

	G701D/N	G701D/G701D	Δ400-408/N	G701D/Δ400-408
Red cell $\text{SO}_4^{2-}$ 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 kanadaptein and characterization of its role on the function of kAE1

In this study we isolated the human kanadaptein 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 kanadaptein is widely expressed in mouse tissues devoid of kAE1 (and also in human tissue, data not shown), some investigators suggested that kanadaptein 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 kanadaptein 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.

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## 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  $\Delta$ 400-408: 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

#### เชิงสาขาวิชานะ

- งานวิจัยนี้ได้เริ่มต้นขึ้นบนพื้นฐานของการสร้างความร่วมมือระหว่างกลุ่มผู้วิจัยทั้งในประเทศไทย ญี่ปุ่นและสหรัฐอเมริกา โดยเฉพาะ Kyorin University School of Medicine และ Harvard University สถาบันทั้งสองนับเป็นสถาบันชั้นนำในการศึกษาวิจัย กลไกการขนส่งสารผ่านเยื่อหุ้มเซลล์ด้วย transporter proteins ในประเทศไทยมีโรคที่เกี่ยวข้องกับความผิดปกติของ gene ของ transport proteins ที่เป็นต้นเหตุทั้งของภาวะ dRTA และโรคอื่น ๆ การศึกษาวิจัยครั้งนี้จึงนับเป็นการวางแผนงานวิจัยในการศึกษา gene ของ transporter proteins ซึ่งจะมีความสำคัญมากขึ้นทั้งในสภาวะปกติ ในสภาวะผิดปกติจากพันธุกรรม หรือในการพัฒนาฯ รักษาโรคให้เกิดความจำเพาะเจาะจงถูกขนส่งเข้าเซลล์เป็น 많이 ให้ออกฤทธิ์ได้ตามที่คาดหวัง จากการวิจัยครั้งนี้ได้เพิ่มความสัมพันธ์และความร่วมมือกับกลุ่มนักวิจัยชั้นนำอย่างเป็นรูปธรรมดังที่สรุปไว้ในกิตติกรรมประกาศ
- โครงการนี้ได้เป็นนำเสนอในที่ประชุมใหญ่ระดับนานาชาติ 2 ครั้ง ในช่วงปีที่ผ่านมา ซึ่งได้ก่อให้เกิดกระแสความสนใจในวงกว้างระดับนานาชาติเพิ่มขึ้น และทำให้มีการติดต่อระหว่างผู้ที่สนใจเกี่ยวกับการศึกษาในด้านนี้ อันจะนำไปสู่การสร้างความร่วมมือดับนานาชาติในอนาคต การประชุมทั้งสองนี้ได้แก่

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  $\Delta$ 400-408: A Basis for Transport Defect in Autosomal Recessive Distal Renal Tubular Acidosis (dRTA). ชี้งในการประชุมครั้งนี้ เรื่องดังกล่าวได้รับการคัดเลือกให้นำเสนอเป็นส่วนหนึ่งของ oral symposium ในหัวข้อ Genetic Disease of Acid Base Regulation เมื่อวันที่ 15 ตุลาคม 2544 และได้รับความสนใจเป็นอย่างมากด้วย

#### เที่ยววิชาการ

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

*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%  $\text{KHCO}_3$  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  $\text{NaCl}$  96 mM,  $\text{KCl}$  2 mM,  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$  1 mM, HEPES 5 mM,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  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 ( $\text{NaCl}$  92.5 mM,  $\text{KCl}$  2 mM,  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$  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 <sup>35</sup>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 <sup>35</sup>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  $\mu$ m in diameter for cRNA microinjection and 15-20  $\mu$ m 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<sub>4</sub>OAC and cRNA was isolated by precipitation.

To precipitate cRNA, 100  $\mu$ l 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  $\mu$ l) was transferred to another vial and 95  $\mu$ l 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  $\mu$ l on ice for at least 10 min with periodic tapping. For determination of cRNA concentration, 1  $\mu$ l of cRNA was diluted with 300  $\mu$ l 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 exercised 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  $\mu$ g/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  $\mu$ l of  $^{36}\text{Cl}$  into ND-58 solution (NaCl 58 mM, KCl 2 mM,  $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$  1 mM, HEPES 5 mM,

CaCl<sub>2</sub>.2H<sub>2</sub>O 1.8 mM, pH 7.4) plus 0.15  $\mu$ l 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  $\mu$ l/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  $\mu$ l 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  $\mu$ l 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  $\mu$ l). 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  $^{36}\text{Cl}$  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  $^{36}\text{Cl}$  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  $^{36}\text{Cl}$  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  $\mu\text{l}$  of efflux medium (ND96 and bumetanide solution). After 15 min. of incubation, a 250  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of Cl-free solution was added. The oocyte was further incubated for another 30 min. A 250  $\mu\text{l}$  Cl-free media was collected. Finally, each oocyte was transferred to a vial for dissolution in 200  $\mu\text{l}$  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  $^{36}\text{Cl}$  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  $\mu\text{l}$  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  $\log(\% \text{ of total cpm injected})$  vs. time. The efflux rate constants were measured from semilog plot linear slopes calculated from the last three time points for each condition.

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Heini Murer & Wolf Frommer**

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

Chairat Shayakul,<sup>1</sup> Surawat Jariyawat,<sup>2</sup> Naparat Kaewkaukul,<sup>1</sup> Samaisukh Sophasan.<sup>2</sup>

<sup>1</sup>*Renal Division, Faculty of Medicine, Siriraj Hospital, 2Department 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

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SEPTEMBER 2001

PROGRAM AND ABSTRACTS ISSUE

World Congress of Nephrology

0047

M1-0004-(PSV)

unctional Rescue of Anion Exchanger 1 (AE1) G701D by  
lycophorin A Is Attenuated by Co-Expression of AE1Δ400-408;  
Basis for Transport Defect in Autosomal Recessive Distal Renal  
Tubular Acidosis (dRTA). Chairat Shayakul,<sup>1</sup> Surawat Jariyawat,<sup>2</sup> Naparat  
Sewkaulkul,<sup>1</sup> Samaisukh Sophasan.<sup>2</sup> <sup>1</sup>Renal Unit, Dept. of Medicine, Siriraj  
Hospital, Bangkok, Thailand; <sup>2</sup>Dept. of Physiology, Fac. of Science,  
Mahidol Univ., Bangkok, Thailand.

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

0048

M2-0018 (PS)

**Increased H-K ATPase in Renal Cortex by Salt Depletion and  $\beta$ -adrenoceptor Activation.** Randi B. Silver. *Physiology, Weill Medical College of Cornell University, New York, NY.*

Intercalated cells (ICs) of the cortical collecting duct (CCD) have a gastric-like SCH 28080-sensitive H-K ATPase (HKAg). In rat, chronic salt depletion enhances the functional activity of HKAg in the ICs of CCD, as evidenced by the rate of K+-dependent pH recovery in 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, 25: F94-F102, 1998). To further characterize the effects of salt-depletion on HKA in the ICs, site-specific antibodies to the  $\alpha$ -subunits of rat gastric (HKAg) and distal colonic HKA (HKAc) were generated. Immunoblotting experiments showed that chronic salt-depletion failed to change the abundance of HKAg protein levels in cortex. Rather, the HKAc protein levels were increased by 40%. These data suggest that HKAg is constitutively expressed in the ICs and is stimulated under conditions of chronic salt-depletion, while HKAc, which is not functional under control conditions in the ICs, is selectively increased with salt depletion. The signal responsible for HKA regulation in the ICs is not known. Acute exposure to the  $\beta$ -adrenoceptor agonist, isoproterenol (1  $\mu$ M) increased HKA activity in ICs from control rats to twice that of controls ( $0.07 \pm 0.01$  pH units/min,  $n = 38$  ICs vs  $0.15 \pm 0.02$  pH units/min +isoproterenol,  $n = 24$  ICs). Furthermore, the increase in HKA activity by isoproterenol was comparable to the enhanced activity measured in ICs from rats on a low salt diet. These results suggest that  $\beta$ -adrenoceptor activation in response to salt depletion may regulate the functional activity of HKA in ICs of rat CCD.

0049

M2-0013 (PS)

**1**olecular Cloning and Characterization of *Atp6nlb*, Encoding a Novel, Kidney-Specific, Fourth Isoform of the Mouse Vacuolar  $\text{H}^+$ -ATPase 116kDa a Subunit. Annabel N. Smith,<sup>1</sup> Mark A.J. Devonald,<sup>1</sup> a Su,<sup>1</sup> Karin E. Finberg,<sup>2</sup> Fiona E. Karet,<sup>1</sup> <sup>1</sup>Medical Genetics, Cambridge University, Cambridge, United Kingdom; <sup>2</sup>HHMI, Yale University, New Haven, CT.

Vacuolar H<sup>+</sup>-ATPases (V-ATPases) acidify intracellular compartments in all eukaryotic cells. They are also present at the plasma membrane of certain specialized cells such as  $\alpha$ -intercalated cells in the kidney where they are critically involved in urine acidification. V-ATPases are composed of a V<sub>0</sub> domain catalysing ATP hydrolysis and a V<sub>1</sub> domain responsible for H<sup>+</sup> translocation. A component of the V<sub>0</sub> domain, the 116kDa  $\alpha$  subunit, has been found to have multiple isoforms. Four  $\alpha$  subunit genes have been identified in man: *ATP6N1A* (a1) is widely expressed; *TJ6* (a2) encodes a putative T cell regulator; *ATP6N1C* (a3) is osteoclast-specific. The fourth, *ATP6N1B*, we have recently shown to encode a kidney-specific isoform expressed at the apical surface of  $\alpha$ -intercalated cells in the distal nephron. This protein is essential for normal urinary acidification, since mutations in *ATP6N1B* cause 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 56% identical to other family members. We have mapped *Atp6n1b* to proximal mouse chr-6 in a region syntenic with the human locus on 2q33-34. RT-PCR and northern

blot analysis demonstrated *Atp6nlb* expression in kidney but not heart. Immunolocalization using a specific antibody revealed high intensity staining on the surface of  $\alpha$ -intercalated cells. In contrast to our previous human studies, the staining pattern may be additional, though lower level, staining in the proximal tubule. These data show that *Atp6nlb* is a novel, kidney-specific, fourth murine V-ATPase 116 kDa subunit.

A0050

## Regulation of AE2-Mediated Cl<sup>-</sup> Transport by Intracellular pH

Abolished by Single Amino Acid Substitutions within Conserved Region of the N-Terminal Cytoplasmic Domain of the Renin Receptor. Andrew K. Stewart,<sup>1</sup> Marina N. Chernova,<sup>1</sup> Sabine W. Alper.<sup>1,2</sup> <sup>1</sup>*Molecular Medicine and Renal Units, Beth Israel Medical Center, Boston, MA;* <sup>2</sup>*Dep't. 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 of transport activity by intracellular pH (pH<sub>i</sub>). We now report that site within the region encompassing AE2 aa310-356 has defined specificity in regulation of AE2-mediated  $^{36}\text{Cl}^-$  efflux by pH. pH<sub>i</sub> was varied extracellular application and removal of permeant weak acid. Wild type  $^{36}\text{Cl}^-$  efflux was inhibited ~80% by low pH, and stimulated by raising pH. Histidine residues have been associated with pH regulation in transporters, the mutations H314A and H317A had no effect on pH<sub>i</sub>. Similarly, mutation of the candidate PKC phosphorylation site T339 had no effect on pH<sub>i</sub>-sensitive AE2-mediated Cl<sup>-</sup> transport. However, systematic mutagenesis within the region aa336-356 identified five amino acids that regulate AE2 by pH<sub>i</sub>. Alanine replacement of W336, E338, W340 resulted in functional proteins no longer sensitive to changes in pH<sub>i</sub> within range of 6.6 - 7.4. The mutation E347D also abolished pH<sub>i</sub>-sensitivity of transport. The conserved region containing these residues comprised about the cytoplasmic face of the AE2 transmembrane domain. We propose interacts directly or indirectly with a proton sensor element in the domain.

A0051

NH<sub>4</sub><sup>+</sup> Transport in Chloride-Depleted C11-MDCK Cells

Tararhuch,<sup>1</sup> Ricardo Fernandez,<sup>2</sup> Gerhard Malnic,<sup>1</sup> <sup>1</sup>*Fisiologia*  
*Inst. Ciencias Biomedicas USP, S. Paulo, SP, Brazil*, <sup>2</sup>  
*Federal do Parana'Curitiba, PR, Brazil.*

In several tissues ammonium ions are able to use transport pathways particularly of  $K^+$ . We investigated this possibility in the C11 clone of MDCK cells to represent intercalated cells, in control and 0  $Cl^-$  conditions. Cells in these cells, grown on permeable supports, by ratiometric fluorescence of the pH indicator BCECF. After preincubating the cells for 20 min in control (by gluconate) Ringer, an ammonium pulse was applied to induce a change in pH. The magnitude of the initial alkalinization ( $DpH$ ) was  $0.24 \pm 0.03$  ( $n=28$ ), which fell to  $0.01 \pm 0.005$  ( $n=22$ ) in 0  $Cl^-$ , suggesting influx of  $NH_4^+$  alkalinization by  $NH_3$ . Addition of 10-3 M bumetanide to the 0  $Cl^-$  medium, which blocks  $Na^+,K^+,2Cl^-$  cotransport, maintained  $DpH$  of  $0.01 \pm 0.01$  ( $n=9$ ). Hexamethylene amiloride, a blocker of  $Na^+/H^+$  exchange, or 3 mM  $Ba^{2+}$  channels, were added to 0  $Cl^-$  medium,  $DpH$  was similar to 0  $Cl^-$  alone.  $Ba^{2+}$ ,  $DpH = 0.09 \pm 0.01$  ( $n=6$ ), ( $P<0.01$  against 0  $Cl^-$ ), corresponding to a  $Na^+/H^+$  exchange rate of 1.0. When  $2.5 \times 10^{-4}$  M ouabain, an inhibitor of  $Na^+-K^+$  ATPase, was used,  $DpH = 0.11 \pm 0.02$  ( $n=6$ ), ( $P<0.01$  against 0  $Cl^-$ ), 46% of control. Preincubation with ouabain, a  $Na^+/K^+$  channel blocker, and 0  $Cl^-$ ,  $DpH = 0.244 \pm 0.04$  ( $n=5$ ), indicating that  $Cl^-$  is not required 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  $^{14}C$ -inulin. In conclusion, both  $Na^+-K^+$  ATPase and  $K^+$  channels appear to be involved in the regulation of  $NH_4^+$  depletion. This phenomenon was not observed in wild type MDCK ( $DpH = 0.31$  and in 0  $Cl^-$  0.21). The observed volume reduction, which is not due to the modification of  $NH_4^+$  flux into cells.

A0052

## H<sup>+</sup>-ATPase Activity in Collecting Duct Segments in

Fed Rats. Role of Angiotensin II on Its Regulation  
Valles,<sup>1</sup> Liliana C. Camizo,<sup>1</sup> Alicia M. Seltzer,<sup>2</sup> Walter A. Mazzoni,<sup>1</sup> and Daniel A. Gómez,<sup>1</sup> <sup>1</sup>División de Endocrinología y <sup>2</sup>División de Fisiopatología, Facultad de Medicina, Universidad Nacional de Mendoza, Argentina; <sup>2</sup>CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Mendoza, Argentina.

Enhanced expression of the genes that encode for various components of the Angiotensin system has been exhibited in low-protein (LP) Objective: Involvement of Angiotensin II (ANG II) through its modulation of H<sup>+</sup>-ATPase activity in cortical and medullary collecting duct segments in LP. Methods: H<sup>+</sup>-ATPase activity in CCD, OMCD and IMCD n:8 (protein 6%) and control rats (NP) n:8 (protein 24%) was evaluated. Baflomycin sensitive H<sup>+</sup>-ATPase activity. Measurement of the AT<sub>1</sub> receptor by ANG II AT<sub>1</sub> receptor binding with quantitative autoradiography.

July 6, 2001



Hairat Shayakul  
Renal Unit, Dept. of Medicine  
Siriraj Hospital  
Prannok Road  
Bangkoknoi Bangkok, 10700  
Thailand

Dear Dr. Shayakul:

We are pleased to inform you that your abstract entitled "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).," has been selected by the ASN/ISN Program Committee for an oral presentation during the ASN/ISN World Congress of Nephrology in San Francisco, California, USA. Now this year, we have selected several outstanding abstracts for presentation during related basic and clinical science symposium. Your abstracts will still be presented in one of the main poster sessions. The details of your poster presentation will come in a separate letter. The details for the oral presentation are outlined below:

Date: Monday, October 15, 2001  
Session Name: Genetic Diseases of Acid Base Regulation  
Presentation Time: Abstract presentations will follow the symposium speakers  
Room: 130/131

Abstract presentations will be 10 minutes in duration with five minutes for discussion. Please note that as the presenting author of your abstract, you are expected to present your work clearly in English and to respond to questions relating to your presentation in English. While one of the purposes of these oral presentations is to provide an opportunity for young investigators to present their work, these sessions also provide a forum for investigators working in your field to hear about and to discuss major research issues. In order to facilitate the depth 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 discussions. This is by no means intended to supersede 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.



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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 Outcomes**

John Daugirdas

University of Illinois at Chicago, Chicago, IL, USA

**Is V the Optimal Way to Normalize Dialysis Treatment?**

Frank Gotch

University of California, San Francisco, San Francisco, CA, USA

**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

MONDAY, OCTOBER 15, 2001

**Inherited Disorders of Renal V-ATPase**

Fiona Karet

Gloucester Truth Centre for Molecular Mechanisms in Disease,  
Cambridge, United Kingdom

**Wolff-Parkinson-White Syndrome with Sensorineural Deafness and Kidney Failure Is Caused by a Novel Gene (BART) Expressed in Kidney**

Heinz Hildebrandt, Edgar Otto, Maria J. Schuermann, Martin Ellmer, 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 Haptophorin A Is Attenuated by Co-Expression of AE1 Δ400-Δ401: 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)

1:30 p.m. – 3:30 p.m.

Symposium

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 genes 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 Pompidou, Paris, France  
Seth Alper

Beth Israel Deaconess Medical Center, Boston, MA, USA

**Molecular Pathogenesis of Proximal Renal Tubular Acidosis**

Takashi Igarashi

The University of Tokyo, Tokyo, Japan

**Mechanisms of Proximal Tubule Na, HCO<sub>3</sub>, and Cl<sup>-</sup> Transport: Lessons from NHE3 Knockout Mice**

Peter Aronson

Yale University School of Medicine, New Haven, CT, USA

**Nephrologists' Accountability for Patient Safety**

Co-Sponsored by the Renal Physicians Association (RPA)

Room 300

*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

**Patient Safety – An Overview on Reducing Medical Errors**

Alan Kliger

Metabolism Associates PC, New Haven, CT, USA

**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