 was increased in the presence of ILK, but the presence of GAPDH and pantophysin did not affect cell surface expression of kAE1.

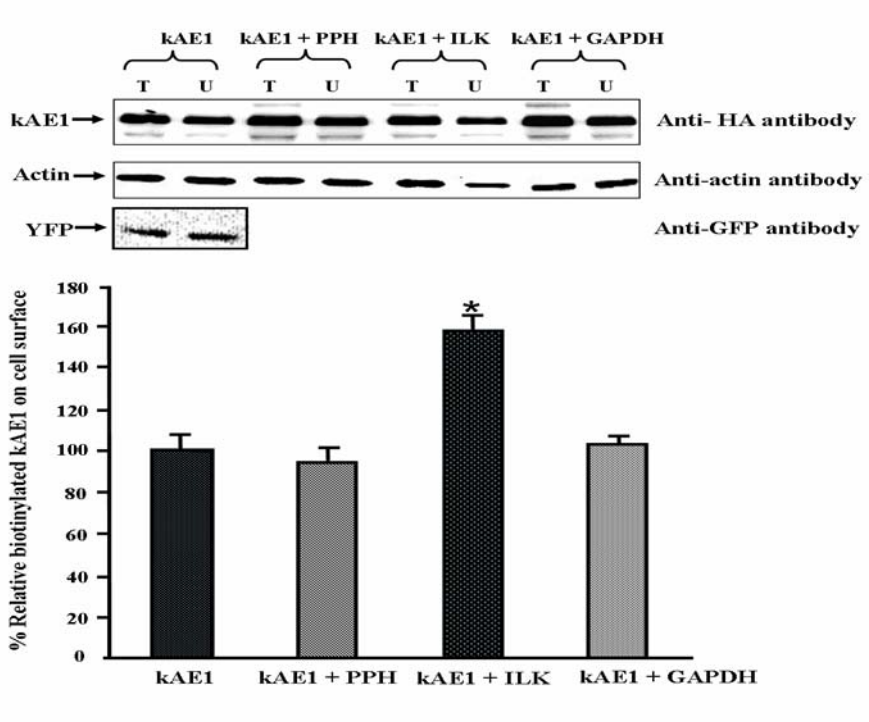

Figure 5 Effect of ILK, GAPDH, and pantophysin on cell surface expression of kAE1.

kAE1 was expressed in HEK 293 cells with or without interacting proteins (ILK, GAPDH and PPH). Cells were incubated with non-penetrating biotinylation reagent at 4°C for 30 min. Biotinylated proteins in lysates were captured using streptavidin resins. Upper panel: Immunoblotting using anti-HA antibody for kAE1 detection shows the amounts of total kAE1 fraction (T) and of unbound fraction (U). Percentage of biotinylated kAE1 on cell surface was calculated with respect to comparable band densities by using (T-U/T) x 100%. HEK 293 cells expressing kAE1 alone was represented as 100% and compared to kAE1 co-expressed with the interacting proteins. Actin was used as an internal control to verify protein expression in each lane while yellow fluorescent protein (YFP) used as a negative control to assess the degree of access of the biotinylation reagent to the cytosol. Lower panel: Quantification of kAE1 expressed on the cell surface, normalized to the fraction of kAE1 expressed alone. The error bar shows mean ± S.D. with n = 4. Asterisk indicates significant difference ($p<0.05$).

7. Interaction between erythrocyte anion exchanger 1 (eAE1) and ILK

kAE1 is identical to eAE1 except that it lacks the first 65 amino acids at the N-terminus. It has been known that the proteins that interact with the N-terminal domain of eAE1 do not interact with kAE1. It was found in the present study that kAE1 binds to ILK by using calponin homology (CH) domain (residues 27-189) present at its N-terminus which is also present in the N-terminal region of eAE1as a binding site. Thus, it is possible that eAE1 also interacts with ILK. To determine whether eAE1 interacts with ILK, co-immunoprecipitation of eAE1 and ILK was carried out by using HEK 293 cells co-expressed these two proteins. eAE1 was able to bind ILK when using anti-His antibody for precipitation (Figure 6). This suggests that the presence of the first 65 amino acids in the N-terminal eAE1 does not affect the ILK binding site. Therefore, the structural feature required for ILK interaction is retained in both kAE1 and eAE1.

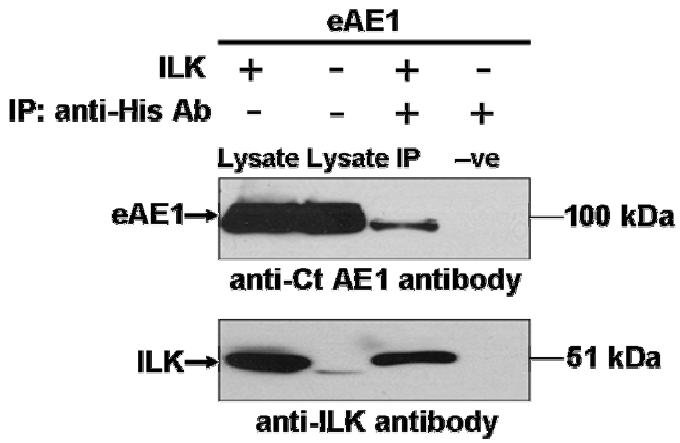

Figure 6 Examination of eAE1 and ILK interaction by co-immunoprecipitation. HEK 293 cells were transiently transfected to express only eAE1 or to co-express both eAE1 and HIS-tagged ILK, as indicated. Cell lysates were either set aside for later analysis (lysate), or immunoprecipitated with anti-His antibody (IP). Blots were probed for eAE1, using anti-AE1 antibody or for ILK using anti-ILK antibody, as indicated. eAE1 and ILK were co-immunoprecipitated, indicating that they could interact.

8. Analysis of kAE1 and eAE1 cell surface expression by fluorescence activated cell sorting (FACS)

When co-expressed with kAE1, ILK could increase cell surface expression of kAE1 as demonstrated by cell surface biotinylation. Since eAE1 was also found to interact with ILK, the question arose whether ILK increase cell surface expression of eAE1. Besides cell surface biotinylation, the amount of cell surface expression of kAE1 and eAE1 was also determined by using fluorescence activated cell sorting (FACS) analysis. HEK 293 cells either transfected with kAE1 HA557 or eAE1 HA557 in the combination with ILK or transfected with kAE1 HA557 or eAE1 HA557 alone, where the HA epitope was inserted at the third extracellular loop (position 557) to allow immunodetection of intact cells expressing the protein at the cell surface. In the presence of ILK and AP1mu1, cell surface expression of kAE1 HA557 was 31.90 ± 5% and 18.4 $\pm$ 3.6%, respectively, when compared to that of kAE1 HA 557 alone (19.88 ± 4%, mean ± SD, n = 5). This indicated that ILK slightly increased the cell surface expression of kAE1. For eAE1 HA557, cell surface expressions of eAE1 HA557 with or without ILK were 25.40 ± 3% and 21.60 ± 5%, respectively. HEK 293 cells transfected with empty vector (pcDNA 3.1) and stained with secondary antibodies were used as negative controls. The results show that ILK interacts with eAE1, but eAE1 trafficking was not affected by the interaction. In contrast, ILK interacted with kAE1 and facilitated kAE1 trafficking to the cell surface. Therefore, this indicates that ILK plays a role in an isoform-specific manner.

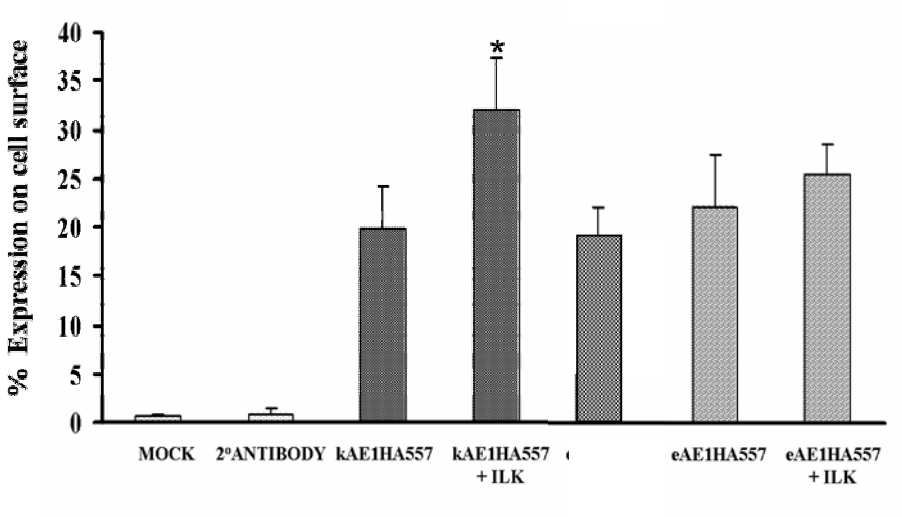

Figure 7 Analysis of cell surface expression of kAE1 and eAE1 by fluorescence activated cell sorting (FACS).

The bar graph showed the percentage of cell surface expression of kAE1 HA557 or eAE1 HA557 co-expressed with ILK or kAE1 HA557 co-expressed with AP1mu1 in transfected HEK 293 cells as indicated. The cells were stained with anti-HA antibody and goat anti-mouse conjugated with Alexa 488. The signal was analyzed by using Beckman-Coulter EPICS Elite Flow Cytometer. The percentage of fluorescence-stained cells was determined to quantify the level of the cell surface expression. The error bar shows the standard deviation (SD) of the results from five experiments. Asterisk indicates significant difference ($p<0.05$).

9. kAE1 anion exchange transport activity

To determine the effect of ILK on kAE1 anion exchange activity, the transport activity of HEK 293 cells expressing untagged kAE1 alone or kAE1 and His-tagged ILK was assessed using a whole cell Cl^-/HCO_3^- exchange assay. The transport activity of kAE1 was monitored using a fluorescence method. Non-charged, non-fluorescent ester form of the BCECF-AM [2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester] was loaded into HEK 293 cells. Non-charged BCECF-AM could rapidly diffuse across membranes to enter HEK 293 cells. Inside the cell, intracellular esterases would cleave the ester bond to release charged BCECF, which produce fluorescent signal according to intracellular pH. This assay measures the rate of Cl^-/HCO_3^- exchange across the plasma membrane mediated by kAE1.

Transfected HEK 293 cells were grown on glass coverslips and loaded with the pH-sensitive dye, BCECF-AM. Cells were perfused in a fluorescence cuvette alternately with Ringer's buffer containing either 140 mM NaCl or 140 mM Na gluconate. In the Cl^- free buffer, Cl^- leaves the cell in exchange for HCO_3^-, causing an alkalinization. Fluorescence changes were monitored by a Photon Technologies International (London, Ontario, Canada) RCR fluorimeter at excitation wavelengths of 440 and 502.5 nm and emission wavelength of 528.7 nm. Calibration with the nigericin/high potassium method was carried out to convert fluorescence measurements to intracellular pH (pHi) with pH values of 6.5, 7.0, and 7.5. Transport activity was calculated as change in pHi/min, and expressed as a percentage of kAE1 transport activity. The transport activity of sham-transfected cells was subtracted from the rate of change to correct for background activity. Statistical analysis was performed using Kaleidagraph software (Synergy Software, Reading, PA, USA).

Transport rates were determined from the initial slope of the alkalinization curve. Transport activity of kAE1 increased 84 ± 28% in the presence of ILK (n=3) (Figure 8). Since ILK promotes kAE1 trafficking to the cell surface, it also increases transport activity of kAE1.

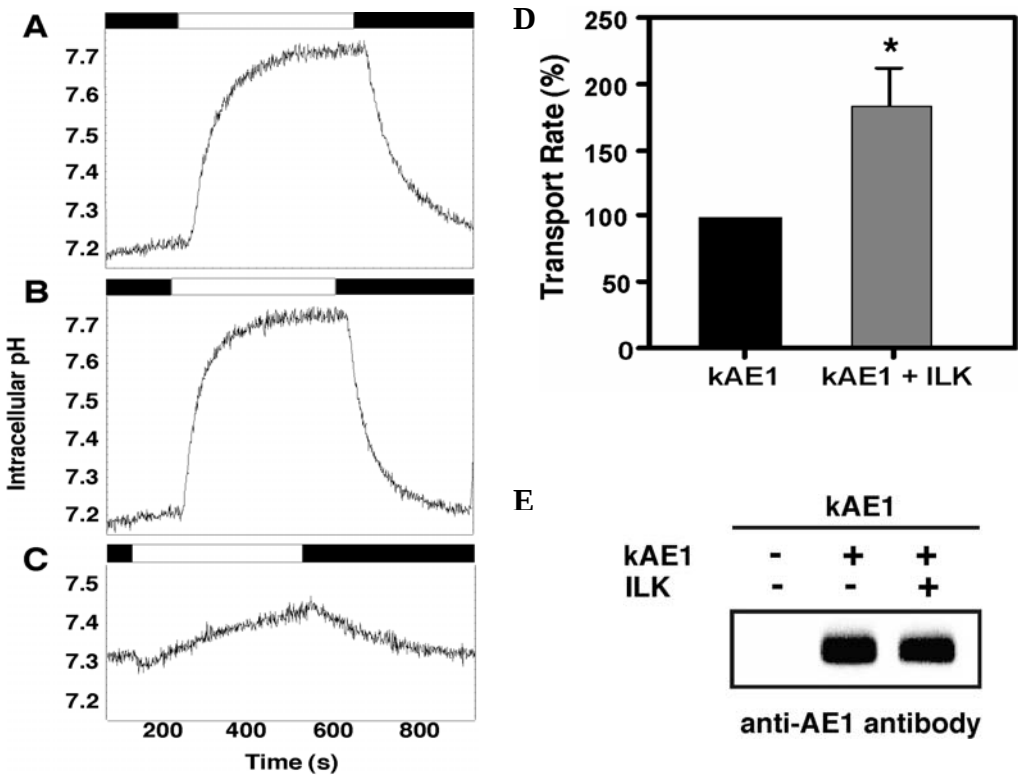

Figure 8 Anion exchange activity of kAE1 in the presence or absence of ILK. Transiently transfected HEK 293 cells were grown on coverslips and then loaded with the pH sensitive dye (BCECF-AM) two days after transfection. Cells were perfused in a fluorescence cuvette alternately with Ringer's buffer containing 140 mM NaCl (black bar) or 140 mM Na gluconate (white bar) as indicated above each panel. Intracellular pH was monitored as detailed in experimental procedures. Cells were transfected to express (A) kAE1, (B) kAE1 + ILK, or (C) with pcDNA3.1 (+) vector alone. (D) Mean anion exchange rate of kAE1. Mean values are expressed relative to transport rate of cells transiently transfected to express kAE1 alone. Transport rates (n = 3) were corrected for the background of HEK 293 cells. *P<0.05, unpaired t-test. (E) Expression of kAE1. HEK 293 cells were harvested from 60 mm dishes that contained the coverslips used for anion exchange assays. Cell lysates were subjected to 10% SDS-PAGE, and transferred to PVDF membrane. kAE1 was detected with monoclonal anti-AE1 antibody (IVF12).

10. Association of kAE1 with actin complex via interacting with ILK

ILK has been reported to be associated with the actin cytoskeleton via interacting with actopaxin, affixin and paxillin. Similarly, ankyrin is an adaptor protein that connects eAE1 to actin cytoskeleton in red blood cells. However, kAE1 is unable to bind to ankyrin. How kAE1 links to the actin cytoskeleton in kidney cells is still unknown. The results of immunofluorescence and cell surface biotinylation experiments led to the hypothesis that ILK might be a linker to connect kAE1 to the actin cytoskeleton. To examine this possibility, the lysates from HEK 293 cells transfected to express kAE1 plus ILK or kAE1 alone, prepared using the lysis buffer containing 0.5% Nonidet P-40, were incubated with Co^{2+} affinity resin. Endogenous paxillin and actopaxin were detected in the lysate of HEK 293 cells using anti-paxillin monoclonal antibody and anti-actopaxin polyclonal antibody, respectively. Proteins were eluted from the resin with 1.5 M imidazole containing buffer. As expected, on immunoblots kAE1 was co-purified with His-tagged ILK. The blot was then stripped and re-probed with anti-paxillin and anti-actopaxin antibodies. These immunoblots revealed that endogenous paxillin (68 kDa) and actopaxin (42 kDa) associated with the complex of ILK/kAE1. No paxillin and actopaxin were detected when kAE1 was expressed without ILK (Figure 9). This suggests that kAE1 interacted with the actin cytoskeleton through a complex containing ILK, paxillin and actopaxin (Figure 10).

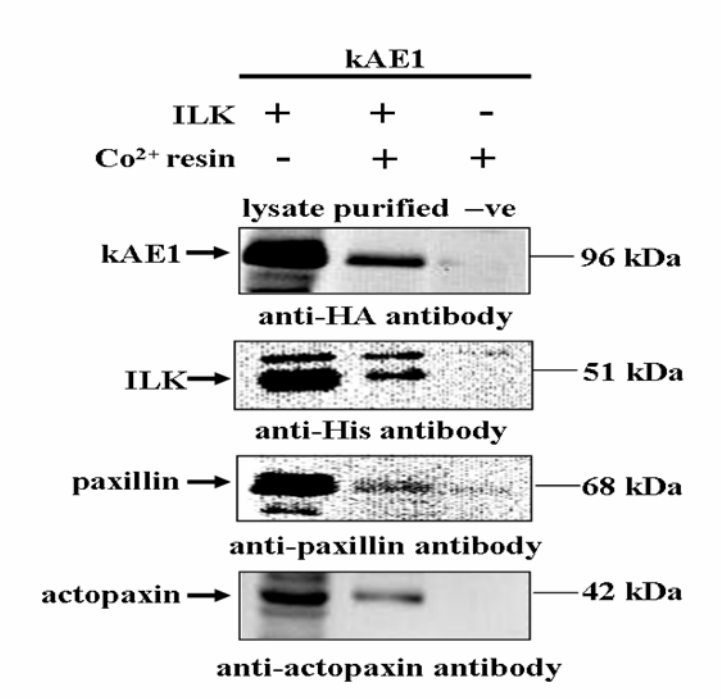

Figure 9 Co-association of kAE1 with actin complex via interacting with ILK.
HEK 293 cells were transiently transfected to express His-tagged ILK alone or HA-tagged kAE1 alone or co-transfected to express both HA-tagged kAE1 and His-tagged ILK, as indicated. Cell lysates were either set aside for later analysis (lysate), or incubated with Co^{2+} affinity resin at 4 $^{\circ}$C. Resin was washed and subsequently eluted with buffer containing 1.5 M imidazole. Samples were subjected to SDS-PAGE and immunoblotting. Blots were probed with anti-HA and anti-His antibodies for kAE1 and ILK detection, respectively, and with anti-paxillin and anti-actopaxin antibodies, as indicated.

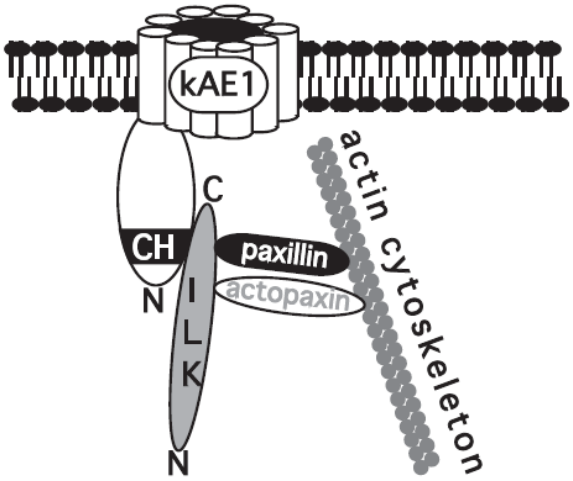

Figure 10 A possible model of association between kAE1-ILK and actin cytoskeletal complex.

kAE1 is composed of an N-terminal cytoplasmic domain and a C-terminal integral membrane domain responsible for anion transport. Near the N terminus of kAE1 is a newly identified calponin homology (CH) domain, which forms the binding site for integrin-linked kinase (ILK). The CH binding region of ILK is near the C terminus of ILK. In turn ILK binds the two actin-binding proteins, paxillin and actopaxin, to form a complex of ILK and kAE1 with the actin cytoskeleton. Cytoskeletal binding may stabilize kAE1 at the cell surface, maximizing cell surface kAE1 levels, and possibly aiding in basolateral targeting in epithelial cells.

Summary

The study in this project is proposed to identify the candidate proteins that interact with the N- and C-terminal domains of kAE1 (NkAE1 and CkAE1). Yeast two-hybrid system was employed to identify the interacting proteins. Recombinant plasmids encoding the GAL4 fusion proteins of cytoplasmic N-terminal domain (amino acids 66-403) and C-terminal domain (amino acids 876-911) of kAE1, namely pNkAE1 and pCkAE1, respectively, were successfully constructed and used as baits to screen cDNA library derived from the human kidney. The yeast transformed with either pNkAE1 or pCkAE1 did not show autonomously transcriptional activation of reporter genes on synthetic dropout (SD) plates. They also expressed GAL4-BD-NkAE1 or GAL4-BD-CkAE1 fusion proteins at molecular weights of 60 kDa or 26 kDa, respectively. According to the suitable properties of bait, the two plasmid constructs, pNkAE1 and pCkAE1, could thus be used as baits for screening of kAE1 interacting proteins from human kidney cDNA library by yeast mating method. The interaction between the N- or C-terminal domains of kAE1 and the interacting proteins was examined by the growth of diploid yeast on SD/-Ade/-His/-Leu/-Trp containing X-α-Gal plate when mating pNkAE1 or pCkAE1 with their interacting proteins. Then, the redundant of positive clones were reduced by using PCR and restriction enzyme analysis. cDNA inserts of the positive clones were sequenced and BLAST searched for homologous proteins in the databases. The identified interacting proteins of the N- and C-terminal kAE1 were classified as proteins involved in metabolism, transport and binding proteins, protein trafficking, apoptosis, membrane associated proteins, and cytoskeleton associated proteins, transcription and translation factors, and hypothetical proteins. After the specificity test of protein-protein interaction in yeast, GAPDH, pantophysin, ILK from N-terminal kAE1-interacting clones and AP1mu1 from C-terminal kAE1-interacting clones were selected selected for further studies to verify whether they are involved in the normal kAE1 trafficking in cultured human kidney cell line. Co-immunoprecipitation, affinity co-purification and immunofluorescence methods were accessed in human embryonic kidney (HEK 293) to confirm the association between kAE1 and interacting proteins. The results of co-immunoprecipitation, co-purification and co-localization revealed that kAE1 associated with AP1mu1, ILK, GAPDH or pantophysin. In addition, cell surface

biotinylation studies were performed to demonstrate the effect of interacting proteins on cell surface expression of kAE1. Interestingly, the result from cell surface biotinylation demonstrated ILK promoted cell surface expression of kAE1 while AP1mu1, GAPDH and pantophysin did not. However, it does not exclude the involvement of AP1mu1, GAPDH and pantophysin in kAE1 trafficking. These proteins may participate in the translocation process between membrane compartments during kAE1 trafficking inside the cells and then kAE1 was eventually facilitated to the cell surface of the α-intercalated cells via interacting with ILK.

The finding that over-expression of ILK promoted processing of kAE1 to the cell surface of HEK 293 cells suggests a role of ILK in kAE1 trafficking. ILK is a serine/threonine kinase, originally discovered as a β_1 integrin cytoplasmic tail-binding protein. Ankyrin repeats at the N-terminus of ILK interact with the LIM-only adaptor protein PINCH, while the C-terminus of ILK interacts with β_1 integrin, paxillin and actopaxin. ILK is a central protein in control of several signaling pathways through interacting with many proteins. ILK also plays a role in focal adhesion during ligand binding to integrin via controlling the reorganization of actin cytoskeleton. Moreover, ILK stimulates the renal epithelial cell morphogenesis and the failure of cell-matrix and cytoskeleton can cause renal diseases. Up to date, there is no report showing interaction between kAE1 and ILK. Our finding also show that ILK interacts with eAE1, which suggests that the removal of the first 65 amino acids at the N-terminus of kAE1 does not alter the folded structure of CH domain in eAE1. However, FACS analysis results revealed that co-expression of ILK did not significantly increase cell surface expression of eAE1 when compared with that of kAE1, suggesting that the interaction between ILK and eAE1 has no biologically relevant effect. It implies that another protein has ILK-like function in red cell membrane. Evidence shows that glycophorin A (GPA) facilitates the movement of eAE1 to the cell surface and has a chaperone-like role, enhancing trafficking during eAE1 biosynthesis; interaction between ankyrin and eAE1 also provides predominant connection between membrane and underlying spectrin/actin cytoskeleton network. Nevertheless, GPA is not found in α-intercalated cells of kidney, where kAE1 is unable to bind ankyrin. Taken together, we propose that for kAE1 in kidney ILK has a function equivalent to GPA and ankyrin acting on eAE1 in the red blood cells.

Cl^-/HCO_3^- exchange assays assessed whether ILK affects the physiological function of kAE1. Results revealed that ILK has an impact on the transport activity of kAE1 since the presence of ILK markedly increased transport activity of kAE1. Accordingly, the increase of kAE1 transport activity showed a correlation to the result of cell surface biotinylation. Since ILK is a linker protein, connecting extracellular matrix and actin cytoskeleton, ILK may promote kAE1 trafficking by connection of kAE1 to the actin cytoskeleton. Many lines of evidence have shown that the association between ILK, paxillin and actopaxin facilitated the complex to the actin cytoskeleton. The studies to examine the role of ILK in kAE1 trafficking revealed that the kAE1-ILK complex can recruit endogenous paxillin and actopaxin from HEK 293 cells, suggesting that kAE1-ILK-paxillin-actopaxin complex links kAE1 to the actin cytoskeleton. In the same way, another finding confirmed that ILK induced increased association of kAE1 with Triton shells, indicative of increased kAE1 association with the actin. It seems likely that actin cytoskeleton involved in kAE1 trafficking via a complex containing ILK, paxillin and actopaxin. Taken together, these present data demonstrated for the first time that ILK is a protein that promotes kAE1 trafficking by mediating interactions with the actin cytoskeleton.

References

[1] Brosius FC, 3rd, Alper SL, Garcia AM, Lodish HF. The major kidney band 3 gene transcript predicts an amino-terminal truncated band 3 polypeptide. The Journal of biological chemistry. 1989 May 15;264(14):7784-7.

[2] Fujinaga J, Tang XB, Casey JR. Topology of the membrane domain of human erythrocyte anion exchange protein, AE1. The Journal of biological chemistry. 1999 Mar 5;274(10):6626-33.

[3] Vince JW, Reithmeier RA. Identification of the carbonic anhydrase II binding site in the Cl(-)/HCO(3)(-) anion exchanger AE1. Biochemistry. 2000 May 9;39(18):5527-33.

[4] Low PS. Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions. Biochimica et biophysica acta. 1986 Sep 22;864(2):145-67.

[5] Tanner MJ. The structure and function of band 3 (AE1): recent developments (review). Molecular membrane biology. 1997 Oct-Dec;14(4):155-65.

[6] Zhang D, Kiyatkin A, Bolin JT, Low PS. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. Blood. 2000 Nov 1;96(9):2925-33.

[7] Rodriguez-Soriano J. New insights into the pathogenesis of renal tubular acidosis--from functional to molecular studies. Pediatric nephrology (Berlin, Germany). 2000 Oct;14(12):1121-36.

[8] Alper SL. Genetic diseases of acid-base transporters. Annual review of physiology. 2002;64:899-923.

[9] Karet FE. Inherited renal tubular acidosis. Advances in nephrology from the Necker Hospital. 2000;30:147-62.

[10] Bruce LJ, Unwin RJ, Wrong O, Tanner MJ. The association between familial distal renal tubular acidosis and mutations in the red cell anion exchanger (band 3, AE1) gene. Biochemistry and cell biology = Biochimie et biologie cellulaire. 1998;76(5):723-8.

[11] Sawasdee N, Udomchaiprasertkul W, Noisakran S, Rungroj N, Akkarapatumwong V, Yenchitsomanus PT. Trafficking defect of mutant kidney anion exchanger 1 (kAE1) proteins associated with distal renal tubular acidosis and Southeast Asian ovalocytosis. Biochemical and biophysical research communications. 2006 Nov 24;350(3):723-30.

[12] Vasuvattakul S, Yenchitsomanus PT, Vachuanichsanong P, Thuwajit P, Kaitwatcharachai C, Laosombat V, et al. Autosomal recessive distal renal tubular acidosis associated with Southeast Asian ovalocytosis. Kidney international. 1999 Nov;56(5):1674-82.

[13] Yenchitsomanus PT. Human anion exchanger1 mutations and distal renal tubular acidosis. The Southeast Asian journal of tropical medicine and public health. 2003 Sep;34(3):651-8.

[14] Quilty JA, Cordat E, Reithmeier RA. Impaired trafficking of human kidney anion exchanger (kAE1) caused by hetero-oligomer formation with a truncated mutant associated with distal renal tubular acidosis. The Biochemical journal. 2002 Dec 15;368(Pt 3):895-903.

[15] Quilty JA, Li J, Reithmeier RA. Impaired trafficking of distal renal tubular acidosis mutants of the human kidney anion exchanger kAE1. American journal of physiology. 2002 May;282(5):F810-20.

[16] Bruce LJ, Cope DL, Jones GK, Schofield AE, Burley M, Povey S, et al. Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. The Journal of clinical investigation. 1997 Oct 1;100(7):1693-707.
[17] Bruce LJ, Wrong O, Toye AM, Young MT, Ogle G, Ismail Z, et al. Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malaysia and Papua New Guinea: loss of up to 95% band 3 transport in red cells. The Biochemical journal. 2000 Aug 15;350 Pt 1:41-51.
[18] Jarolim P, Shayakul C, Prabakaran D, Jiang L, Stuart-Tilley A, Rubin HL, et al. Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) Cl-/HCO3- exchanger. The Journal of biological chemistry. 1998 Mar 13;273(11):6380-8.
[19] Tanphaichitr VS, Sumboonnanonda A, Ideguchi H, Shayakul C, Brugnara C, Takao M, et al. Novel AE1 mutations in recessive distal renal tubular acidosis. Loss-of-function is rescued by glycophorin A. The Journal of clinical investigation. 1998 Dec 15;102(12):2173-9.
[20] Cordat E, Li J, Reithmeier RA. Carboxyl-terminal truncations of human anion exchanger impair its trafficking to the plasma membrane. Traffic (Copenhagen, Denmark). 2003 Sep;4(9):642-51.
[21] Devonald MA, Smith AN, Poon JP, Ihrke G, Karet FE. Non-polarized targeting of AE1 causes autosomal dominant distal renal tubular acidosis. Nature genetics. 2003 Feb;33(2):125-7.
[22] Cordat E, Kittanakom S, Yenchitsomanus PT, Li J, Du K, Lukacs GL, et al. Dominant and recessive distal renal tubular acidosis mutations of kidney anion exchanger 1 induce distinct trafficking defects in MDCK cells. Traffic (Copenhagen, Denmark). 2006 Feb;7(2):117-28.
[23] Kittanakom S, Cordat E, Akkarapatumwong V, Yenchitsomanus PT, Reithmeier RA. Trafficking defects of a novel autosomal recessive distal renal tubular acidosis mutant (S773P) of the human kidney anion exchanger (kAE1). The Journal of biological chemistry. 2004 Sep 24;279(39):40960-71.
[24] Kittanakom S, Cordat E, Reithmeier RA. Dominant-negative effect of Southeast Asian ovalocytosis anion exchanger 1 in compound heterozygous distal renal tubular acidosis. The Biochemical journal. 2008 Mar 1;410(2):271-81.
[25] Groves JD, Ring SM, Schofield AE, Tanner MJ. The expression of the abnormal human red cell anion transporter from South-East Asian ovalocytes (band 3 SAO) in Xenopus oocytes. FEBS letters. 1993 Sep 13;330(2):186-90.
[26] Toye AM. Defective kidney anion-exchanger 1 (AE1, Band 3) trafficking in dominant distal renal tubular acidosis (dRTA). Biochemical Society symposium. 2005(72):47-63.
[27] Chen J, Vijayakumar S, Li X, Al-Awqati Q. Kanadaptin is a protein that interacts with the kidney but not the erythroid form of band 3. The Journal of biological chemistry. 1998 Jan 9;273(2):1038-43.
[28] Hubner S, Bahr C, Gossmann H, Efthymiadis A, Drenckhahn D. Mitochondrial and nuclear localization of kanadaptin. European journal of cell biology. 2003 May;82(5):240-52.
[29] Hubner S, Jans DA, Xiao CY, John AP, Drenckhahn D. Signal- and importin-dependent nuclear targeting of the kidney anion exchanger 1-binding protein kanadaptin. The Biochemical journal. 2002 Jan 15;361(Pt 2):287-96.

[30] Kittanakom S, Keskanokwong T, Akkarapatumwong V, Yenchitsomanus PT, Reithmeier RA. Human kanadaptin and kidney anion exchanger 1 (kAE1) do not interact in transfected HEK 293 cells. Molecular membrane biology. 2004 Nov-Dec;21(6):395-402.
[31] Wongthida P, Akkarapatumwong V, Limjindaporn T, Kittanakom S, Keskanokwong T, Eurwilaichitr L, et al. Analysis of the interaction between human kidney anion exchanger 1 and kanadaptin using yeast two-hybrid systems. Genetics and Molecular Biology. 2006;29(1):14-22.
[32] Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, et al. Current protocols in molecular biology. United States: John Wiley and Sons Inc.,New York, NY 1998.

Outputs

Publications

1. Sawasdee N, Udomchaiprasertkul W, Noisakran S, Rungroj N, Akkarapatumwong V, Yenchitsomanus P. Trafficking defect of mutant kidney anion exchanger 1 (kAE1) proteins associated with distal renal tubular acidosis and Southeast Asian ovalocytosis. Biochem Biophys Res Commun. 2006;350(3):723-30.
2. Keskanokwong T, Shandro HJ, Johnson DE, Kittanakom S, Vilas GL, Thorner P, Reithmeier RA, Akkarapatumwong V, Yenchitsomanus PT, Casey JR. Interaction of integrin-linked kinase with the kidney chloride/bicarbonate exchanger, kAE1. J Biol Chem. 2007;282(32):23205-18.
3. Ngaojanlar P, Limjindaporn T, Akkarapatumwong V, Thongnoppakhuu W, Noisakran S, Sawasdee N, Sanchatjate S, Sangiambut S, Yenchitsomanus P. AP-1 mu1A as a novel binding protein of human kidney anion exchanger 1 (Manuscript submitted 2008).

Presentations

1. Sawasdee N, Udomchaiprasertkul W, Noisakran S, Rungroj N, Akkarapatumwong V, Yenchitsomanus P. Trafficking defect of mutant kidney anion exchanger 1(kAE1) proteins associated with distal renal tubular acidosis. Molecular Medicine 2006: From Research to Clinical Practice, November 28-30, 2006, Institute of Science and Technology for Research and Development, Mahidol university, Salaya campus, Thailand
2. Sawasdee N, Udomchaiprasertkul W, Noisakran S, Rungroj N, Akkarapatumwong V, Yenchitsomanus P. Trafficking defect of mutant kidney anion exchanger 1(kAE1) proteins associated with distal renal tubular acidosis. First annual symposium of the protein society of Thailand "Challenges in Protein Research in Thailand", October 24-25, 2006, Chulabhorn Research Institute Conference Center, Bangkok, Thailand.
3. Yenchitsomanus P, Ngaojanlae P, Keskanokwong T, Limjindaporn T, Akkarapatuwong V. Identification of proteins binding to anion exchanger 1 in human kidney. Young Research Scholars Meet TRF-Senior Research Scholars Conference, October 12-14, 2006, The Regent Cha-Am Beach Resort, Cha-am, Petchburi.

4. Keskanokwong T, Shandro HJ, Kittanakom S, Reithmeier RAF, Yenchitsomanus P, Casey JR, Akkarapatumwong V. A new insight into anion exchanger 1 (AE1) trafficking in human kidney. Gordon Research Conference on Membrane Transport Protein, August 13-18, 2006, University of New England, Biddeford, Maine, USA.
5. Keskanokwong T, Akkarapatumwong V, Shandro HJ, Kittanakom S, Reithmeier RAF, Yenchitsomanus P, Casey JR. Integrin-linked Kinase Promotes Intracellular Trafficking of Kidney Anion (Cl-/HCO3-) Exchanger 1 (kAE1). RGJ-Ph.D. Congress VII, April 20-22, April 2006, Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand.
6. Keskanokwong T, Shandro HJ, Kittanakom S, Reithmeier RA, Akkarapatumwong V, Yenchitsomanus P, Casey JC. Integrin-linked Kinase Promotes Intracellular Trafficking of Kidney Anion (Cl-/HCO3-) Exchanger 1 (kAE1). Siriraj-Rama Congress, April 18-21, 2006. Queen Sirikit National Convention Center, Bangkok, Thailand.
7. Ngaojanlar P, Limjindaporn T, Sa-ngiumbut S, Thongnoppakhun W, Akkarapatumwong V, Yenchitsomanus P. Identification of proteins interacting with human kidney anion exchanger 1 (kAE1). Siriraj-Ram a Congress, April 18-21, 2006. Queen Sirikit National Convention Center, Bangkok, Thailand.
8. Sawasdee N, Udomchaiprasertkul W, Noisakran S, Rungroj N, Akkarapatumwong V, Yenchitsomanus P. Molecular defect of distal renal tubular acidosis caused by compound heterozygous *anion exchanger 1* mutations. Siriraj-Rama Congress, April 18-21, 2006. Queen Sirikit National Convention Center, Bangkok, Thailand.
9. Ngaojanlar P, Limjindaporn T, Sa-ngiambut S, Thongnoppakhun W, Akkarapatumwong V, Yenchitsomanus P. Identification of protein interacting to anion exchanger 1 in human kidney by yeast two-hybrid system. The Second Protein Research Network Symposium on: “Proteins: Structure, Function, and Proteomics”, September, 22-23, 2005, Chulabhorn Research Institute, Bangkok, Thailand.

Theses and graduate students

1. Isolation and identification of protein binding to anion exchanger 1 (AE1) in human kidney.
   Miss Thitima Keskanokwong, Ph.D. (Molecular Genetics and Genetic Engineering), Faculty of Graduate Studies, Mahidol University, 2007.
2. Search for protein interacting with kidney isoform of anion exchanger 1 (kAE1) by Gal4-based yeast two-hybrid system.
   Miss Phuttawadee Phuengcharoen, M.Sc. (Molecular Genetics and Genetic Engineering), Faculty of Graduate Studies, Mahidol University, 2007.
3. Identification of human kidney proteins interacting with the carboxyl terminus of anion exchanger 1.
   Miss Peingpaga Ngaojanlar, M.Sc. (Immunology), Faculty of Graduate Studies, Mahidol University. 2006.
4. Interaction, trafficking and subcellular localization of mutant kidney anion exchanger 1 (kAE1) proteins in cultured human embryonic kidney 293 (HEK 293) cells.
   Miss Nunghathai Sawasdee, M.Sc. (Immunology), Faculty of Graduate Studies, Mahidol University. 2006.

Appendix