รายงานฉบับสมบูรณ์

โครงการ

การศึกษาพยาธิวิทยาในระดับโมเลกุลของการกลายพันธุ์

ในยีนแอนไอออนเอกซ์เชนเจอร์ วัน

อันเป็นสาเหตุของภาวะไตขับกรดไม่ได้ในประเทศไทย

Molecular Pathologic Studies of the Anion Exchanger 1 Mutation

as the Cause of Distal Renal Tubular Acidosis in Thailand

หัวหน้าโครงการ : นายชัยรัตน์ ฉายากุล

หน่วยงาน: ภาควิชาอายุรศาสตร์

คณะแพทยศาสตร์ศิริราชพยาบาล ม.มหิดล

สนับสนุนโดย

วิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา

สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(1 พฤศจิกายน 2542 ถึง 31 ตุลาคม 2544)

รายงานฉบับสมบูรณ์

โครงการ

การศึกษาพยาธิวิทยาในระดับโมเลกุลของการกลายพันธุ์ ในยีนแอนไอออนเอกซ์เชนเจอร์ วัน อันเป็นสาเหตุของภาวะไตขับกรดไม่ได้ในประเทศไทย

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สนับสนุนโดย
ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา
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ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์
เลขที่ 979/17-21 ถนนพหลโยธิน แขวงสามเสนใน
เขตพญาไท กรุงเทพฯ 10400
โทร.298-0455 โทรสาร 298-0476
Home page : http://www.trf.or.th
E-mail : trf-info@trf.or.th

กิตติกรรมประกาศ

งานวิจัยครั้งนี้คงจะเกิดขึ้นไม่ได้หากมิได้รับการสนับสนุนจากบุคคลที่ความสำคัญและหน่วยงานหลายๆฝ่าย ดังนี้

1) ศ.พรชัย มาตังคสมบัติ อดีตคณบดี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลที่ได้สนับสนุนโครงการ โดยเฉพาะครุภัณฑ์พื้นฐานในการเลี้ยงกบ *Xenopus levis* ซึ่งอาจจะเป็นแห่งแรกในประเทศไทย

2) Prof. Seth Alper แห่ง Molecu ar Medicine และ Renal Unit, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA ที่ได้อนุญาตให้ใช้ cDNA clone ต่าง ๆ ของ AE 1 variants, antibodies ที่จำเป็นในการวิจัย รวมทั้งให้คำแนะนำที่มีประโยชน์อย่างยิ่งระหว่างการดำเนินการวิจัย

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4) Prof. Hitoshi Endou แห่ง Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, JAPAN ที่ได้ให้ *Xenopus levis* ตลอดการทดลอง รวมทั้งอุปกรณ์เครื่องมือบางอย่างที่จำเป็นสำหรับการวิจัยในครั้งนี้

และท้ายที่สุด 5) สำนักงานกองทุนสนับสนุนการวิจัย ที่ได้สนับสนุนการวิจัยมาตลอดระยะเวลา 2 ปี

กลุ่มผู้วิจัยใคร่ขอขอบคุณทุกท่านเป็นอย่างสูง รวมทั้งใคร่ขอขอบคุณหน่วยงานต้นสังกัด สาขาวิชาวักกะวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล และภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่ได้ให้การสนับสนุนโครงการวิจัยนี้เป็นอย่างดีตลอดมา

บทคัดย่อ

ภาวะไตขับกรดไม่ได้ (classical distal renal tubular acidosis) เป็นปัญหาที่สำคัญทางสาธารณสุขอย่างหนึ่งในประเทศ ปัจจุบันทราบว่าภาวะไตขับกรดไม่ได้ที่เป็นแต่กำเนิดและมีการถ่ายทอดทางพันธุกรรมนั้น มีสาเหตุจากยีนที่ผิดปรกติอย่างน้อย 4 ชนิด แต่ที่พบบ่อยและมีรายงานในประเทศเกิดจากการกลายพันธุ์ของยีน Anion Exchanger 1 (AE1 หรือ Band 3) ซึ่งกำหนดโครงสร้างของโปรตีนที่ทำการแลกเปลี่ยน HCO_3^- ภายในเซลล์กับ Cl^- ภายนอกทั้งในเซลล์ขับกรดในไตและในเม็ดเลือดแดง นอกจากนั้น AE1 ยังทำหน้าที่ร่วมกับโปรตีนอื่นในการกำหนดรูปทรงของเม็ดเลือดแดงด้วย ดังนั้น การศึกษาหน้าที่ของ AE1 จึงมีความสำคัญเพื่อพิสูจน์ว่าโปรตีนที่เกิดจากการกลายพันธุ์นั้นเป็นสาเหตุของการเกิดโรคจริง

ในโครงการนี้ คณะผู้ศึกษาได้พัฒนาวิธีศึกษาหน้าที่ และโครงสร้างของโปรตีนที่แสดงออกบนผิวเซลล์ (membrane protein) ขึ้น โดยใช้ระบบการแสดงออกของโปรตีนในไข่กบ (*Xenopus laevis* oocyte expression system) ทำให้สามารถทดสอบหน้าที่และโครงสร้างของโปรตีน AE1 ที่เกิดจากการกลายพันธุ์ได้ นอกจากนั้น ยังสามารถทดสอบโปรตีนอื่นที่เคยรายงานว่ามีปฏิสัมพันธ์กับ AE1 เช่น glycophorin A (GpA) และ protein 4.2 ผลการศึกษาที่สำคัญที่ได้ในส่วนนี้ คือ 1) ทดสอบยืนยันว่า GpA มีความสำคัญอย่างยิ่งในการทำหน้าที่ของ AE1 G701D ที่พบในผู้ป่วยไตขับกรดไม่ได้ที่มีการถ่ายทอดแบบ recessive, 2) พิสูจน์ว่า protein 4.2 ไม่มีผลสำคัญต่อการทำหน้าที่แลกเปลี่ยนสารของ AE1 ในระบบที่ทำการศึกษา, และ 3) พบว่า AE1 Δ400-408 ยับยั้งการฟื้นหน้าที่ของ AE1 G701D โดย GpA ในลักษณะ dominant negative effect ซึ่งสามารถอธิบายการเปลี่ยนแปลงที่ไตและเม็ดเลือดแดงของผู้ป่วยไตขับกรดไม่ได้ร่วมกับ ovalocytosis ที่มีการกลายพันธุ์แบบ compound heterozygosity ของ AE1 Δ400-408 / G701D

การศึกษาอีกส่วนหนึ่ง คือการ clone หา human kanadaptin จากไต ซึ่งสามารถทำได้สำเร็จด้วยวิธี reverse transcription / polymerase chain reaction โดยโปรตีนดังกล่าวถูกรายงานใน mouse ว่ามีปฏิสัมพันธ์กับ AE1ในไต และอาจมีความสำคัญต่อกระบวนการขับกรดในเซลล์ที่ท่อไตส่วนปลาย ผลการวิเคราะห์ลักษณะการเรียงตัวของกรดอะมิโนของ human kanadaptin พบมีลักษณะพิเศษหลายอย่างที่น่าจะเกี่ยวข้องกับ sorting ของโปรตีนที่ทำปฏิสัมพันธ์ด้วย ซึ่งเมื่อทำการศึกษาในไข่กบ พบว่า kanadaptin ในขนาดสูงสามารถยับยั้งการทำหน้าที่ของโปรตีน AE1 ปรกติ และ AE1-R589H ที่สัมพันธ์กับภาวะไตขับกรดไม่ได้ชนิด autosomal dominant ได้บางส่วน

ABSTRACT

Classical distal renal tubular acidosis (dRTA), one of the major health problems in Thailand, results from urinary acid excretion defects, causing metabolic acidosis, hypokalemic paralysis, metabolic bone disease and nephrocalcinosis / nephrolithiasis. It is currently known that dRTA may be inherited in either autosomal dominant or recessive form, and mutations in at least 4 genes have been identified. Thai patients have recently been shown to have mutations in the anion exchanger 1 (AE1, band 3) gene that seem to be unique. This gene encodes for the Cl^- / HCO_3^- exchanger located at the basolateral membrane of the kidney acid secreting eells and red blood cells. AE1 mutations have also been found in association with many red cell defects including ovalocytosis and spherocytosis but these patients do have normal urinary acidification function. Thus, it is important to know the transport function of mutant AE1 proteins in order to prove their significance as the cause of dRTA.

In this study, we developed the heterologous expression system of *Xenopus laevis* oocyte to study function and structure of AE1 mutant variants associated with dRTA and potential interaction with AE1 associated proteins. The important results include 1) confirmation that glycophorin A (GpA) is crucial for the function of AE1 G701D, mutant found in association with recessive dRTA, 2) co-expression of protein 4.2 does not result in significant change of AE1-mediated anion transport in this system, and 3) AE1 Δ400-408 attenuates the rescued effect of AE1 G701D by GpA in a dominant negative fashion. The latter finding explains the distinct red blood cell and kidney phenotypes in patients with ovalocytosis and recessive dRTA.

Another part of this study is to isolate human kanadaptin, a protein known to interact with kAE1 in mouse, from kidney tissue. By reverse transcription and polymerase chain reaction human kanadaptin cDNA was cloned. The coding sequence predicts a 796 amino acid residue protein with multi-domain structure that shares 80% identity to mouse kanadaptin. Co-expression of high dose kanadaptin results in partial inhibition of anion transport function mediated by both wild-type kidney AE1 and AE1-R589H, a mutant found in association with autosomal dominant dRTA.

สารบัญ

บทนำ

Classical distal renal tubular acidosis (dRTA) is a clinical syndrome characterized by hyperchloremic metabolic acidosis secondary to impairment in urinary acid excretion by the distal nephron. Clinical manifestations in these patients may vary from growth retardation, hypokalemic muscle weakness, osteomalacia and nephrocalcinosis or nephrolithiasis causing pyelonephritis to eventual renal failure. Many patients with classical dRTA have the condition in association with a systemic illness (acquired form), while the disease also occurs as an isolated defect inherited as either autosomal dominant or autosomal recessive trait.

In search of the genetic defects underlying inherited dRTA, studies in the past few years have persistently identified mutations in the anion exchanger 1 (*AE1*, SLC4A1) gene associated with autosomal dominant dRTA. AE1 is a member of the anion exchanger family that is only known to be expressed in the erythrocyte (eAE1) and in the basolateral membranes of acid secreting type A intercalated cells of collecting ducts of the kidney (kAE1). eAE1 functions to increase the ability of blood to carry carbon dioxide from the tissue to the lung and maintains the red cell stability and integrity by anchoring the sub-membrane protein skeleton to the lipid bilayer. kAE1, which lacks the amino-terminal 65 amino acid residues of eAE1, plays role in acid secretion by providing the exit route of HCO_3^- in exchange for Cl^- in the process of distal renal acidification. Its defect should result in excessive intracellular alkalinization and impaired distal renal acidification machinery. The pathogenesis of dominantly inherited dRTA, however, is still unclear since functional analysis of the common mutation, R589X, revealed only a 40-50%

reduction in anion transport function, and without dominant negative effects as might be expected from an AD trait.

Studies in patients with autosomal recessive dRTA revealed conflicting but remarkable results. Genetic linkage analysis studies exclude AE1 as the cause of dRTA in recessive patients of Western extraction but its association with AE1 G701D mutation has been reported in Thai kindred originally in its homozygous form. The G701D mutation has since been detected in compound heterozygote state in families also harboring Southeast Asian ovalocytosis (SAO), a heterozygous in-frame deletion of amino acid residues 400 through 408 not yet itself found as a homozygous mutation.

Given its complexity, molecular pathology of dRTA is at present unclear. Nevertheless, available information suggests that virtual mechanism leading to renal acidification defect might be attributed to defective membrane insertion of mutant kAE1 in the kidney type A intercalated cell. This polarized epithelium requires for fully effective AE1 function complements of specific kAE1 binding proteins that differ from those (i.e. glycophorin A and protein 4.2) in red blood cells. For example, the loss-of-function phenotype of kAE1 G701D associated with recessive dRTA appears secondary to its inability to reach the cell surface properly in the absence of *specific red cell chaperonin, glycophorin A*. As for *AE1* mutation associated with dominant dRTA, it is believe that AE1 R589 proteins may be incorrectly targeted, or inadequately retained at the basolateral membrane of particular type A intercalated cells, but not in red cells. This raises an important question that R589 kAE1 mutant is differentially regulated in the intercalated cells by components for membrane sorting mechanisms that are not present in the red blood

cells. One logical candidate protein involved in this process is the novel protein *kanadaptin*, which has been shown to *specifically interact with kAE1* but not eAE1 in mouse. Preliminary data suggest that kanadaptin might involve in membrane sorting and targeting of kAE1 polypeptides to the basolateral membrane. Isolation of the human kanadaptin cDNA will allow us to investigate its interaction with several kAE1 mutant variants and its role in pathogenesis of some forms of dRTA.

The present study was designed to investigate the effects of particular AE1 mutations and the role of AE1 binding proteins as the cause of dRTA in Thailand. Functional and structural analyses of AE1 have been performed individually and in combination using the heterologous expression system of *Xenopus* oocytes. We also tested the functional effects of glycophorin A (the AE1 chaperonin), protein 4.2 (the eAE1 binding protein), and the kAE1 binding protein kanadaptin, by co-expression studies.

วิธีการทดลอง

Preparation of AE1 variants, *c-myc* tagged AE1 and AE1 binding proteins cDNAs

- The plasmids containing several variants of AE1 cDNA were provided by Dr.Seth Alper, our collaborator at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA. These clones were re-constructed and subcloned in the same Xenopus oocyte expression vector containing at the 5' and 3' flanking regions the corresponding untranslated regions from Xenopus globin mRNA. These clones include:-

1. wild-type erythroid AE1
2. wild-type kidney AE1
3. R589H erythroid AE1
4. R589H kidney AE1
5. M31T/K56E/G701D erythroid AE1
6. G701D kidney AE1
7. Δ400-408 erythroid AE1
8. Δ400-408 kidney AE1
9. human glycophorin A
10. human protein 4.2

- In order to test interactions between two AE1 variants in the co-expression studies, tagging of one human AE1 construct was done by insertion of a '*c-myc* reporter epitope' at the carboxyl terminus using PCR-based technique. The *c-myc* tags encoding PCR primers were as follows: Sense, 5' ACC TGG CGC ATG CAC TTA TTC 3'; Antisense, 5' CTC CTA ACA CAG GTA GGT CTT CCT CAG AGA TCA GCT TCT GCT CCA TGG CCA CTT CGT CGT ATT C 3'. These primers contain the *c-myc* epitope (EQKLISEEDL) and, respectively, an *Sph*I and *Eco*NI site for subcloning. The clones derived designated

- cmyc-tagged erythroid AE1
- cmyc-tagged kidney AE1

- An AE1 mutation with deletion in the C-terminal 11 amino acids was constructed in both the erythroid and kidney isoform using PCR-based technique. This mutant AE1 variant has been found in a family with autosomal dominant dRTA but the effect of this mutation on the anion transport function remains to be determined. The PCR primers used in this study were as follows: Sense: 5'-TGG CGC ATG CAC TTA TTC-3' and Antisense 5'-CTC CTA ACA TAG GTC ATC CTT CCT CCT CAT C-3'. The PCR products were purified, doubly digested with *Sph*I / *EcoN*I , and subcloned into the similarly cleaved cmyc-tagged clone. The sequence was then confirmed by DNA sequencing. The clones derived designated

- C-deleted cmyc-tagged eAE1

- C-deleted cmyc-tagged kAE1

Cloning of human kanadaptin

- Total RNA extracted from human kidney cortex and medulla is used as the template for cloning of human kanadaptin by RT-PCR approach. The PCR primers used in this study were designed based on alignment of nucleotide sequence of mouse kanadaptin with sequences derived from human expressed sequence tagged (EST) database. Two overlapping PCR primer pairs were used: a) 5'-CGA TCC CGT TGT AAC CTC TAA A-3' and 5'-GCC GAA GCA ATC CCA AAG T-3', and b) 5'- GTA ACA CAG TTG AAG GAA TTG CG-3' and 5'-GGT TTT CTT TTG GGA TTC TGA A-3'. The respective RT-PCR products of 1.55 and 1.42 kb were cloned to the pGEM-T vector (Promega) while combination of both clones were done by *BamH*I / *Sal*I double digestion and ligation. The nucleotide sequence of human Kanadaptin was analyzed by DNA sequencing on both strands.

RNA transcription

- Capped cRNA was transcribed in vitro from linearized plasmid DNA using the Ambion (Austin, TX) Megascript Kit according to manufacturer's instruction.

Functional expression of AE1 in *Xenopus* ooycytes

- The mature female *Xenopus laevis* (South African clawed frogs) and food were kindly supplied without charge throughout the study period by Prof. Hitoshi Endou, Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan. The methods used for oocyte preparation and microinjection of cRNA have been described in detail previously (see Addendum). In brief, caped cRNA was micro-injected into collagenase treated and manually defolliculated oocytes, and allowed to express the protein for 2-3 days at 18 °C.

Isotopic chloride influx assays

- The isotopic influx medium was prepared by adding 3 µl of ^{36}Cl (ICN) into 147 µl of ND-58 solution (NaCl 58 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, $CaCl_2$ 1.8 mM, pH 7.4) plus 0.15 µl of 20 mM bumetanide to block endogenous Na,K,2Cl cotransporter. The final chloride concentration in the influx medium was 96 mM, with a calculated final osmolality of 212 mOsm. The influx assay was initiated by transfer group of 8-10 control (water injected) oocytes or cRNA injected oocytes into a microtiter 96-well plate containing 150 µl of the above isotopic influx medium (one well/group). Using manual pipette, isotopic influx medium was pipette up and down for mixing oocytes and isotopic medium. Furthermore, 10 µl of isotopic medium was removed from that medium for counting external medium of isotopic influx medium. Oocytes were incubated at room temperature for 15 to 30 min according to the study condition. To

terminate the reaction, ice-cold Cl-free washing solution (Na Isethionate 96 mM, K gluconate 2 mM, Mg gluconate 1 mM, HEPES 5 mM, Ca gluconic acid 1.8 mM, pH 7.4) was added onto the oocytes, and rapidly transferred from 96-well plate to a petri dish containing ice-cold washing solution. The oocytes were rapidly washed 5 more times in a similar manner, and individual oocytes were then transferred to the vial containing 1% SDS (200 µl). The oocyte was shaked for 30 min to dissolve its membrane and solubilize the content. The scintillation fluid (2 ml, contain: 0.5 gm%PPO, 0.1 gm% POPOP, 33% triton X-100, and 66% toluene) was added into each vial and thoroughly mixed. Radioactivity of the lysates was counted using scintillation counter to determine the transport rate. All experiment were repeated 2-3 times using different oocyte batches. The mean values were compared using conventional statistical analysis.

Isotopic chloride efflux assays

- After 2-3 days of cRNA microinjection, the selected AE1-expressing oocytes were sorted for ^{36}Cl microinjection. These oocytes were transferred to Cl-free ND-96 solution containing bumetanide. One oocyte was microinjected at 1 min interval, at room temperature with 50 nl of 123 mM ^{36}Cl by Drummond Digital Microdispenser. After ^{36}Cl microinjection, individual oocyte was transferred into 96-well plate containing the same solution. After 10-15 min, when there was no detectable radioactivity leakage, the oocytes were washed thrice and transferred into a 48-well plate containing 300 µl of efflux medium containing ND96 plus bumetanide solution. At 10-15 min interval, a 250 µl efflux medium was removed for scintillation counting and equal volume of fresh efflux medium was replaced for a total period of approximately 1 h. To terminate chloride efflux, the oocytes were washed thrice with a Cl-free solution. In order to test for

leakage, oocytes were incubated in 300 µl of Cl-free solution for another 30 min and 250 µl of the media was collected. Finally, each oocyte was transferred to a vial for dissolution in 200 µl of 1% SDS. The collected medium were counted using scintillation counter to measure efflux rate of each oocyte. All experiment were repeated 2-3 times using different oocyte batches. Data were presented as percentage of total cpm injected at particular time interval or as log (% of total cpm injected) vs. time. The efflux rate constants were also calculated from semilog plot linear slopes calculated from the last three time points for each condition.

Structural Analysis of heterologous AE1 polypeptides

- Metabolic labeling and immunoprecipitation

Groups of 20-30 cRNA injected or water injected oocytes were incubated for 12 h after injection and then metabolic labeled by microinjection of ^{35}S-methionine (ICN). The labeled oocytes were incubated further for 24-36 h and homogenized in 100 µl of ice-cold oocyte lp buffer containing 100 mM NaCl, 2 mM EDTA, 100 mM Tris pH 8.0, 1% Triton X-100, 1 mM phenlymethylsulfonylchloride (PMSF), and 1 x Complete proteinase inhibitor (Boehringer Mannheim, Indianapolis). The homogenate was incubated with shaking at 4 °C for 30 min, then centrifuged in a microfuge for 10 min twice. The resultant supernatants were brought up to 500 µl and incubated with either polyclonal C-terminal AE1 antibody (kindly gifted by Dr. Seth Alper) or monoclonal *c-myc* antibody overnight at 4 °C, followed by protein A-Sepharose precipitation for 1 h. The sepharose pellets were washed six times in 500 µl of ice-cold oocyte lp buffer, eluted and then analyzed by SDS-PAGE autoradiography.

- Cell surface biotinylation

Oocytes metabolically labeled for 2 days with ^{35}S-Methionine were pre-incubated in 10mM NaIO4 at 4°C for 30 min, washed, and labeled at 4°C for 1 h with 2 mM biotin hydrazide (Sigma) in 100 mM NaOAc. Biotinylated oocytes were washed and quenched in a solution containing 5 mM glycine for 10 min, and whole cell extracts were subjected to AE1 immunoprecipitation. Immune complexes were released from Protein A Sepharose by incubation in acid glycine solution containing 1 % Triton X-100 for 25 min at 20°C, and the neutralized supernatant was incubated 1 h at 4°C with avidin-agarose beads (Pierce, Rockford, IL). The beads were washed and resuspended in SDS sample buffer for 1 h at room temperature. Eluted immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

- Immunocytochemistry

Cryosections (3-5 µm) of *Xenopus* oocytes previously injected with AE1 cRNA plus or minus GPA cRNA were cut at –20°C and post-fixed in acetone for 5 min. After 1 h preincubation with PBS/1% BSA, sections were incubated 1 h at 20°C with affinity-purified rabbit polyclonal anti-mouse AE2 antibody cross-reactive with the AE1 C-terminal dodecapeptide or mouse monoclonal anti-*cmyc* antibody, washed thrice with PBS, and incubated 1 h with 1:200 Cy3-labeled goat anti-rabbit or anti-mouse Ig, respectively (Zymed laboratories, San Francisco, CA). Specificity of the staining was tested in water-injected oocytes.

ผลการทดลอง

Effects of AE1 binding proteins on AE1-mediated anion transport function

- Glycophorin A (GpA)

We examined the stilbene disulphonate-sensitive Cl^- transport activity of wild-type AE1 and mutant constructs associated with dRTA in our country, i.e. G701D AE1 and Δ400-408 AE1, in the presence and the absence of GpA (Figure 1). As previously reported, no Cl^- transport activity was found by G701D and Δ400-408 AE1 variant in the absence of GpA indicating that both of them are the loss-of-function mutations. Co-expression of GpA enhances the Cl^- transport activity mediated by wild-type AE1 ~ 1.5 times, and rescues the transport function of G701D variant almost to the level of wild-type. GpA, however, does not effect the Cl^- transport activity mediated by Δ400-408 AE1.

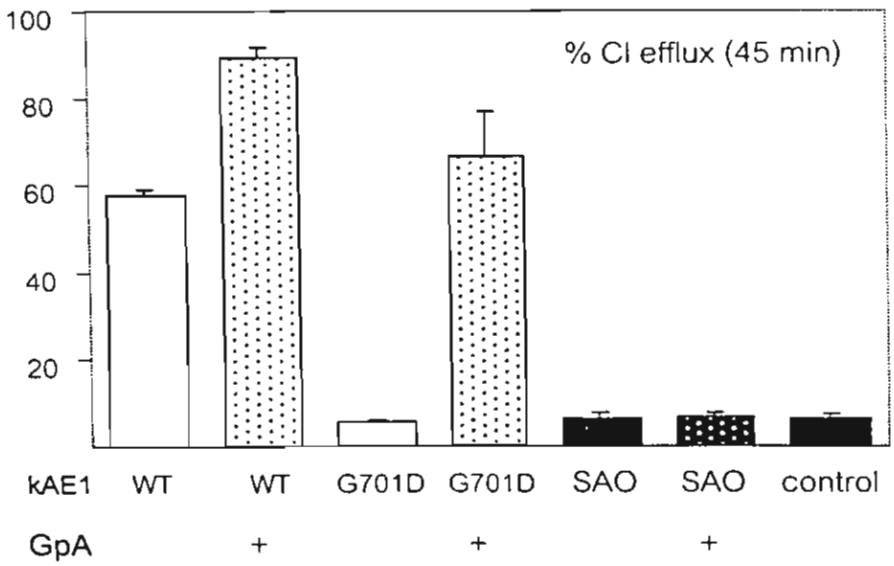

Figure 1. Functional effect of Glycophorin A (GpA) on AE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with 2 ng of each kAE1 variants either in the absence or presence of 10 ng of GpA, and incubated at 18°C for three days. The $^{36}Cl^-$ solution was preloaded into the oocytes by

micro-injection and effluxes were measured in isotonic ND96 solution after 45 min. The values represent mean ± SEM of one representative experiment with 6-8 oocytes per group.

- Protein 4.2

Since it is suggested that interactions of protein 4.2 with the erythroid band 3 occur at the cytoplasmic domain, the functional effect of protein 4.2 was thus examined by co-expression of protein 4.2 with the erythroid isoform of AE1. As shown in Figure 2, co-expression of protein 4.2 resulted in modest reduction of Cl^- transport activity mediated by eAE1 both in the absence and presence of GpA. The inhibitory effects, however, are not statistically significant ($p>0.05$) even with increasing dosage of protein 4.2 cRNA for micro-injection from 2 to 50 ng.

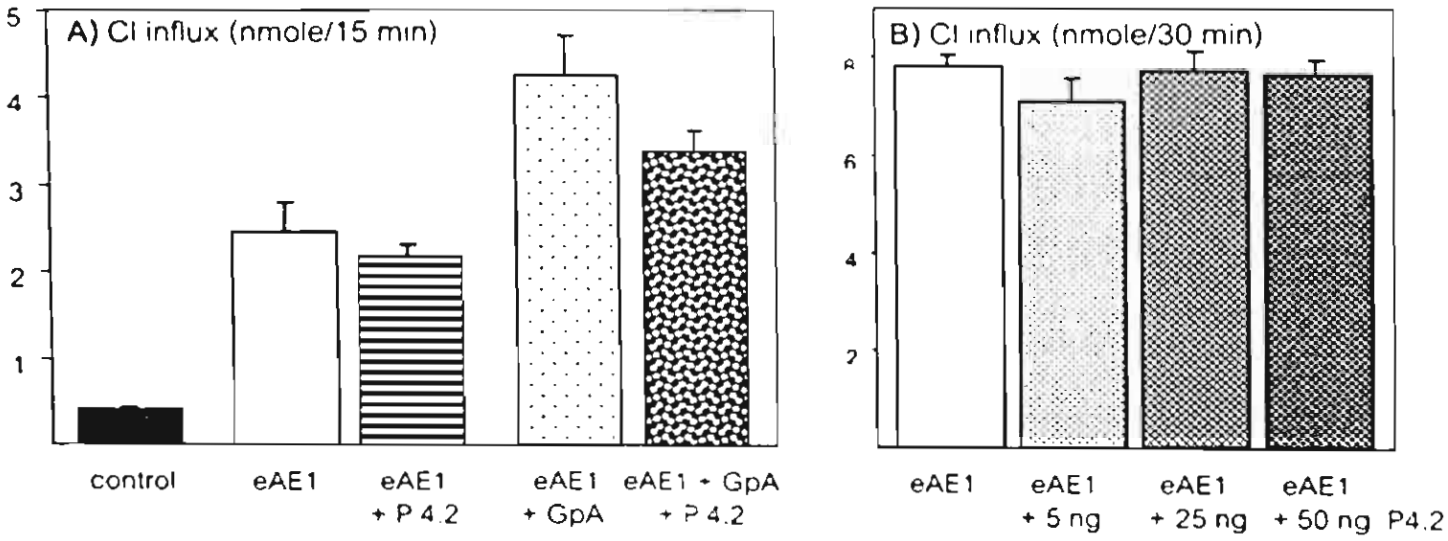

Figure 2. Functional effect of Protein 4.2 (P 4.2) on eAE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with 2 ng of wild-type eAE1 ± 10 ng GpA in the absence or presence of 2 ng of P4.2 (3A), or 2 ng of eAE1 + various dosage of P4.2 as indicated (3B). The $^{36}Cl^-$ influxes were measured three days later in isotonic ND96 solution. The values represent mean ± SEM of the representative experiment with 6-8 oocytes per group.

Cloning of Human Kanadaptin

By RT-PCR approaches, the full-length human kanadaptin cDNA was isolated from the kidney tissue. It consists of ~ 3-kb nucleotides and contains an open reading frame of 2,388 nucleotides. The human kanadaptin coding sequence predicts a 796 amino acid residue peptide with 80% identity to mouse kanadaptin, and a predicted molecular weight of 89 kDa. Motif scan in the protein sequence using PROSITE database revealed a multidomain structure with an SH3 domain binding proline-rich sequences, leucine zipper domain, forkhead-associated domain, nuclear localization signal, and glutamic acid-rich region of unknown function (Figure 3).

To test the interaction and potential effects on the Cl^- transport activity of kidney AE1, human kanadaptin was subsequently subcloned into the oocyte expression vector pXT7, and synthesized cRNA was used for co-expression studies with kAE1. Figure 4 showed that co-expression of human kanadaptin with wild-type kAE1 resulted in partial inhibition of chloride influxes only with higher expression level of kAE1, i.e. at the cRNA dosage of 10 ng not 1 ng. This effect was also observed when kanadaptin was co-expressed with kAE1-R589H variant, the known mutant associated with autosomal dominant distal renal tubular acidosis (data not shown).

Analysis of the Human Kanadaptin Amino Acid Sequence

```
MLAPLRNAPG REGATSPSPP TDATGSLGEW DVDRNVKTEG WVSKERISKL   50
HRLRMADILS QSETLASQDL SGDFKKPALP VSPAARSKAP ASSSSNPEEV  100
QKEGPTALQD SNSGEPDIPP PQPDCGDFRS LQEEQSRPPT AVSSPGGPAR  150
APPYQEPPWG GPATAPYSLE TLKGGTILGT RSLKGTSYCL FGRLSGCDVC  200
LEHPSVSRYH AVLQHRASGP DGECDSNGPG FYLYDLGSTH GTFLNKTRIP  250
PRTYCRVHVG HVVRFGGSTR LFILQGPEED REAESELTVT QLKELRKQQQ  300
ILLEKKMLGE DSDEEEEMDT SERKINAGSQ DDEMGCTWGM GEDAVEDDAE  350
ENPIVLEFQQ EREAFYIKDP KKALQGFFDR EGEELEYEFD EQGHSTWLCR  400
VRLPVDDSTG KQLVAEAIHS GKKKEAMIQC SLEACRILDT LGLLRQEAVS  450
RKRKAKNWED EDFYDSDDDT FLDRTGLIEK KRLNRMKKAG KIDEKPETFE  500
SLVAKLNDAE RELSEISERL KASSQVLSES PSQDSLDAFM AETKSGSTLD  550
GVSRKKLHLR TFELRKEQQR LKGLIKIVKP AEIPELKRTE TQTTGAENKA  600
KKLTLPLFGA MKGGSKFKLK TGTVGKLPPK RPELPPTLMR MKDEPEVEEE  650
EEEEEEEEKE KEEHEKKKLE DGSLSRPQPE IEPEAAVQEM RPPTDLTHFK  700
ETQTHENMSQ LSEEEQNKDY QDCSKTTSLC AGPSASKNEY EKSRGELKKK  750
KTPGPGKLPP TLSSKYPEDD PDYCVWVPPE GQSGDGRTHL NDKYGY.796
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X = Proline rich region

X = Glutamic acid-rich region

\- = FHA

X = Forkhead-associated (FHA) domain profile

X = Bipartite nuclear localization signal

\- = Double-stranded RNA binding motif

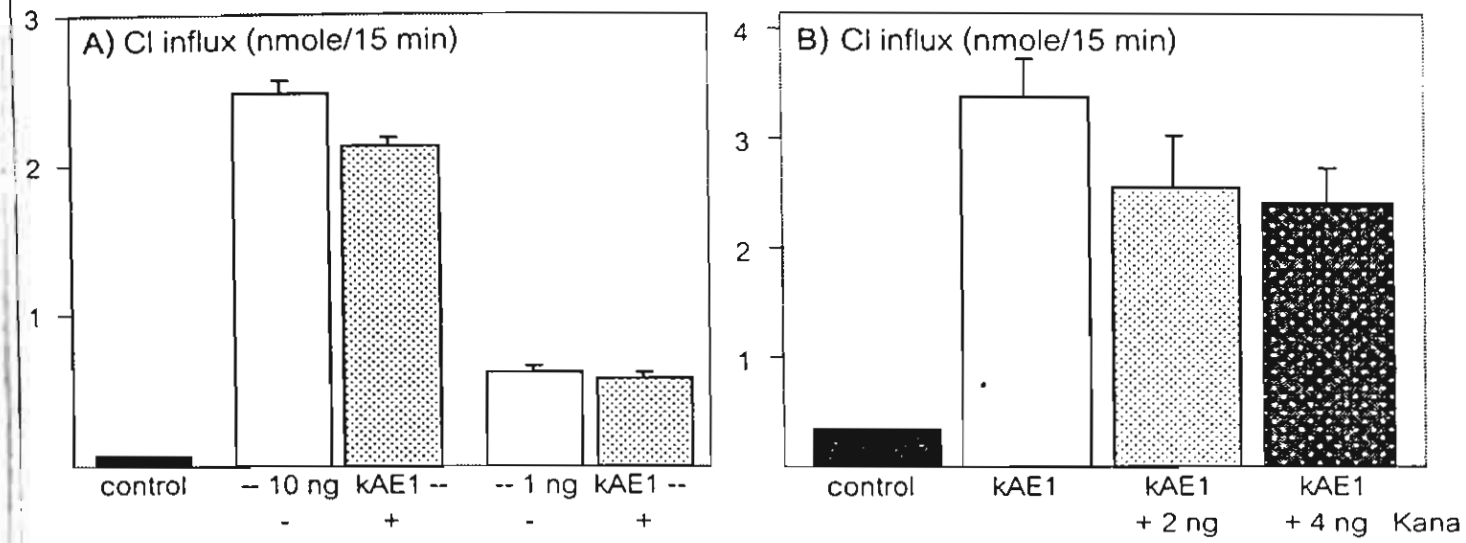


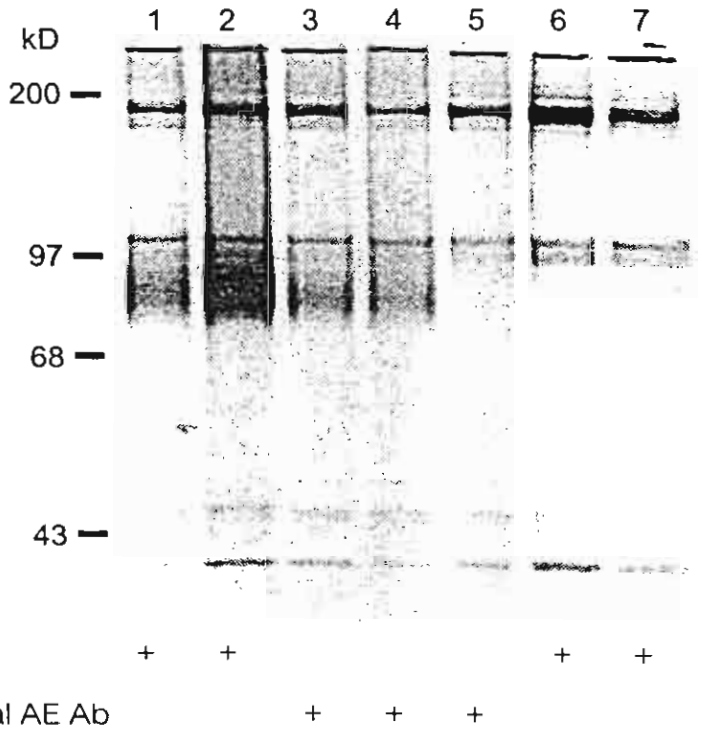
Figure 4. Functional effect of Human Kanadaptin (Kana) on kAE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with indicated amount of wild-type kAE1 ± various dosage of Kana as indicated. The $^{36}Cl^-$ influxes were measured three days later in isotonic ND96 solution. The values represent mean ± SEM of the representative experiment with 6-8 oocytes per group.

Interaction between AE1 G701D and Δ400-408 variants

The kidney and erythroid phenotypes in SAO patients with dRTA are distinct. While these individuals lost their ability to acidify urine which implied that kidney AE1 loss the anion exchanger function, the DIDS sensitive sulfate flux in their red blood cells reduced to only ~30% of normal. In this study, we used *c-myc* epitope tagged AE1 G701D to investigate heteromeric formation with AE1 Δ400-408 variant in the absence or presence of GpA, thus mimic conditions in the kidney intercalated cells and red blood cells, respectively. Immunoprecipitation studies in Figure 5 showed that both kAE1 G701D cmyc and kAE1 Δ400-408 proteins expressed properly and in similar amount in the whole oocyte lysate. Functional effects of co-expression studies were shown in Figure 6. Without GpA, both AE1 G701D cmyc and AE1 Δ400-408 lost the anion

transport activity, and co-expression of AE1 Δ400-408 could not rescue the functional loss of AE1 G701D-cmyc. Remarkably, co-expression of AE1 Δ400-408 (Fig. 7) led to inhibition of the rescued chloride transport activities of AE1 G701D-cmyc by GpA (60% reduction at a 1:1 molar ratio). This inhibitory effect did not depend on GpA dosage but increased with increasing amount of AE1 Δ400-408. These results indicate that AE1 Δ400-408 should form heteromers with AE1 G701D-cmyc, and have a dominant negative effect on AE1 G701D. The findings are consistent with the transport defects found in these patients.

The cell surface expressions of AE1 Δ400-408 and AE1 G701D-cmyc were shown by biotinylation and immunocytochemical methods in Figure 8.



1. kAE1 G701D-cmyc
2. kAE1 G701D-cmyc + GpA
3. kAE1 Δ400-408
4. kAE1 Δ400-408 + GpA
5. kAE1 G701D-cmyc
6. kAE1 Δ400-408
7. control

Figure 5. Autoradiography of the whole oocyte lysates. Metabolically labeled oocytes previously injected with cRNA (indicated on the left) were lysed, and immunoprecipitated with specific antibody (indicated below). Proteins from ~ 10 oocytes were loaded each lane.

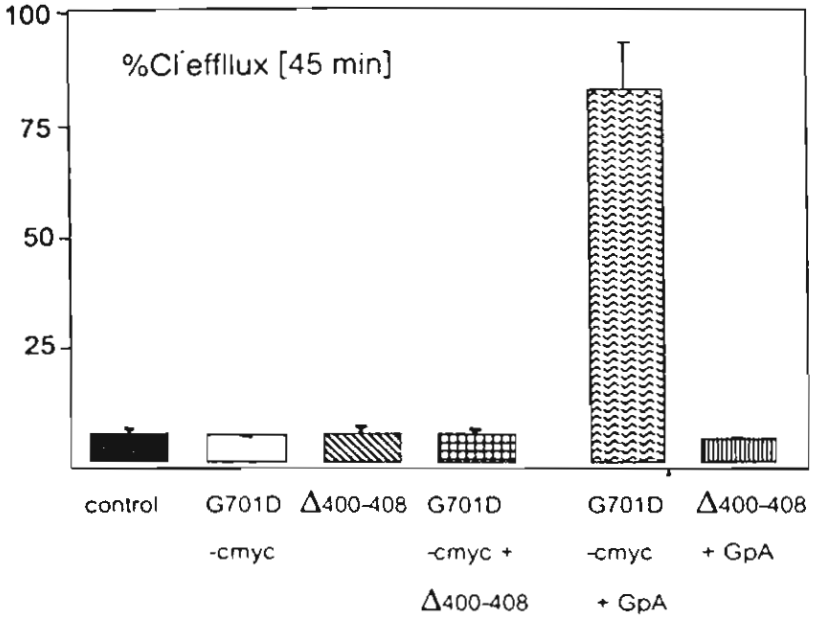


Figure 6. Functional characterization of kAE1-G701D-cmyc and kAE1 Δ400-408 variant. Each construct was expressed either alone, in combination (2 ng each), or with 10 ng of GpA co-expression. The $^{36}Cl^-$ effluxes were measured at 45 min in ND96 media.

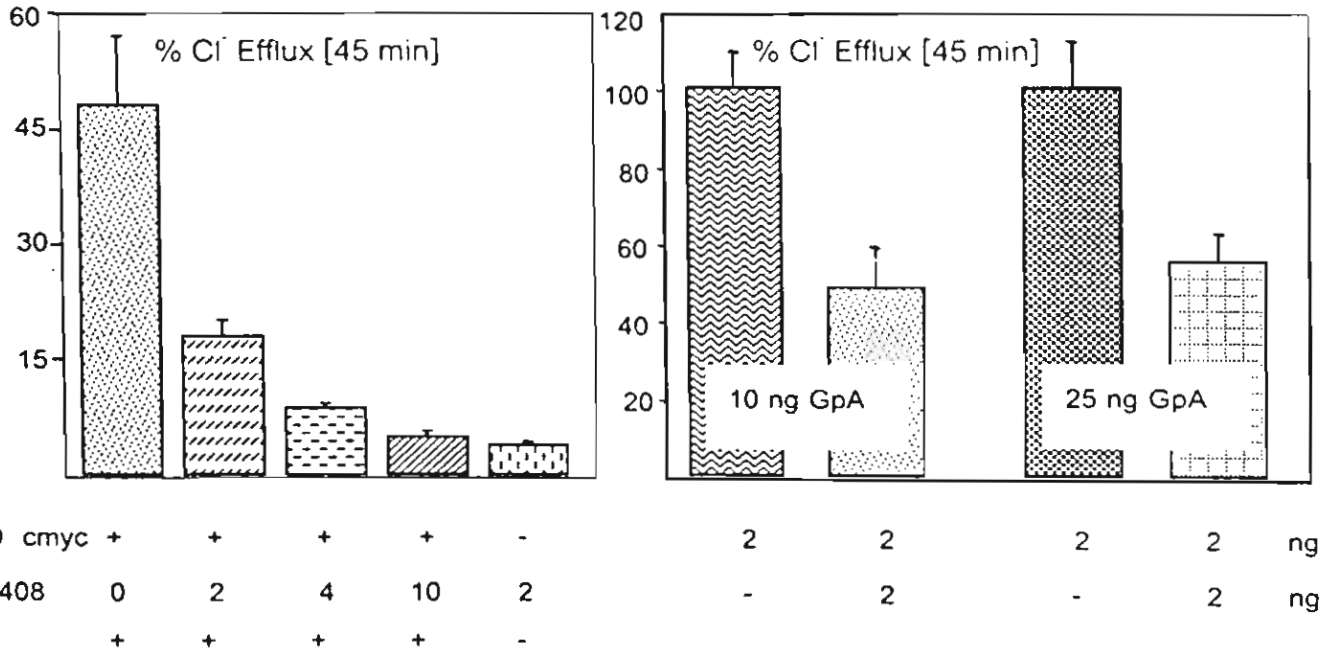


Figure 7. Co-expression studies of kAE1 Δ400-408 and kAE1 G701D-cmyc. The kAE1 Δ400-408 attenuated the rescued effects of kAE1 G701D-cmyc by GpA in a dose dependent fashion as demonstrated by $^{36}Cl^-$ effluxes at 45 min. This inhibitory effect was not due to lack of GpA since increasing dosage of GpA had no effects on percentage of inhibition.

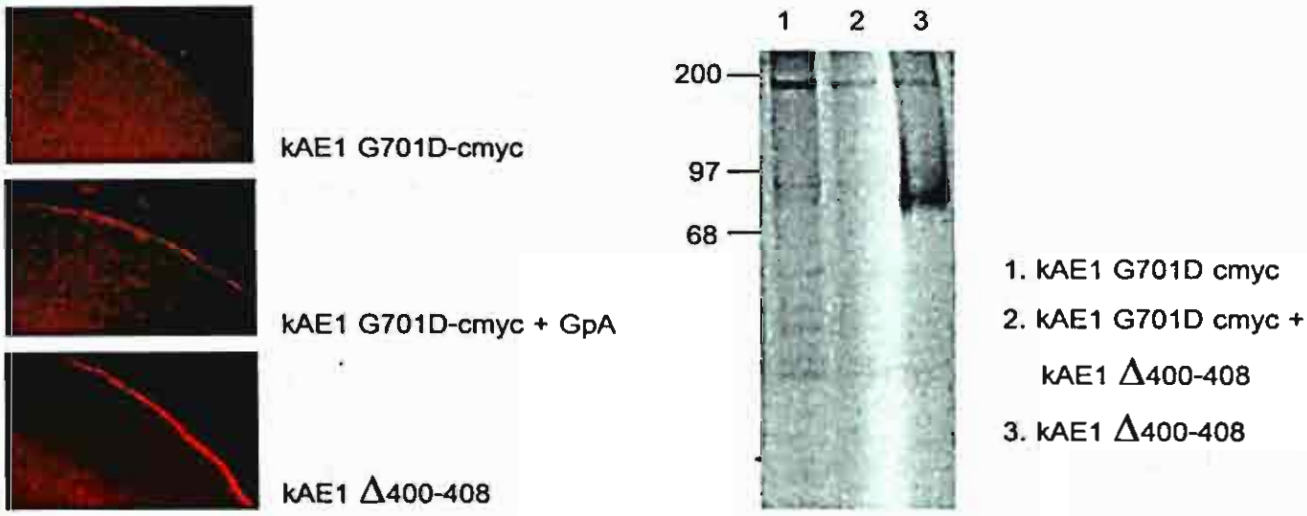


Figure 8. Expression of AE1 mutant variants associated with autosomal recessive dRTA on the plasma membrane as demonstrated by A) immunocytochemistry and B) surface biotinylation methods (see methods). The antibody used for the oocytes expressing AE1 Δ400-408 is AE-C terminal Ab, and that used for the oocytes expressing AE1 G701D-cmyc and AE1 G701D-cmyc/ Δ400-408 is monoclonal *cmyc* Ab.

บทวิจารณ์

Development of the *Xenopus* oocyte expression system

Over the past 2 years, we were able to achieve our first study objective, i.e. to develop the heterologous expression system of *Xenopus* oocytes to investigate function and regulation of integral membrane proteins. Expression of these proteins of interest, including receptors, channels and transporters, can be achieved by micro-injection of such cRNA. The good quality oocytes have machineries for translation and post-translational modifications so the proteins can be studied both at the functional and structural levels. For example, in our case, the anion exchanger activities of AE1 were investigated by measurement of radioactive $^{36}Cl^-$ influx or efflux rates. The expression level of wild-type or mutant AE1 proteins can be studied either by Western blot analysis or metabolic labeling with immunoprecipitation techniques. Moreover, the cell compartment of which the proteins are expressed can be investigated by cell surface biotinylation or immunocytochemical studies. All of these procedures were used in the past study period to study the function and structure of wild-type and various mutant AE1 variants associated with dRTA, as illustrated in Figure 1-8.

Transport function of dRTA associated *AE1* mutations

The mutation known to be associated with dRTA in our country is AE1 G701D in its homozygous form or compound heterozygosity with AE1 Δ400-408. Both mutant kAE1 proteins were expressed in *Xenopus* oocytes to assess their transport activities in the absence of GpA (to mimic conditions in the kidney acid secreting intercalated cells) and the presence of GpA (to mimic red blood cell conditions). As previously demonstrated, the kAE1 G701D did not mediate any $^{36}Cl^-$ flux in the oocytes in the absence of GpA but the transport activities recovered to the levels of wild-type in the presence of GpA. In parallel, expression of AE1 G701D at the plasma membrane was demonstrated only in the presence of GpA co-expression. All of these findings can explain distinct red blood cell and kidney transport phenotypes in the patients.

Our novel findings that AE1 Δ400-408 attenuated the rescued effect of AE1 G701D by GpA are distinct from the previous reports. The red blood cell isoform of wild-type AE1 forms heterodimers with the SAO proteins but no dominant negative effects on the anion transport function have been investigated. The findings of dosage-dependent inhibitory effects of AE1 Δ400-408 suggest that the G701D mutant protein might interact with SAO protein only in the presence of GpA, and forms heteromers. These observations remain to be further investigated by other methods including structural analysis. However, the findings can also explain the kidney phenotype of acidification defect and ~ 60% sulfate fluxes observed in patients' red blood cell.

สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์
เลขที่ 979/17-21 ถนนพหลโยธิน แขวงสามเสนใน
เขตพญาไท กรุงเทพฯ 10400
โทร.298-0455 โทรสาร 298-0476
Home page : http://www.trf.or.th
E-mail : trf-info@trf.or.th