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

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
ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา
สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(1 พฤศจิกายน 2542 ถึง 31 ตุลาคม 2544)

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

3) Prof. Matthias A. Hediger แห่ง Harvard Institute of Medicine, Boston, MA, USA. ที่ได้เป็นที่ปรึกษาและให้คำแนะนำในการเตรียมโครงการวิจัย

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	หน้า 1 - 3
วิธีการทดลอง	หน้า 4 - 9
ผลการทดลอง	หน้า 10 - 17
บทวิจารณ์	หน้า 18 - 20
หนังสืออ้างอิง	หน้า 21 - 26
Output ที่ได้	หน้า 27
การนำไปใช้ประโยชน์	หน้า 28 - 29
ภาคผนวก	หน้า 30 - 38
เอกสารที่เกี่ยวข้อง	หน้า A - F

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.

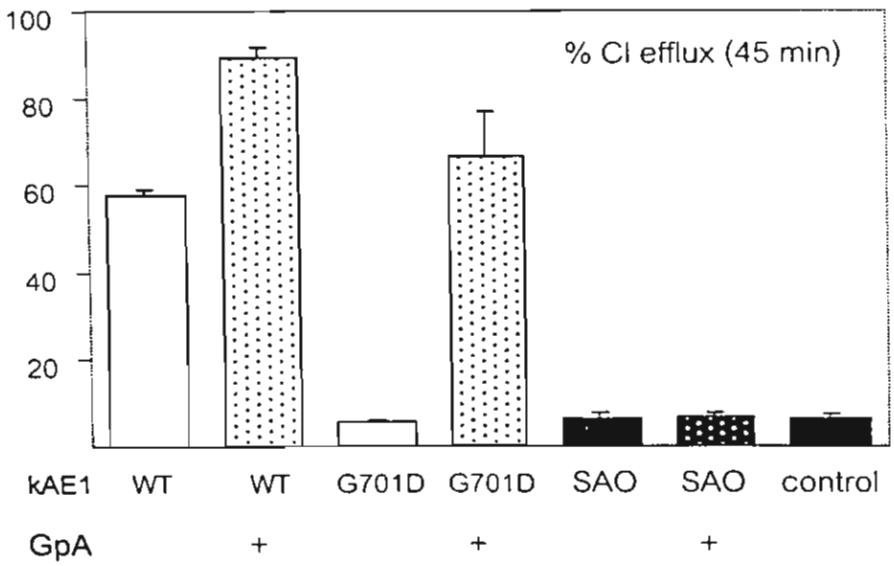


Figure 1. Functional effect of Glycophorin A (GpA) on AE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with 2 ng of each kAE1 variants either in the absence or presence of 10 ng of GpA, and incubated at 18°C for three days. The $^{36}Cl^-$ solution was preloaded into the oocytes by

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.

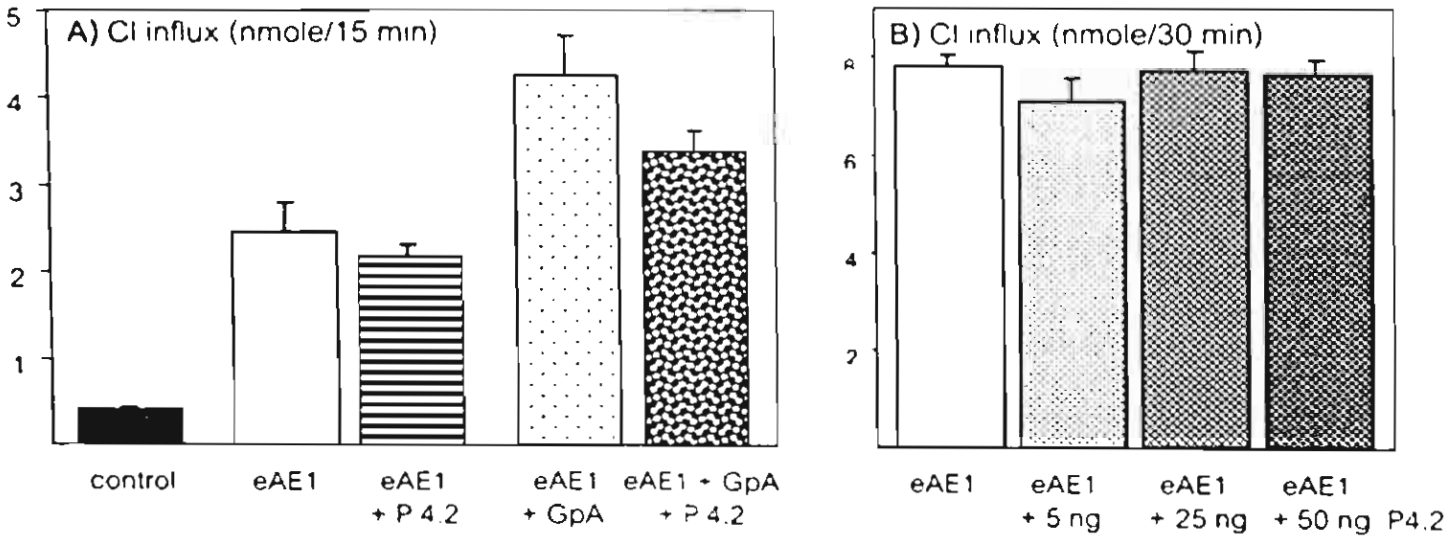


Figure 2. Functional effect of Protein 4.2 (P 4.2) on eAE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with 2 ng of wild-type eAE1 ± 10 ng GpA in the absence or presence of 2 ng of P4.2 (3A), or 2 ng of eAE1 + various dosage of P4.2 as indicated (3B). The $^{36}Cl^-$ influxes were measured three 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
```

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

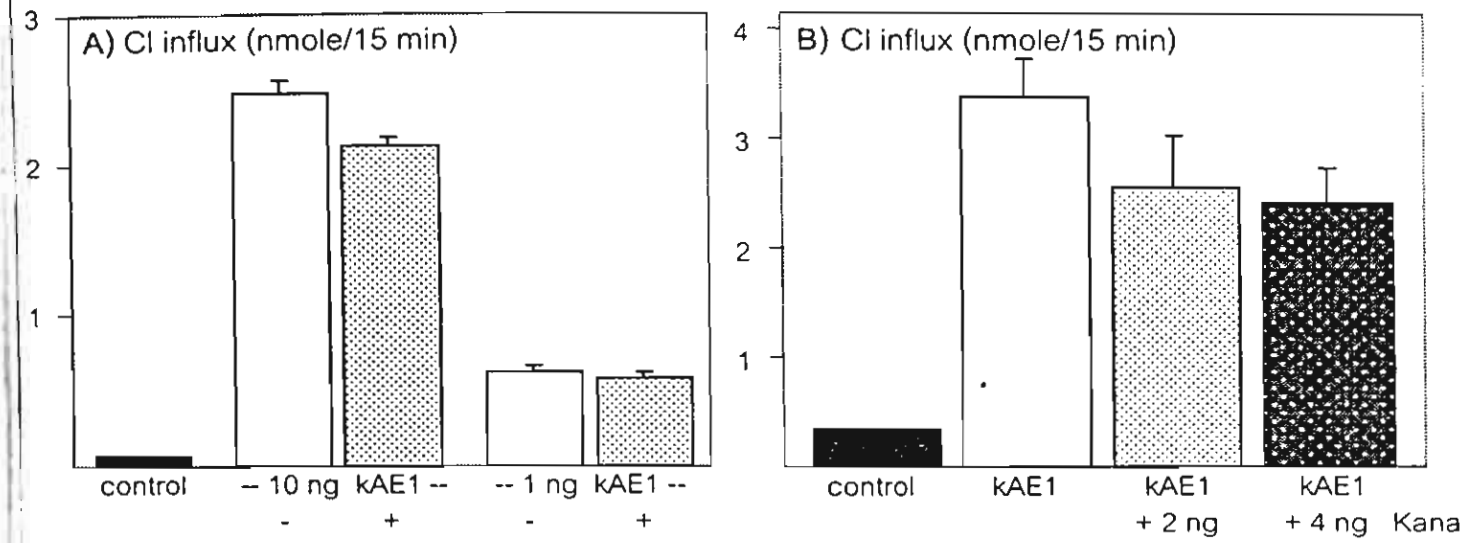


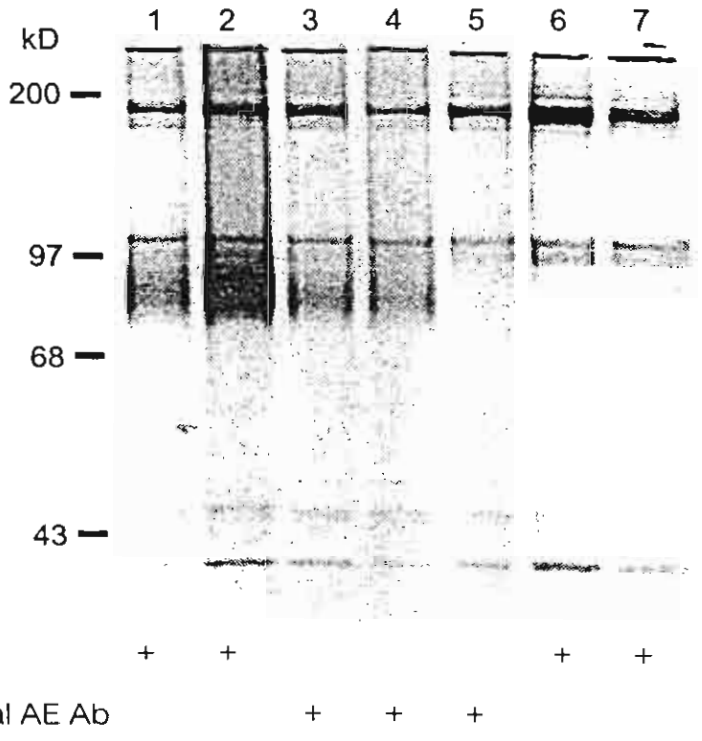
Figure 4. Functional effect of Human Kanadaptin (Kana) on kAE1-mediated $^{36}Cl^-$ flux. Oocytes were injected with indicated amount of wild-type kAE1 ± various dosage of Kana as indicated. The $^{36}Cl^-$ influxes were measured three days later in isotonic ND96 solution. The values represent mean ±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

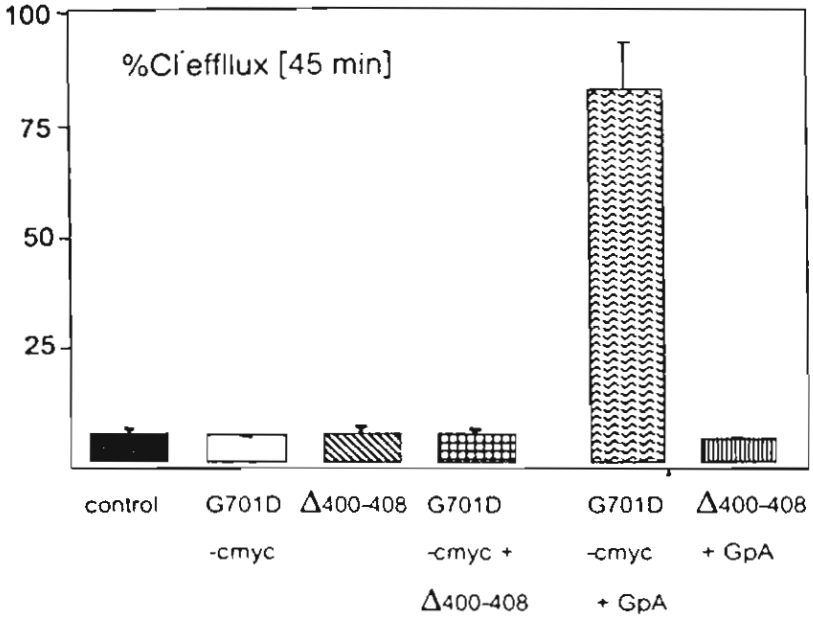


Figure 6. Functional characterization of kAE1-G701D-cmyc and kAE1 Δ400-408 variant. Each construct was expressed either alone, in combination (2 ng each), or with 10 ng of GpA co-expression. The $^{36}Cl^-$ effluxes were measured at 45 min in ND96 media.

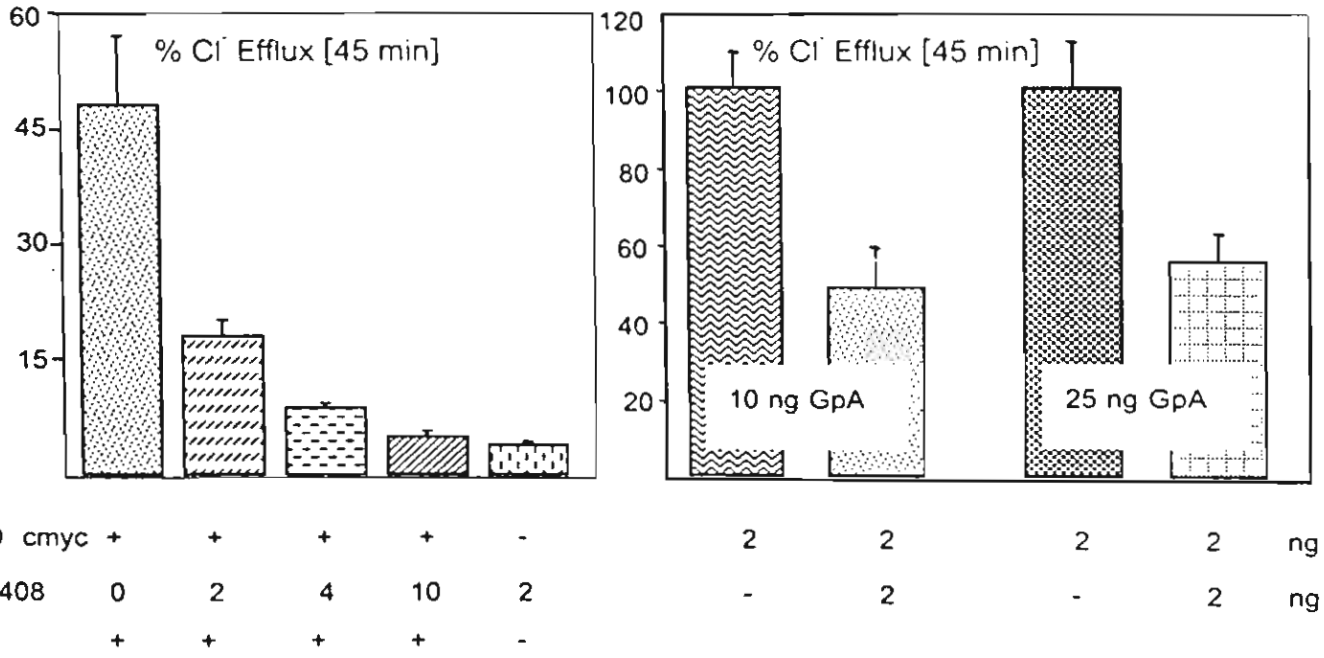


Figure 7. Co-expression studies of kAE1 Δ400-408 and kAE1 G701D-cmyc. The kAE1 Δ400-408 attenuated the rescued effects of kAE1 G701D-cmyc by GpA in a dose dependent fashion as demonstrated by $^{36}Cl^-$ effluxes at 45 min. This inhibitory effect was not due to lack of GpA since increasing dosage of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

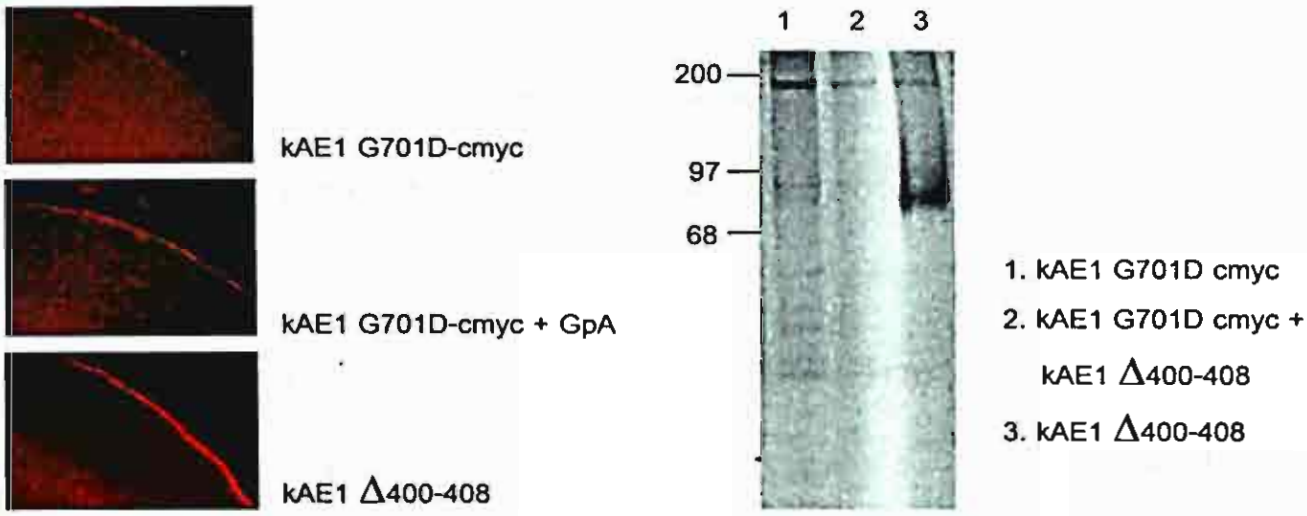


Figure 8. Expression of AE1 mutant variants associated with autosomal recessive dRTA on the plasma membrane as demonstrated by A) immunocytochemistry and B) surface biotinylation methods (see methods). The antibody used for the oocytes expressing AE1 Δ400-408 is AE-C terminal Ab, and that used for the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

	G701D/N	G701D/G701D	Δ400-408/N	G701D/Δ400-408
Red cell SO_4^{2-} flux	N	N	40%	50%
Express (+GpA)	~ N	~ N	~ 60%	~ 40%
Kidney acidification	N	X	N	X
Express (-GpA)	~ N	X	~ 60%	X

Identification of human kanadaptin and characterization of its role on the function of kAE1

In this study we isolated the human kanadaptin cDNA from the kidney tissue and showed that it is a multidomain protein including an SH3 domain-binding sequence, which is a proline-rich sequences with the core sequence "PXXP" (where "X" is any amino acid other than cystine), a glutamic acid-rich domain (E-domain) of unknown function, a forkhead associated domain, bipartite nuclear localization signal and RNA binding motif, etc. As kanadaptin is widely expressed in mouse tissues devoid of kAE1 (and also in human tissue, data not shown), some investigators suggested that kanadaptin might involve in cross-talk between the nucleus and the transporter proteins. This hypothesis remain to be determined in the expression system of mammalian cell not *Xenopus* oocytes.

However, the observed inhibitory effects in the oocyte expression system suggested that kanadaptin might also interact with kAE1 protein in other cell compartments. Since the effect was observed in kAE1-R589H, an autosomal dominant dRTA mutant, its significance in the pathogenesis of dRTA requires further investigations.

หนังสืออ้างอิง

1. Bruce LJ, Cope DL, Jones GK, Schofield AE, Burley M, Povey S, Unwin RJ, Wrong O, Tanner MJ: Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. J Clin Invest 100:1693-707, 1997

2. Bruce LJ, Wrong O, Toye AM, Young MT, Ogle G, Ismail Z, Sinha AK, McMaster P, Hwaihwanje I, Nash GB, Hart S, Lavu E, Palmer R, Othman A, Unwin RJ, Tanner MJ: 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. Biochem J 350:41-51, 2000

3. Chaabani H, Hadj-Khlil A, Ben-Dhia N, Braham H: The primary hereditary form of distal renal tubular acidosis: clinical and genetic studies in 60-member kindred. Clin Genet 45:194-9, 1994

4. 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. J Biol Chem 273:1038-43, 1998

5. Chernova MN, Humphreys BD, Robinson DH, Stuart-Tilley AK, Garcia AM, Brosius FC, Alper SL: Functional consequences of mutations in the transmembrane domain and the carboxy-terminus of the murine AE1 anion exchanger. Biochim Biophys Acta 1329:111-23, 1997

6. Chernova MN, Jarolim P, Palek J, Alper SL: Overexpression of AE1 Prague, but not of AE1 SAO, inhibits wild-type AE1 trafficking in Xenopus oocytes. J Membr Biol 148:203-10, 1995

7. Dubose TD, Jr., Alpern RJ: The Metabolic and Molecular Bases of Inherited Disease (ed 6th). New York, McGraw-Hill, 1995, p3655-3689.

8. Holmes TC, Fadool DA, Ren RB, Levitan IB: Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. Science 274:2089-2091, 1996

9. Inaba M, Yawata A, Koshino I, Sato K, Takeuchi M, Takakuwa Y, Manno S, Yawata Y, Kanzaki A, Sakai J, Ban A, Ono K, Maede Y: Defective anion transport and marked spherocytosis with membrane instability caused by hereditary total deficiency of red cell band 3 in cattle due to a nonsense mutation. J Clin Invest 97:1804-17, 1996

10. Jarolim P, Murray JL, Rubin HL, Taylor WM, Prchal JT, Ballas SK, Snyder LM, Chrobak L, Melrose WD, Brabec V, Palek J: Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood 88:4366-74, 1996

11. Jarolim P, Palek J, Amato D, Hassan K, Sapak P, Nurse GT, Rubin HL, Zhai S, Sahr KE, Liu SC: Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. Proc Natl Acad Sci U S A 88:11022-6, 1991

12. Jarolim P, Shayakul C, Prabakaran D, Jiang L, Stuart-Tilley A, Rubin HL, Simova S, Zavadil J, Herrin JT, Brouillette J, Somers MJ, Seemanova E, Brugnara C, Guay-Woodford LM, Alper SL: 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. J Biol Chem 273:6380-8, 1998

13. Jennings ML, Gosselink PG: Anion exchange protein in southeast asian ovalocytosis: heterodimer formation between normal and variant subunits. Biochemistry 34: 3588-3595, 1995

14. Karet FE, Gainza FJ, Gyory AZ, Unwin RJ, Wrong O, Tanner MJ, Nayir A, Alpay H, Santos F, Hulton SA, Bakkaloglu A, Ozen S, Cunningham MJ, di Pietro A, Walker WG, Lifton RP: Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. Proc Natl Acad Sci U S A 95:6337-42, 1998

15. Kelleher CL, Buckalew VM, Frederickson ED, Rhodes DJ, Conner DA, Seidman JG, Seidman CE: CLCN5 mutation Ser244Leu is associated with X-linked renal failure without X-linked recessive hypophosphatemic rickets. Kidney Int 53:31-7, 1998

16. McDonald FJ, Welsh MJ: The proline-rich region of the epithelial Na+ channel binds SH3 domains and associates with specific cellular proteins. Biochem J 312:491-497, 1995

17. Nilwarangkur S, Nimmannit S, Chaovakul V, Susaengrat W, Ong-aj-Yooth S, Vasuvattakul S, Pidetcha P, Malasit P: Endemic primary distal renal tubular acidosis in Thailand. Q J Med 74:289-301, 1990

18. Nimmannit S, Malasit P, Susaengrat W, Ong-Aj-Yooth S, Vasuvattakul S, Pidetcha P, Shayakul C, Nilwarangkur S: Prevalence of endemic distal renal tubular acidosis and renal stone in the northeast of Thailand. Nephron 72:604-10, 1996

19. Popov M, Tam LY, Li J, Reithmeier RAF: Mapping the ends of transmembrane segments in a polytopic membrane protein. Scanning N-glycosylation mutagenesis of extracytosolic loops in the anion exchanger, band 3. J Biol Chem 272:18325-18332, 1997

20. Romero MF, Kanai Y, Gunshin H, Hediger MA: Expression cloning using Xenopus laevis oocytes. Method Enzymol 296:17-52, 1998

21. Rysava R, Tesar V, Jirsa M, Jr., Brabec V, Jarolim P: Incomplete distal renal tubular acidosis coinherited with a mutation in the band 3 (AE1) gene. Nephrol Dial Transplant 12:1869-73, 1997

22. Shayakul C, Tanphaichitr VS, Sumboonnanonda A, Ideguchi H, Brugnara C, Takao M, Veerakul G, Alper SL: Autosomal recessive distal renal tubular acidosis associated with a novel loss-of-function mutation in the gene encoding the AE1 (Band 3) Cl^-/HCO_3^- exchanger of type A intercalated cells. J Amer Soc Nephrol 9:12A, 1998

23. Shayakul C, Jarolim P, Alper SL: Autosomal dominant distal renal tubular acidosis and the AE1 gene (Authors' reply). Am. J. Kidney Dis. 33:1194-1197, 1999.

24. Shayakul C, Breton S, Brown D, Alper SL: Gene therapy of inherited renal tubular disease. Am. J. Kidney Dis. 34:374-377, 1999.

25. Shayakul C and Alper SL: Inherited renal tubular acidosis. Curr. Opin. Nephrol. Hypertens.; 9:541-546, 2000

26. Sitprija V, Tungsanga K, Tosukhowong P, Leelhaphunt N, Kruerklai D, Sriboonlue P, Saew O: Metabolic problems in northeastern Thailand: possible role of vanadium. Miner Electrolyte Metab 19:51-6, 1993

27. Tanner MJA: Molecular and cellular biology of the erythrocyte anion exchanger. Sem Hematol 30:34-57, 1993

28. Tanphaichitr VS, Sumboonnanonda A, Ideguchi H, Shayakul C, Brugnara C, Takao M, Veerakul G Alper SL: Novel AE1 mutations in distal renal tubular acidosis: loss-of-function is rescued by glycophorin A. J. Clin. Invest. 102:2173-2179, 1998

29. Vasuvattakul S, Nimmannit S, Chaovakul V, Susaengrat W, Shayakul C, Malasit P, Halperin ML, Nilwarangkur S: The spectrum of endemic renal tubular acidosis in the northeast of Thailand. Nephron 74:541-7, 1996

30. Vasuvattakul S, Yenchitsomsnus PT, Vachuanichsanong P, Thuwajit P, Kaitwatcharachai C, Laosombat V, Malasit P, Wilairat P, Nimmannit S: Autosomal recessive distal renal tubular acidosis associated with Southeast Asian ovalocytosis. Kidey Int. 56:1674-1682, 1999.

31. Vince JW, Reithmeier RAF: Structure of the band 3 transmembrane domain. Mol and Cell Biol 42:1041-1051, 1996

32. Yang B, Verkman AS: Water and glycerol permeabilities of aquaporins 1-5 and MIP determined quantitatively by expression of epitope-tagged constructs in Xenopus oocytes. J Biol Chem 272:16140-16146, 1997

Output ที่ได้

1. Chairat Shayakul, Surawat Jariyawat, Naparat Kaewkaukul, Samaisukh Sophasan. Molecular Characterization of Human Kanadaptin, and Its Effect on Human Kidney Anion Exchanger 1 Transport Activities in Xenopus Oocytes. PharmaConference 2001. August 5-10, 2001; Interlaken congress Center, Interlaken, Switzerland (abstract p 74).
2. Chairat Shayakul, Surawat Jariyawat, Naparat Kaewkaukul, Samaisukh Sophasan. Functional Rescue of Anion Exchanger 1 (AE1) G701D by Glycophorin A is Attenuated by Co-expression of AE1 Δ400-408: A Basis for Transport Defect in Autosomal Recessive Distal Renal Tubular Acidosis (dRTA). J Am Soc Nephrol 2001; 12:10A (abstract).
3. Two manuscripts currently under preparation for submission to peer reviewed international journals.

การนำไปใช้ประโยชน์

การประยุกต์ใช้ผลงานวิจัย (โปรดระบุ ผู้ใช้/หน่วยงาน, ช่วงเวลา, สถานที่ ที่นำผลงานไปใช้)

1. การนำไปใช้ประโยชน์

☐ เชิงสาธารณะ

- งานวิจัยนี้ได้เริ่มต้นขึ้นบนพื้นฐานของการสร้างความร่วมมือระหว่างกลุ่มผู้วิจัยทั้งในประเทศญี่ปุ่นและสหรัฐอเมริกา โดยเฉพาะ Kyorin University School of Medicine และ Harvard University สถาบันทั้งสองนับเป็นสถาบันชั้นนำในการศึกษาวิจัย กลไกการขนส่งสารผ่านเยื่อหุ้มเซลล์ด้วย transporter proteins ในประเทศไทยมีโรคที่เกี่ยวข้องกับความผิดปกติของ gene ของ transport proteins ที่เป็นต้นเหตุทั้งของภาวะ dRTA และโรคอื่น ๆ การศึกษาวิจัยครั้งนี้จึงนับเป็นการวางรากฐานของงานวิจัยในการศึกษา gene ของ transporter proteins ซึ่งจะมีความสำคัญมากขึ้นทั้งในสภาวะปกติ ในสภาวะผิดปกติจากพันธุกรรม หรือในการพัฒนายารักษาโรคให้เกิดความจำเพาะเจาะจงถูกขนส่งเข้าเซลล์เป้าหมาย ให้ออกฤทธิ์ได้ตามที่คาดหวัง จากการวิจัยครั้งนี้ได้เพิ่มความสัมพันธ์และความร่วมมือกับกลุ่มนักวิจัยข้างต้นอย่างเป็นรูปธรรมดังที่สรุปไว้ในกิตติกรรมประกาศ
- โครงการนี้ได้ไปนำเสนอในที่ประชุมใหญ่ระดับนานาชาติ 2 ครั้งในช่วงปีที่ผ่านมา ซึ่งได้ก่อให้เกิดกระแสความสนใจในวงกว้างระดับนานาชาติเพิ่มขึ้น และทำให้มีการติดต่อระหว่างผู้ที่สนใจเกี่ยวกับการศึกษาในด้านนี้ อันจะนำไปสู่การสร้างความร่วมมือระดับนานาชาติในอนาคต การประชุมทั้งสองนี้ ได้แก่

1. PharmaConference 2001. August 5-10, 2001; Interlaken congress Center, Interlaken, Switzerland ในรูปของโปสเตอร์ เรื่อง Molecular Characterization of Human Kanadaptin, and Its Effect on Human Kidney Anion Exchanger 1 Transport Activities in Xenopus Oocytes.
2. The ASN/ISN World Congress of Nephrology. October 10-17, 2001; San Francisco, California, USA ในรูปของโปสเตอร์ เรื่อง Functional Rescue of Anion Exchanger 1 (AE1) G701D by Glycophorin A is Attenuated by Co-expression of AE1 Δ400-408: A Basis for Transport Defect in Autosomal Recessive Distal Renal Tubular Acidosis (dRTA). ซึ่งในการประชุมครั้งนี้ เรื่องดังกล่าวได้รับการคัดเลือกให้นำเสนอเป็นส่วนหนึ่งของ oral symposium ในหัวข้อ Genetic Disease of Acid Base Regulation เมื่อวันที่ 15 ตุลาคม 2544 และได้รับความสนใจเป็นอย่างมากด้วย

☐ เชิงวิชาการ

นอกจากนี้การวิจัยครั้งนี้มีส่วนสำคัญในการพัฒนานักวิจัยในด้านนี้ขึ้นใหม่ นักศึกษาปริญญาโทและเอกของภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ ม.มหิดล หลายคนในปัจจุบันได้เรียนรู้งานวิจัยด้านนี้ นอกจากนี้ยังมีนักศึกษาอีก 3 คนจากภาควิชาสรีรวิทยา ได้มีโอกาสไปเพิ่มประสบการณ์และเรียนรู้งานวิจัยใหม่ๆในแนวทางนี้ที่ Kyorin University School of Medicine ทั้งนี้โดยมิได้ใช้งบประมาณประเทศไทยเลย จึงนับเป็นการช่วยพัฒนานักวิจัยรุ่นใหม่ได้เป็นอย่างดี

ภาคผนวก

Xenopus laevis oocyte expression system

- Supply and Food

The lab-conditional mature female *Xenopus laevis* (South African clawed frogs) and food were kindly supplied without charge through out the study period from Japan by Prof. Hitoshi Endou, Department of Pharmacology and Toxicology, Kyorin University School of Medicine. Altogether, 90 frogs were supplied. These Xenopus were in healthy conditions, however, only about 65 % were beneficial, as the others never produced good quality oocytes for experimental use. Furthermore, those who produced good oocytes may not maintain the quality in the subsequent batches and may have to be terminated later.

The *Xenopus laevis* were maintained in three glass tanks using clean water at 18 °C to achieve high quality of the oocytes. The temperature was controlled by intermittent pumping of chilled water through the lower chamber of each tank. The chilled water was generated by special designed water cooler, maintaining at 12 °C. The flow of chilled water through each tank was individually controlled by feed back loop of the temperature sensor. At one time each tank will hold not more than 20 frogs per tank. This temperature control system was maintained by the Department of Physiology, Faculty of Science, Mahidol University. The frogs were fed twice a week and, after feeding, the tanks were cleaned and filled up with fresh de-ionized water. Small amount of table salt was added. A record was kept for each frog, on conditions, operation with quantity and quality of obtained oocytes.

- Oocyte preparation

For each set of experiment, a frog was anesthetized in solution containing 0.1% MS-222 and 0.3% $KHCO_3$ pH 7.5 for 30-45 min and place on ice for operation. Under aseptic condition, an incision of 0.5-1 cm was made on one side of the lower belly, and 4-5 lobes or 2/3 of ovary was harvested each time. The incisions, muscle and skin were closed by suture. The operated frog was kept in an isolated container till recovery and then returned to its storage tank. In certain frogs, the oocytes can be repeatedly harvested up to 5 times with approximate 1-month interval. In most cases oocytes may be harvested successfully for 3 times. If after 3 operation, good quality oocytes were not obtained, the frog will be terminated. The percentage of dead frog is about 16%. Frogs treated in this way usually recover without subsequent morbidity. However, Some frogs became infected after the operation and may die from infection a few weeks post-operatively. For each experiment, normally 1-5 frogs were operated, since not every frog will produce quality oocytes.

Xenopus laevis oocytes defolliculation

Harvested oocytes were kept in ND96 solution, containing NaCl 96 mM, KCl 2 mM, $MgCl_2.H_2O$ 1 mM, HEPES 5 mM, $CaCl_2.2H_2O$ 1.8 mM pH 7.4 Each sag was carefully cut into small clumps, a rather time consuming procedure. This is to ensure the homogeneous enzymatic digestion of follicles. The small clumps were placed in a vial containing OR2 solution (NaCl 92.5 mM, KCl 2 mM, $MgCl_2.H_2O$ 1 mM, HEPES 5 mM pH 7.5) and oocytes were washed 5 times with this OR2 solution. Oocytes were then treated with collagenase 2 mg/ml in OR2 solution to partially digest the follicular layer. These oocytes were mixed by rotating the bottle

continuously for 40-50 min. At the end of digestion, oocytes were washed with OR2 solution 4-5 times. These oocytes were transferred to petri dish containing ND-96 solution. Each oocyte was manually separated from its surrounding follicle under stereomicroscope. The mature oocytes of stage V-VI about 1-1.2 mm in diameter were selected and stored in ND-96 solution at 18oC till ready for microinjection of capped poly (A+) RNA. In each experiment, 5-10 groups of oocytes were needed. For each group, 40-50 oocytes will be microinjected with RNA. Therefore, around 250-500 good quality oocytes were need. In general, under optimal digestion condition without damaging to the oocytes, about 100 oocytes may be defolliculated in an hour.

For simultaneous structural evaluation and functional analysis of the expressed proteins, the same batch of oocytes was used for each experiment. In this experiment, at least 60 oocytes were needed per group. Thus 300-600 oocytes were required for each experiment. Half of these microinjected oocytes were kept for structural analysis and the remaining were used for functional analysis. In structural analysis experiment, 50 nl of ^{35}S methionine (1000 Ci/mM) was microinjected into each oocyte, about 1 hr. after the microinjection of cRNA. This procedure was carried out on the same day of oocyte preparation, defolliculation and cRNA injection to assure that ^{35}S methionine would be incorporated into newly synthesized transporter.

Since the oocytes were kept 2-4 days before analysis, some oocytes did not survived the first or second day after microinjection. The percentage of dead oocytes depended on several factors from the quality of oocytes, stage, enzymatic digestion, defolliculation and

microinjection. It is well known that the quality of oocytes was not optimal especially during summer. For these reasons, large quantity of oocytes must be prepared.

Microinjection of AE1 cRNA into oocyte

- Glass micropipette preparation

Twenty-centimeter long glass pipette was pulled by micropipette puller yielding 2 glass micropipettes of 10 cm in length. Pulled pipette were ground by micropipette grinder until the tip was 30-40 μm in diameter for cRNA microinjection and 15-20 μm for ^{36}Cl and ^{35}S microinjection. Ground glass pipettes were washed using water and acetone. These grounded micropipettes were made RNAse free by baking at 200 °C for at least 6 hr in the morning of the microinjected experiment. These glass micropipettes were also kindly supplied by Prof. Hitoshi Endou.

- cRNA synthesis

After the preparation of cDNA template, the corresponding cRNA was synthesized. To prevent any contamination of RNAse, all vial and micropipette were kept RNAse free. All procedures were carried out under stringent condition to avoid contamination, as RNAse is quite stable and difficult to inactivate, whereas RNA is easily destroyed.

In vitro cRNA synthesis was carried out using Ambion kit. The synthetic reaction was performed by adding following chemicals sequentially, 2X Ribonucleotide Mix, 10X Transcription buffer, linearized DNA template and 10X enzyme mix. The reaction was incubated at 37 °C for 1hr. Then the cDNA template was eliminated by adding RNAse-free DNAse and

further incubated at 37 °C. for 15 min. The reaction was stopped by adding 5M NH_4OAC and cRNA was isolated by precipitation.

To precipitate cRNA, 100 µl of phenol/chloroform was added to the reaction mixture. After vigorous mixing, the samples were centrifuged at 12,000 rpm for 2 min. at room temperature. The supernatant phase (around 95 µl) was transferred to another vial and 95 µl of chloroform/isoamyl alcohol was added. The sample was centrifuged at the same speed and temperature. The supernatant was precipitated by adding isopropanol. Precipitation was performed at -20 °C for at least 24 hr. After precipitation, cRNA pellet was separated from the solution by centrifugation at 15,000 rpm at 4 °C for 20 min. The supernatant was discarded and 70% ethanol (-20 °C) was added to wash out the remaining salt. The supernatant was discarded after centrifugation at 15,000 rpm at 4 °C for 15 min. The pellet was dried by vacuum pump and dissolved with RNAse-free water 20 µl on ice for at least 10 min with periodic tapping. For determination of cRNA concentration, 1 µl of cRNA was diluted with 300 µl distilled water. The absorption of diluted cRNA was recorded by spectrophotometer (Jasco Model 7850) from the wavelength of 320 to 220 nm. The peak of absorption from the curve was used for calculation of cRNA concentration. The dissolved cRNA was kept at -80 °C for oocyte injection. cRNA integrity was confirmed by RNA gel electrophoresis. Agarose RNA gel showed one band of cRNA at position size of that cRNA.

- Preparation for cRNA microinjection

A glass micropipette was back filled with mineral oil using a long flexible needle. During this step, care must be exercise to avoid the formation of air bubbles in the micropipette, as

these bubbles will cause inaccuracy in microinjection. The oil filled micropipette was mounted on to a Drummond microdispensor. Then cRNA was loaded on to a parafilm and was sucked into the micropipette by the Drummond microdispensor, which was mounted on a Narishige micromanipulator. This Drummond microdispensor was also donated by Prof. Hitoshi Endou.

- Co-expression

The previously defolliculated oocytes were place in a petri-dish with glued nylon mesh screen on the bottom to prevent oocyte freely movement during microinjection. The cRNA in range of 2 to 50 ng in a final volume of 50 nl was microinjected into the cytoplasm of defolliculated oocytes using microdispenser under stereomicroscope. For co-expression experiment, two or three different cRNA were mixed at appropriate concentration to obtain the required final concentration for microinjection of 50 nl of the mixed cRNA into each oocyte. After injection, the oocytes were incubated for 2-4 days in ND96 solution containing gentamicin 50 μg/ml & sodium pyruvate 2.5 mM. The oocytes were maintained in the incubator at a constant temperature of 18 °C. The dead and bad oocytes were removed and the incubating medium was changed every day till the time of functional studies.

Functional analysis of AE1 proteins

- Uptake experiment

After 2-4 days, the transport protein of interest can be detected functionally by radioactive tracer flux measurement. The isotopic influx medium was prepared by adding 3 μl of ^{36}Cl into ND-58 solution (NaCl 58 mM, KCl 2 mM, $MgCl_2.2H_2O$ 1 mM, HEPES 5 mM,

$CaCl_2.2H_2O$ 1.8 mM, pH 7.4) plus 0.15 µl of 20 mM bumetanide. Bumetanide was used to inhibit endogenous chloride channel of oocytes. The isotopic influx medium was added into 96-well plate (150 µl/well). Group of 8-10 control (water injected) oocytes and cRNA injected oocytes were sorted and placed on a 24-well plate (one well/group) and washed with ND-96 solution. Then the oocytes were transferred into prepared 96-well plate containing 150 µl of 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 for 15 to 30 min. To stop the influx reaction, cold Cl-free ND96 solution (Na Isothionate 96 mM, K gluconate 2 mM, Mg gluconate 1 mM, HEPES 5 mM, Ca gluconic acid 1.8 mM, pH 7.4) was added to the oocytes in 96-well plate. The oocytes were then washed by transferring the oocytes from 96-well plate to a petri-dish containing cold Cl-free solution. The oocytes were rapidly washed 5 more times in cold Cl-free solution in a similar manner. Each oocyte was then transferred to the vial containing 1% SDS (200 µl). The oocytes were shaked for 30 min. to dissolve its membrane and solubilized its content. The scintillation fluid, 2 ml (contain: 0.5 gm%PPO, 0.1 gm% POPOP, 33% triton X-100, and 66% toluene) was added to each vial and the solution was thoroughly mixed. Radioactivity of the lysates were counted using scintillation counter to yield the transport rate of each oocyte. All experiment were repeated 2-3 times using different oocyte batches. The mean values were compared using conventional statistical analysis.

- Efflux experiment

After 2-4 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 Injection. The site of injection was just above the border between the two colors on the yellow side of the oocytes (cytoplasm site). After ^{36}Cl microinjection, individual oocyte was transferred into 96-well plate containing Cl-free ND-96 and bumetanide solution. Time of about 15 min. was allowed for the oocyte to seal the opening hole of the injection. This step was tested several times, using visual observation under microscope and counting the radioactivity of the injected oocyte. After 10-15 min. there was no detectable leakage of radioactivity into the medium. The microinjected oocytes were washed 3 times and transferred to 48-well plate containing 300 µl of efflux medium (ND96 and bumetanide solution). After 15 min. of incubation, a 250 µl efflux medium was collected for scintillation counting. An equal volume of the fresh efflux medium was added back and oocytes were incubated for 15 min. more. Another 250 µl of efflux medium was collected. (2 samples of efflux medium/oocyte). The oocytes were then washed 3 times with Cl-free solution to stop the efflux experiment. To test if oocyte is leaky, 300 µl of Cl-free solution was added. The oocyte was further incubated for another 30 min. A 250 µl Cl-free 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.

- Efflux kinetics

After 2-4 days of transporter expression, the AE1-expressing oocytes were sort and microinjected with ^{36}Cl as in the net efflux experiment. However, the medium was collected at 10 min. interval for a total duration of 1 hr .(6 samples/oocyte) before testing for the oocyte leakage. During this phase 2 samples of Cl-free efflux medium were collected per oocyte for a total duration of 30 min. Finally, each oocyte was transferred to a vial and dissolved in 200 µl of 1% SDS. The collected medium were counted using scintillation counter. All experiment were repeated 2-3 times using different oocyte batches. Data were presented as log(% of total cpm injected) vs. time. The efflux rate constants were measured from semilog plot linear slopes calculated from the last three time points for each condition.

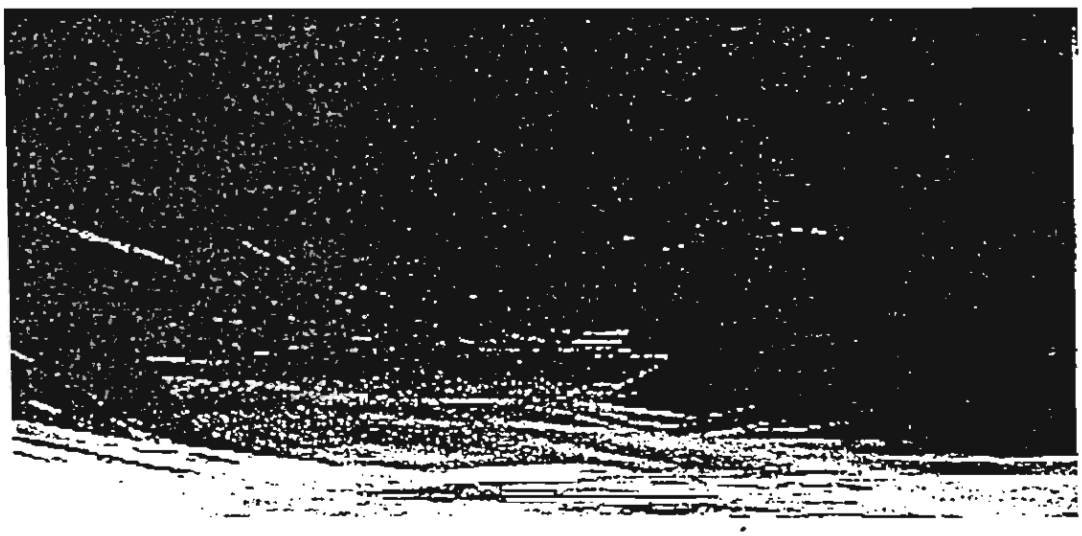
2nd International Research Conference

PharmaConference 2001

"Where Science Meets Industry"

Membrane Transporters: From Identification to Drug Discovery

Includes the Hepatobiliary Transport Satellite Meeting



August 5-10, Interlaken Congress Center, Interlaken, Switzerland

Organizer: Matthias A. Hediger
Coorganizers: Peter Meier-Abt, Robert Burrier,
Heini Murer & Wolf Frommer

P28

Molecular characterization of human kanadaptin, and its effect on human kidney anion exchanger 1 transport activities in Xenopus oocytes

Chairat Shayakul,[1] Surawat Jariyawat,[2] Naparat Kaewkaukul,[1] Samaisukh Sophasan.[2]
[1]*Renal Division, Faculty of Medicine, Siriraj Hospital,* [2]*Department of Physiology, Faculty of Science, Mahidol University, Bangkok, THAILAND*

Kanadaptin, a protein known to interact with the cytoplasmic domain of kidney anion exchanger 1 (kAE1), was first isolated in mouse by a yeast two hybrid screen. Though kanadaptin was located to the kAE1 containing vesicles in the cytoplasm of acid secreting type A intercalated cells, its function remains largely speculative. One possibility is that kanadaptin involves in acid secretion in distal nephron by targeting kAE1 vesicles to the basolateral membrane.

To verify this hypothesis, we conducted the study to isolate kanadaptin from human kidney and tested its effect on human kAE1 function using the Xenopus oocyte expression system. Isolation of human kanadaptin cDNA was performed based on homology search for the mouse kanadaptin on EST database. Several overlapping sequences were aligned and subsequently used to design primers for the polymerase chain reaction using human kidney cDNA as the template. The RT-PCR products were sequenced, and full-length human kanadaptin cDNA was constructed and subcloned into the Xenopus oocyte expression vector pXT7. It consists of ~ 3-kb nucleotides and contains an open reading frame of 2388 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 kD. 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. Over-expression of human kanadaptin in Xenopus oocytes resulted in partial inhibition of chloride fluxes mediated by wild-type kAE1. The effect was also observed after coexpression with kAE1-R589H variant, known mutant associated with autosomal dominant distal renal tubular acidosis. Detailed regulatory mechanisms of human kAE1 by kanadaptin and its significance are currently under investigation.

This work was supported by the Thailand Research Fund

Journal of the

American Society of Nephrology

Programs and Abstracts

ASN/ISN World Congress of Nephrology

October 10–17, 2001 ~ San Francisco, California, USA

Abstracts Available with On-line Discussion Forum at www.renalweek.org

Accredited Sponsor: The American Society of Nephrology

ASN 2001 ISN

World Congress of Nephrology

VOLUME 12

SEPTEMBER 2001

PROGRAM AND ABSTRACTS ISSUE

0047 M1-0004-(PS)

unctional Rescue of Anion Exchanger 1 (AE1) G701D by lycophorin A Is Attenuated by Co-Expression of AE1 Δ400-408: Basis for Transport Defect in Autosomal Recessive Distal Renal ubular Acidosis (dRTA). Chairat Shayakul,[1] Surawat Jariyawat,[2] Naparat ewkaukul,[1] Samaisukh Sophasan.[2] *[1]Renal Unit, Dept. of Medicine, Siriraj ospital, Bangkok, Thailand; [2]Dept. of Physiology, Fac. of Science, ahidol Univ., Bangkok, Thailand.*

A certain group of patients with autosomal recessive dRTA in Southeast Asia is associated th G701D mutation of AE1 gene, either homozygosity or compound heterozygosity th Southeast Asian Ovalocytosis mutation AE1 Δ400-408. The renal and erythroid enotypes in these patients, however, are distinct as demonstrated by normal or ~30% duction in red cell sulfate flux respectively. Our previous studies showed that kidney E1 G701D does not mediate anion transport activities but the function is regained by co-pression of the erythroid-specific AE1-associated protein glycophorin A (GpA). GpA is ential to facilitate AE1 G701D to the plasma membrane though comprehensive chanisms remain to be determined. In this study we used epitope tagged AE1 to investigate teromeric formation between AE1 G701D and wild-type or Δ400-408 variants in the sence of GpA. When expressed in Xenopus oocytes, the carboxyl terminus c-Myc tagged E1 G701D (AE1 G701D-cMyc) mediated Cl efflux to the comparable level of wild-type E1 only when GpA was co-expressed. Immunoblot and immunocytochemical studies ng cMyc antibodies also showed that expression of AE1 G701D-cMyc in the plasma mbrane was increased by GpA. Triple expression of wild-type AE1, AE1 G701D-cMyc d GpA did not result in alteration of Cl transport activities and cell surface expression of E1 G701D-cMyc. In contrast, co-expression of AE1 Δ400-408 with AE1 G701D-cMyc d GpA led to marked inhibition of Cl transport activities (60% reduction at a 1:1 molar io), and no Cl flux was observed in the absence of GpA. These results indicate that AE1 01D and AE1 Δ400-408 variants form heteromers that loss the anion exchanger properties nsistent with the transport defects found in these patients.

0048 M2-0018 (PS)

creased H-K ATPase in Renal Cortex by Salt Depletion and β-drenoceptor Activation. Randi B. Silver. *Physiology, Weill Medical ollege of Cornell University, New York, NY.*

Intercalated cells (ICs) of the cortical collecting duct (CCD) have a gastric-like SCH 080-sensitive H-K ATPase (HKAg). In rat, chronic salt depletion enhances the functional tivity of HKAg in the ICs of CCD, as evidenced by the rate of K+-dependent pHi recovery response to an acid load. Salt depletion also induces a separate SCH 28080-independent, abain-sensitive HKA, presumed to be the distal colonic HKA (HKAc) (Silver et al, AJP, 5:F94-F102, 1998). To further characterize the effects of salt-depletion on HKA in the Is, site-specific antibodies to the α-subunits of rat gastric (HKAg) and distal colonic KA (HKAc) were generated. Immunoblotting experiments showed that chronic salt-pletion failed to change the abundance of HKAg protein levels in cortex. Rather, the KAc protein levels were increased by 40%. These data suggest that HKAg is nstitutively expressed in the ICs and is stimulated under conditions of chronic salt pletion, while HKAc, which is not functional under control conditions in the ICs, is aptively increased with salt depletion. The signal responsible for HKA regulation in dney is not known. Acute exposure to the β-adrenoceptor agonist, isoproterenol (1μM), creased HKA activity in ICs from control rats to twice that of controls (0.07 ±0.01 pH its/min, n = 38 ICs vs 0.15 ±0.02 pH units/min+isoproterenol, n = 24 ICs). Furthermore, e increase in HKA activity by isoproterenol was comparable to the enhanced activity easured in ICs from rats on a low salt diet. These results suggest that β-adrenoceptor tivation in response to salt depletion may regulate the functional activity of HKA in ICs rat CCD.

0049 M2-0013 (PS)

olecular Cloning and Characterization of *Atp6n1b*, Encoding a ovel, Kidney-Specific, Fourth Isoform of the Mouse Vacuolar [+]-ATPase 116kDa a Subunit. Annabel N. Smith,[1] Mark A.J. Devonald,[1] a Su,[1] Karin E. Finberg,[2] Fiona E. Karet.[1] *[1]Medical Genetics, Cambridge niversity, Cambridge, United Kingdom; [2]HHMI, Yale University, New aven, CT.*

Vacuolar H+-ATPases (V-ATPases) acidify intracellular compartments in all eukaryotic ells. They are also present at the plasma membrane of certain specialized cells such as α-ntercalated cells in the kidney where they are critically involved in urine acidification. V-ATPases are composed of a V_1 domain catalysing ATP hydrolysis and a V_0 domain responsible or H+ translocation. A component of the V_0 domain, the 116kDa a subunit, has been found o have multiple isoforms. Four *a* subunit genes have been identified in man: *ATP6N1A* (*a1*) s widely expressed; *TJ6* (*a2*) encodes a putative T cell regulator; *ATP6N1C* (*a3*) is steoclast-specific. The fourth, *ATP6N1B*, we have recently shown to encode a kidney-pecific isoform expressed at the apical surface of α-intercalated cells in the distal nephron. This protein is essential for normal urinary acidification, since mutations in *ATP6N1B* ause autosomal recessive distal renal tubular acidosis. Orthologs of *ATP6N1A*, *TJ6* and *ATP6N1C* have been identified in a number of species but to date none has been described for *ATP6N1B*. We now report the cloning of *Atp6n1b*, the murine ortholog of *ATP6N1B*. *Atp6n1b* has 20 coding exons, with complete conservation of intron/exon boundaries between mouse and human. The 833 amino acid murine protein is 85% identical to human ATP6N1B and ≤61% identical to other family members. We have mapped *Atp6n1b* to proximal mouse chr. 6 in a region syntenic with the human locus on 7q33-34. RT-PCR and northern blot analysis demonstrated *Atp6n1b* expression in kidney but no Immunolocalization using a specific antibody revealed high intensity surface of α-intercalated cells. In contrast to our previous human st be additional, though lower level, staining in the proximal tubule. *Atp6n1b* as a novel, kidney-specific, fourth murine V-ATPase 116k show that it is the ortholog of human *ATP6N1B*.

A0050

Regulation of AE2-Mediated Cl⁻ Transport by Intr Abolished by Single Amino Acid Substitutions within Conserved Region of the N-Terminal Cytopla Andrew K. Stewart,[1] Marina N. Chernova,[1] Sabine Wi Alper.[1,2] *[1]Molecular Medicine and Renal Units, Beth Medical Center, Boston, MA; [2]Depts. of Medicine, Harvard Medical School, Boston, MA.*

We have previously defined a critical region within the N-terminal of the murine AE2 anion exchanger which is required for regulation transport activity by intracellular pH (pH_i). We now report that site within the region encompassing AE2 aa310-356 has defined specific in regulation of AE2-mediated ^{36}Cl efflux by pH_i. pH_i was vari extracellular application and removal of permeant weak acid. Wild typ ^{36}Cl efflux was inhibited ~80% by low pH_i and stimulated by raising pH_i histidine residues have been associated with pH regulation in transporters, the mutations H314A and H317A had no effect on pH_i Similarly, mutation of the candidate PKC phosphorylation site T339 effect on pH_i-sensitive AE2-mediated Cl⁻ transport. However, syst mutagenesis within the region aa336-356 identified five amino acids regulation of AE2 by pH_i. Alanine replacement of W336, E338, W resulted in functional proteins no longer sensitive to changes in pH_i wi range of 6.6 - 7.4. The mutation E347D also abolished pH_i-sensitivity transport. The conserved region containing these residues comprises abut the cytoplasmic face of the AE2 transmembrane domain. We pro interacts directly or indirectly with a proton sensor element in the domain.

A0051

NH4+ Transport in Chloride-Depleted C11-MDCK Tararthuch,[1] Ricardo Fernandez,[2] Gerhard Malnic.[1] *[1]Fisiolo Inst. Ciencias Biomedicas USP, S. Paulo, SP, Brazil; [2]Federal do Parana'Curitiba, PR, Brazil.*

In several tissues ammonium ions are able to use transport pat particularly of K+. We investigated this possibility in the C11 clone of to represent intercalated cells, in control and 0 Cl- conditions. Cell these cells, grown on permeable supports, by ratiometric fluorescence pH indicator BCECF. After preincubating the cells for 20 min in control by gluconate) Ringer, an ammonium pulse was applied to induce c magnitude of the initial alkalinization (DpH) was 0.24 ± 0.03 (n=28) which fell to 0.01 ± 0.005 (n=22) in 0 Cl-, suggesting influx of alkalinization by NH3. Addition of 10-3 M bumetanide to the 0 Cl Na+,K+,2Cl- cotransport, maintained DpH of 0.01 ± 0.01 (n=9). hexamethylene amiloride, a blocker of Na+/H+ exchange, or 3 mM Ba channels, were added to 0 Cl- medium, DpH was similar to 0 Cl- alone. Ba+, DpH= 0.09 ± 0.01 (n=6), (P<0.01 against 0Cl-), correspondi When 2.5 x 10-4 M ouabain, an inhibitor of Na+-K+ ATPase, was us 0.11 ± 0.02 (n=6), (P<0.01 against 0 Cl-), 46% of control. Preincubat channel blocker, and 0 Cl-, DpH = 0.244 ± 0.04 (n=5), indicating that C for the 0 Cl- effect on alkalinization by the ammonium pulse. In 0 Cl- these cells underwent a mean volume reduction of 36% as measured by In conclusion, both Na+-K+ ATPase and K+ channels appear to be depletion. This phenomenon was not observed in wild type MDCK (D = 0.31 and in 0 Cl- = 0.21). The observed volume reduction, which impli mediate the modification of NH4+ flux into cells.

A0052

H⁺-ATPase Activity in Collecting Duct Segments in Fed Rats. Role of Angiotensin II on Its Regulation Valles,[1] Liliana C. Camizo,[1] Alicia M. Seltzer,[2] Walter A. Mam *de Fisiopatologia, Facultad de Medicina, Universidad Na Mendoza, Argentina; [2]CONICET (Consejo Nacional de Cientificas y Tecnicas), Mendoza, Argentina.*

Enhanced expression of the genes that encode for various compo Angiotensin system has been exhibited in low protei (LP). Objective: Involvement of Angiotensin II (ANG II) through its modulation of H⁺-ATPase activity in cortical and medullary collecting segments in LP. Methods: H⁺-ATPase activity in CCD, OMCD and IM n:8 (protein 6%) and control rats (NP) n:8 (protein 24%) was eval Bafilomycin sensitive H⁺-ATPase activity. Measurement of the AT_1 expr by ANG II AT_1 receptor binding with quantitative autoradiography

uly 6, 2001

hairat Shayakul
enal Unit, Dept. of Medicine
iriraj Hospital
Prannok Road
angkoknoi Bangkok, 10700
hailand

ear Dr. Shayakul:

Ve are pleased to inform you that your abstract entitled "Functional Rescue of Anion Exchanger 1 (AE1) G701D y Glycophorin A Is Attenuated by Co-Expression of AE1 Δ400-408: A Basis for Transport Defect in Autosomal ecessive Distal Renal Tubular Acidosis (dRTA).," has been selected by the ASN/ISN Program Committee for an ral presentation during the ASN/ISN World Congress of Nephrology in San Francisco, California, USA. New this ear, we have selected several outstanding abstracts for presentation during related basic and clinical science ymposium. Your abstracts will still be presented in one of the main poster sessions. The details of your poster resentation will come in a separate letter. The details for the oral presentation are outlined below:

ate:	Monday, October 15, 2001
ession Name:	Genetic Diseases of Acid Base Regulation
resentation Time:	Abstract presentations will follow the symposium speakers
oom:	130/131

bstract presentations will be 10 minutes in duration with five minutes for discussion. Please note that as the resenting author of your abstract, you are expected to present your work clearly in English and to respond to uestions relating to your presentation in English. While one of the purposes of these oral presentations is to rovide an opportunity for young investigators to present their work, these sessions also provide a forum for nvestigators working in your field to hear about and to discuss major research issues. In order to facilitate the epth and quality of these discussions, we are asking that senior investigators who have contributed to this paper whether or not they are the actual presenters) attend the session and participate with you in the question and answer iscussions. This is by no means intended to supercede the participation of young investigators in these discussions. However, we believe that more participation by senior investigators will enhance the overall quality of the sessions for all of the participants.

The room will be set with one 35mm slide projector, so please prepare one set of clear and concise 2" x 2" slides for your presentation.

You should make every effort to avoid detailed tables and cluttered diagrams. Since the purpose of your presentation is to communicate new information in a brief time, please pay particular attention to the quality and quantity of your slides. Please use a font size above 22 for greatest visibility.

Please check your slides the morning of your presentation in the Speaker Ready Room, located in room 228-230 at the Moscone Center. When you check in, pay particular attention to the mock podium setup with timer, microphone, pointer, etc. An audiovisual technician will be available to assist you in the Speaker Ready Room.

World Congress of Nephrology

c/o American Society of Nephrology • 2025 M Street, NW, Suite 800 • Washington, DC 20036-3309 • USA

MONDAY, OCTOBER 15, 2001

Moderators:
Thomas Parker
Dallas Nephrology Associates, Dallas, TX, USA
Claudio Ronco
St. Bortolo Hospital, Vicenza, Italy

Body Mass and its Determinants and Dialysis Outc
John Daugirdas
University of Illinois at Chicago, Chicago, IL, USA

Is V the Optimal Way to Normalize Dialysis Treatme
Frank Gotch
University of California, San Francisco, San Francisco, CA

Role of V in Peritoneal Dialysis Adequacy
Bengt Lindholm
Baxter Novum and Renal Medicine Karolinska Institute
Stockholm, Sweden

Hemodialysis Practice Patterns and Outcomes in the Countries of DOPPS
Eric Young
VA Medical Center, Ann Arbor, MI, USA

1:30 p.m. – 3:30 p.m. Symposium

Genetic Diseases of Acid Base Regulation

Room 130/131

Extensive physiological studies have functionally identified acid-base regulating membrane proteins in the kidney and speculated their role in whole kidney acidification ability. Molecular technology in the last 10 years has confirmed the presence of hypothesized transporters, pumps, and channels. Generation of knockout mice and identification of human diseases caused by mutations of acid-base transport proteins have now allowed the evaluation of the roles of each transport protein in overall kidney acid-base function. This symposium will focus on acid-base transport genes and reevaluate their functions from molecular to whole body.

Moderators:
Michel Paillard
Hospital European Georges Pompidov, Paris, France
Seth Alper
Beth Israel Deaconess Medical Center, Boston, MA, USA

Molecular Pathogenesis of Proximal Renal Tubular Acid
Takashi Igarashi
The University of Tokyo, Tokyo, Japan

Mechanisms of Proximal Tubule Na, HCO3, and Cl Transport: Lessons from NHE3 Knockout Mice
Peter Aronson
Yale University School of Medicine, New Haven, CT, USA

MONDAY, OCTOBER 15, 2001

Inherited Disorders of Renal V-ATPase
Fiona Karet
Wellcome Trust Centre for Molecular Mechanisms in Disease, Cambridge, United Kindgom

Syndrome with Sensorineural Deafness and Kidney Failure Is Caused by a Novel Gene (*BART*) Expressed in Kidney
Helm Hildebrandt, Edgar Otto, Maria J. Schuermann, Martin Ulmer, Eva-Maria Ruf, Irina Maier-Lutz, Frank Beekmann, Andrea Fekete, Nikola Jeck, Martin Konrad, Ralf Birkenhager.
Freiburg, Germany.
(Abstract A2874)

Functional Rescue of Anion Exchanger 1 (AE1) G701D by Stomatin A Is Attenuated by Co-Expression of AE1 Δ400-A Basis for Transport Defect in Autosomal Recessive Distal Renal Tubular Acidosis (dRTA)
Chairat Shayakul, Surawat Jariyawat, Naparat Kaewkaukul, Samaisukh Sophasan.
Bangkok, Thailand.
(Abstract A0047)

p.m. – 3:30 p.m. Symposium

Nephrologists' Accountability for Patient Safety

Co-Sponsored by the Renal Physicians Association (RPA)

Room 300

...rs in medicine and patient safety have become an important ... of regulatory agencies and legislative bodies, and this concern is ...ected by the year-long theme that was developed by the RPA ...gram Committee on the topic of patient safety as an important ...ponent of quality care.

Moderators:
William F. Owen, Jr., M.D.
Duke Institute of Renal Outcomes
Research & Health Policy, Durham, NC, USA
Robert Provenzano, M.D.
St. John Hospital & Medical Center,
Detroit, MI, USA

...tient Safety – An Overview on Reducing Medical Errors
Alan Kliger
...Metabolism Associates PC, New Haven, CT, USA

...p Starting Patient Safety: Lessons Learned from the Pharmaceutical Industry on Errors
Lou Diamond
The Medstar Group, Washington, DC, USA

Errors from Variations in the Practice of Medicine
Paul Miles
Nashville, TN, USA