



## Analysis of the interaction between human kidney anion exchanger 1 and kanadaplin using yeast two-hybrid systems

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### Abstract

Kidney anion exchanger adaptor protein (Kanadaplin) is a protein which interacts with the cytoplasmic N-terminal domain of kidney anion exchanger 1 (kAE1) and was first detected in mice using the yeast two-hybrid system and was also found to co-localize with kAE1 in rabbit  $\alpha$ -intercalated cells. Impaired trafficking of human kAE1 can result in the kidney disease-distal renal tubular acidosis (dRTA), and defective interaction between human kAE1 and kanadaplin may cause this trafficking impairment and be the basis for dRTA pathogenesis. However, it is unknown whether kAE1 can really interact with kanadaplin in humans. We have thus investigated the interaction between human kAE1 and human kanadaplin by using both Gal4 and LexA yeast two-hybrid systems. It was found that co-expression of Gal4DBD fused to the cytoplasmic N-terminal domain of kAE1 and Gal4AD fused to kanadaplin could not activate the transcription of the *ADE2*, *HIS3* and *lacZ* reporters in the Gal4 system. A similar result was obtained for the interaction between B42AD fused to the cytoplasmic N-terminal domain of kAE1 and LexA fused to kanadaplin in activation of *lacZ* transcription in the LexA system. The absence of interaction between the fusion proteins in both yeast two-hybrid systems raises the possibility that kAE1 may not interact with kanadaplin in human cells. Considerably different structures of both kAE1 and kanadaplin in mice and humans may lead to different binding properties of the proteins in these two species.

**Key words:** kidney anion exchanger 1, kanadaplin, yeast two-hybrid system.

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### Introduction

Human anion exchanger 1 (AE1), an integral membrane protein with 911 amino acid residues (Drickamer *et al.*, 1978; Jennings *et al.*, 1989), consists of two isoforms, erythroid AE1 (eAE1 or band 3) and kidney AE1 (kAE1). Erythroid AE1 is the major integral protein of the erythrocyte plasma membrane where it is responsible for the dual roles of the electroneutral exchange of  $\text{Cl}^-/\text{HCO}_3^-$  across the plasma membrane and cytoskeletal anchorage (Showe *et al.*, 1987). Kidney AE1 is located on the basolateral membrane of acid-secreting  $\alpha$ -intercalated cells of the distal tubule and collecting duct of the nephron and is identical to

eAE1 except that transcription of kAE1 occurs from an alternative initiation site within intron 3 leading to a protein with a truncated N-terminus. Mouse kAE1 lacks 79 N-terminal amino acid residues and human kAE1 65, because of which kAE1 no longer binds to ankyrin, protein 4.1, or glycolytic enzymes (Drenckhahn and Merte, 1987; Brosius *et al.*, 1989; Kollert-Jons *et al.*, 1993; Zhang *et al.*, 2000).

Mutations in the human *AE1* gene may lead to the kidney disease known as distal renal tubular acidosis (dRTA) (Bruce *et al.*, 1997; Rysava *et al.*, 1997; Jarolim *et al.*, 1998; Karet *et al.*, 1998; Tanphaichitr *et al.*, 1998; Vasuvattakul S. *et al.*, 1999; Bruce *et al.*, 2000; Yenchitsomanus *et al.*, 2002) characterized by defective urinary acidification in the distal nephron, which is unable to lower urine pH to less than 5.5 in the presence of metabolic acidosis, frequently accompanied by hypokalemia,

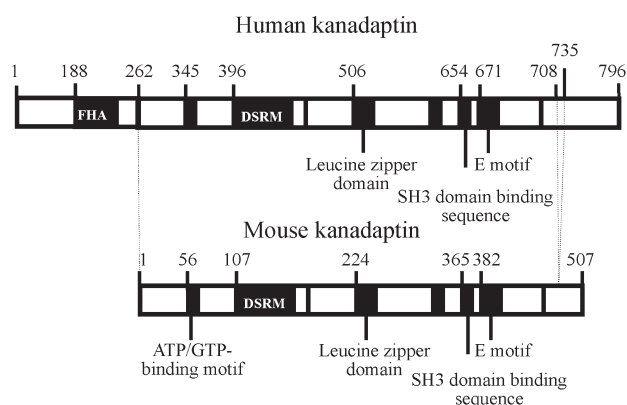
nephrocalcinosis and metabolic bone disease (Morris and Sebastian, 1983). Unlike wild-type kAE1, the mutant proteins exhibited intracellular retention when they were studied in non-polarized cells (Quilty *et al.*, 2002a; Quilty *et al.*, 2002b; Toye *et al.*, 2002; Kittanakom *et al.*, 2004a). However, in polarized cells, dRTA mutations cause either intracellular retention (Toye *et al.*, 2004) or apical mistargeting of the proteins (Devonald *et al.*, 2003; Toye *et al.*, 2004; Rungroj *et al.*, 2004). The correct trafficking of kAE1 may require both N- and C-termini (Devonald *et al.*, 2003; Toye *et al.*, 2004). Tyrosine-based sorting signals locating at these regions may interact with adaptor-protein complexes (Bonifacino and Dell'Angelica, 1999; Mostov *et al.*, 2000). However, studies using the proximal renal tubular cell line LLC-PK1 lacking the  $\mu$ 1B subunit have indicated that the  $\mu$ 1B subunit of the AP1B adaptor protein may not be involved in kAE1 trafficking to the basolateral membrane (Devonald *et al.*, 2003; Toye *et al.*, 2004) and that other adaptor proteins may be involved. In red cells, glycophorin acts as a chaperone in eAE1 trafficking (Young *et al.*, 2000) but in kidney cells there is as yet no information as to the protein involved in kAE1 trafficking or the checkpoint for normal kAE1 trafficking nor is there any indication as to how kAE1 trafficking fails. Thus, to understand kAE1 transport, targeting, and regulation, it will be necessary to identify the proteins that bind to kAE1.

Kidney anion exchanger adaptor protein (kanadapтин) was first isolated from mice using the Gal4 yeast two-hybrid system. A cytoplasmic N-terminal kAE1 domain was used as a bait to screen its interactor in mouse kidney cDNA library and kanadapтин was identified (Chen *et al.*, 1998). Mouse kanadapтин consists of 507 amino acid residues and has a multi-domain structure with three distinctive putative nuclear localizing sequences (NLSs). Northern blot analysis demonstrated that mouse kanadapтин was widely expressed in lung, liver, brain, testis, heart, skeletal muscle and kidney tissues and that it was co-localized with kAE1 in intracellular vesicles but not in the basolateral membrane of rabbit  $\alpha$ -intercalated cells (Chen *et al.*, 1998). This suggests that kanadapтин might play a role in the targeting of kAE1-containing vesicles to basolateral membrane of  $\alpha$ -intercalated cells. However, immunofluorescence and subcellular fractionation studies have demonstrated that kanadapтин is localized within the nuclei of various epithelial and non-epithelial cultured cell types (Hubner *et al.*, 2002) and immuno-staining revealed strong kanadapтин signals in association with the mitochondria of proximal and distal tubule cells, cerebella cortex cells, enterocytes, hepatocytes and pancreatic acinar cells (Hubner *et al.*, 2003).

Human kanadapтин cDNA (AY028435) was cloned and characterized by our group. The human kanadapтин protein contains 796 amino acids, has 262 amino acids longer than mouse kanadapтин at its N-terminus and has the extra 28 amino acid residues (codons 708 to 735) inserted

near the C-terminus (Figure 1). However, the role of kanadapтин in human cells has not yet been demonstrated but the evidence from mouse and rabbit cells indicate that human kanadapтин may have a similar function in the targeting of kAE1-containing vesicles to their final destination. Since it is known that kAE1 defect results in dRTA, the failure of interaction between kAE1 and kanadapтин may lead to impaired trafficking of kAE1 to the basolateral membrane of the  $\alpha$ -intercalated cells where it mediates  $\text{Cl}^-/\text{HCO}_3^-$  exchange thereby producing dRTA.

The yeast two-hybrid system is a powerful genetic tool to rapidly select uncharacterized proteins specifically interacting with a target protein of interest from a suitable cDNA library or to determine whether two known proteins interact with one another (Finley, 1995). This technique takes advantage of the modular domain structure of eukaryotic transcription factor. Eukaryotic transcription activators have at least two distinct functional domains, the DNA-binding domain that directly binds to the specific DNA sequences and the activation domain that activates transcription (Fields and Song, 1989). To study protein-protein interaction, protein X is first fused to the DNA-binding domain (DBD) and then protein Y is fused to activation domain (AD), the interaction between proteins X and Y leading to the reconstitution of a functional transcription factor and activation of a reporter gene. The most commonly used yeast two-hybrid systems are the Gal4 system (Fields and Song, 1989) in which the Gal4 transcriptional activator involved in galactose metabolism is reconstituted and the LexA system (Gyuris *et al.*, 1993) which uses the operator sequence and binding domain (BD) from the *E. coli* lexA repressor protein. The use of two different systems with the same protein as a bait sometimes produce different results because the different fusion proteins may affect protein folding and interfere with the protein-protein



**Figure 1** - Differences in structure between human and mouse kanadapтин. The upper picture shows human kanadapтин and the lower picture mouse kanadapтин. Human kanadapтин has 262 amino acids more than mouse kanadapтин at the N-terminal region containing forkhead-associated (FHA) domain and an extra 28 amino acid residues (positions 708 to 735) inserted near the C-terminal region which is not present in mouse kanadapтин. DSRM = double-stranded RNA-binding motif, E motif = glutamic acid motif and SH3 = Src homology domain 3.

interaction (Crieke and Beyaert, 1999). Some proteins may show interaction in the LexA system but not in the Gal4 system, e.g. the interaction between SopA and SopB of F plasmid (Kim and Shim, 1999).

In this study both the Gal4 and LexA yeast two-hybrid systems were used to investigate the interaction between the cytoplasmic N-terminal domain of human kAE1 and human kanadap-  
tin. In the Gal4 system, Gal4DBD was fused to kAE1 and Gal4AD to kanadap-  
tin, while in the LexA system, B42AD was fused to kAE1 and LexA to kanadap-  
tin. In both systems, the interactions were studied by mating and co-transformation procedures.

## Materials and Methods

### Materials

The yeast, *Saccharomyces cerevisiae*, strains AH109 (*MAT $\alpha$* , *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4 $\Delta$* , *gal80 $\Delta$* , *LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3*, *GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2* *URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>* -*lacZ MEL1*), and Y187 (*MAT $\alpha$* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3, 112*, *gal4 $\Delta$* , *gal80 $\Delta$* , *met-*, *URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ MEL1*), the plasmid pGBKT7, pGADT-7, monoclonal anti-LexA, monoclonal anti-Gal4DBD and polyclonal anti-HA antibodies were purchased from Clontech (Franklin Lakes, USA). Yeast (*S. cerevisiae*) strains RFY231 (*MAT $\alpha$*  *ura3-1 his3 trp1 $\Delta$ ::hisG* 3LexAop-*LEU2::leu2*), and RFY206 (*MAT $\alpha$*  *his3 $\Delta$ 200 leu2-3 lys2 $\Delta$ 201 ura3-52 trp1 $\Delta$ hisG*) and the plasmid pNLex and pJZ4 were kindly donated by Associate Professor Russell L. Finley Jr., Wayne State University, School of Medicine, Michigan. The propagation host for the recombinant DNA plasmid was *Escherichia coli* (*E. coli*) strain DH5 $\alpha$  (*supE44  $\Delta$ lacU169* ( $\phi$ 80 *lacZ  $\Delta$ M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) from a stock culture maintained in our laboratory. Affinity-purification used ProBond<sup>TM</sup> Ni<sup>2+</sup> beads from Invitrogen (Calsbad, USA). X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) was purchased from ROTH (Karlsruhe, Germany) and O-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) was purchased from Sigma Chemical Co. (St. Louis, USA).

### Cloning of human kanadap- tin cDNA

Human kanadap-  
tin cDNA was amplified and isolated by the polymerase chain reaction (PCR) from cDNAs prepared from human kidney mRNA. Total RNA was prepared from autopsied kidney tissue homogenate using TRIzol<sup>®</sup> reagent (Life Technologies, Gaithersburg, MD, USA). The full-length human cDNAs were synthesized using the oligo(dT) 12-18 primer and SUPERScript<sup>TM</sup> Pre-amplification System for First Strand cDNA Synthesis (Life Technologies). Human kanadap-  
tin primers for two-step (nested) PCRs were designed from human expressed sequence tags (ESTs) searched by using the mouse kanadap-  
tin

mRNA sequence. The PCR was performed in a Gene-Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA) for 30 cycles, each cycle consisting of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min (10 min for the final cycle). The primary PCR product was then used as a template for nested PCR under the same conditions. The PCR product was detected by gel electrophoresis and purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany) for sequencing analysis using the ABI PRISM Dye Terminal Cycle Sequencing Ready Reaction Kit (ABI, USA) and ABI-PRISM 310 Genetic Analyzer (ABI, USA). The human kanadap-  
tin sequence was deposited in GenBank (AY028435). The isolated human kanadap-  
tin cDNA was cloned into the *XhoI* and *EcoRI* sites of prokaryotic (pTrcHisA) vector and transformed into *E. coli* cells strain DH5 $\alpha$ . The recombinant plasmids were screened by plasmid mini-preparation and restriction endonuclease digestion. Human kanadap-  
tin protein was expressed in *E. coli* and purified for polyclonal antibody production.

### Production of mouse polyclonal anti-human kanadap- tin antibody

Mouse polyclonal anti-sera directed against human kanadap-  
tin was generated using 6xHis-kanadap-  
tin fusion protein as an immunogen. The recombinant human kanadap-  
tin protein was expressed as a fusion protein with a 6xHis tag at the amino-terminus in *E. coli* using the pTrcHisA vector. Expression of 6xHis-kanadap-  
tin fusion protein was induced by adding 0.25 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at 20 °C for 6 h. The bacterial cells were sonicated in the presence of protease inhibitor, centrifuged to generate soluble and particulate fractions. To verify protein expression in each fraction, samples were fractionated by SDS-PAGE and Western blotting was carried out using an anti-6xHis antibody. Affinity-purification with ProBond<sup>TM</sup> Ni<sup>2+</sup> beads was used to purify 6xHis-kanadap-  
tin eluted with 100 mM imidazole. To generate polyclonal antibody against human kanadap-  
tin, we immunized mice with affinity-purified 6xHis-kanadap-  
tin. In the first immunization, 6xHis-kanadap-  
tin (20-60  $\mu$ g protein) was combined with equal volume of complete Freund's adjuvant and then subcutaneously injected into mice. After 2 weeks, anti-kanadap-  
tin antibody was raised and the antibody titer was checked by dot-blot enzyme immunoassay. Mice were boosted for two weeks with 6xHis-kanadap-  
tin without any adjuvant after which they were sacrificed and the heart blood centrifuged to collect serum, which was then stored at -20 °C. Specificity of antibody was confirmed by Western blot analysis.

### Yeast strains and growth condition

For the Gal4 yeast two-hybrid experiments, we used *S. cerevisiae* strains AH109 and Y187 while for the LexA system we used *S. cerevisiae* strains RFY231 and RFY206. The yeasts were grown at 30 °C in yeast extract potato dex-

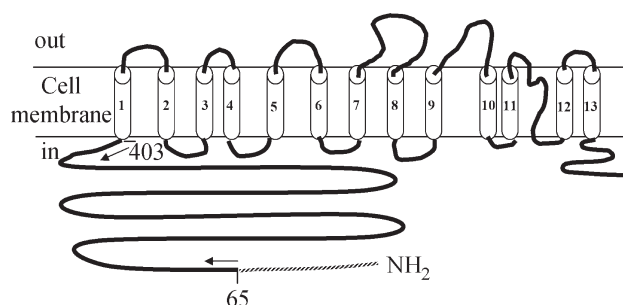
rose medium (YPD) or in a synthetic defined (SD) minimal medium supplemented with amino acids and glucose.

### Construction of recombinant plasmids

A recombinant plasmid (pADkAE1) containing the cytoplasmic N-terminal domain of human kAE1 cDNA (encoding amino acids 66-403 of AE1, Figure 2) was constructed by amplifying kAE1 cDNA available in our laboratory and digesting the purified PCR product with *EcoRI* and *XhoI*, followed by ligation into the *EcoRI* and *XhoI* sites of plasmid pJZ4 (expressing the nuclear localizing signal, B42 activation domain and the HA-epitope tag). Plasmid pGalkAE1 containing the cytoplasmic N-terminal domain of kAE1 cDNA fused to Gal4BD was constructed by amplifying the kAE1 sequence (encoding amino acids 65-403) and inserting it into the pGBKT7 plasmid. Plasmid pLexKd containing full-length human kanadaptn cDNA (2.4 kb) in pNLex (expressing the fusion protein, nuclear localizing signal and LexA protein) was constructed by amplifying kanadaptn cDNA and digesting the purified PCR product with *EcoRI* and *SalI*. After digestion the purified fragment was ligated with *EcoRI/SalI*-digested pNLex. Plasmid pGalKd was constructed by digesting pLexKd with *EcoRI* and *SalI*. The kanadaptn cDNA fragment was sub-cloned into *EcoRI/XhoI*-digested pGADT4 (Gal4 binding domain fused to SV40). All recombinant plasmids were verified by DNA sequencing.

### Western blot analysis

Protein expression in yeasts was examined by Western blot analysis. The yeast cells were lysed by resuspending in 1 volume of 2x breaking buffer (50 mM Tris-HCl pH 8.0, 0.1% v/v Triton® X-100 and 0.05% v/v SDS) in the presence of 1 mM PMSF purchased from Sigma Chemical Co. (St. Louis, USA) to prevent protease activities. The protein samples were separated in SDS-PAGE gels and transferred to nitrocellulose membranes. In the Gal4 system, the expression of the Gal4DBD-kAE1 fusion protein



**Figure 2** - Schematic representation of human erythroid and kidney anion exchanger 1 (AE1) protein structure. Human eAE1 is composed of three domains, the cytoplasmic N-terminal domain, the transmembrane domain and the cytoplasmic C-terminal domain. Human kAE1 lacks 65 amino acids present at the N-terminus of eAE1. The region of 66-403 amino acids of the cytoplasmic N-terminal domain of AE1 was fused to either Gal4DBD or B42AD to study their interactions with human kanadaptn in the Gal4 and LexA yeast two-hybrid systems, respectively.

was detected using monoclonal anti-Gal4DBD as primary antibody and HRP-conjugated anti-mouse IgG as secondary antibody. The expression of the Gal4AD-kanadaptn fusion protein was detected using polyclonal anti-human kanadaptn as primary antibody and HRP-conjugated anti-mouse IgG as secondary antibody.

In the LexA system, the expression of the B42AD-kAE1 fusion protein was detected using polyclonal anti-HA-tag as primary antibody and HRP-conjugated anti-rabbit IgG as secondary antibody. The LexA-kanadaptn fusion protein was detected using either polyclonal anti-human kanadaptn or monoclonal anti-LexA as primary antibody followed by adding HRP-conjugated anti-mouse IgG.

The binding of secondary antibody to primary antibody was subsequently detected using the Sigma ECL plus Western Blotting Detection System by incubating for 5 min. To detect chemiluminescence the membrane was exposed to X-ray film in a cassette for 30 min and the film developed normally.

### Analysis of protein-protein interaction using the Gal4 yeast two-hybrid system

To study kAE1 and kanadaptn interaction, yeast strain AH109 containing pkAE1 was mated with yeast strain Y187 containing pGalKd or the plasmids pGalKd and pGalkAE1 were co-transformed into yeast strain AH109. Transformants were selected on *glu/trp<sup>-</sup>leu<sup>-</sup>* dropout plates. The interaction between the two proteins was determined by activation of three reporter genes, *HIS3*, *ADE2* and *lacZ* on *glu/trp<sup>-</sup>leu<sup>-</sup>his<sup>-</sup>ade<sup>-</sup>* dropout plates and by assaying the activity of  $\beta$ -galactosidase in the transformant yeasts.

### Analysis of protein-protein interaction using the LexA yeast two-hybrid system

The interaction between kAE1 and kanadaptn was also examined by mating yeast strain RFY231 containing pkAE1M0 with yeast strain RFY206 containing pBDKdM0 and pSH18-34 (the *lacZ* reporter) or by co-transformation of the plasmids pADkAE1 and pLexKd into yeast strain RFY231 containing pSH18-34. Transformants were selected on *glu/ura<sup>-</sup>his<sup>-</sup>trp<sup>-</sup>* dropout plates. The interaction between the two proteins was determined by activation of two reporter genes, *LEU2* and *lacZ* on *gal/raf/ura<sup>-</sup>his<sup>-</sup>trp<sup>-</sup>leu<sup>-</sup>*, *glu/ura<sup>-</sup>his<sup>-</sup>trp<sup>-</sup>leu<sup>-</sup>*, *gal/raf/ura<sup>-</sup>his<sup>-</sup>trp<sup>-</sup>* X-gal and *glu/ura<sup>-</sup>his<sup>-</sup>trp<sup>-</sup>* X-gal plates. The activity of  $\beta$ -galactosidase in the transformant yeasts containing both recombinant plasmids was also determined.

### Assay of $\beta$ -galactosidase activity

To measure  $\beta$ -galactosidase activity, a single yeast colony was grown in 10 mL glucose dropout medium suitable for each yeast transformant. The cultures were incubated at 30 °C with shaking (~250 rpm) for 16 h and then transferred to 40 mL of the same dropout medium and incu-

bated at 30 °C with shaking (~250 rpm) until the culture reached an optical density (OD) of ~ 0.7 at 600 nm. The cells were washed twice with 10 mL of sterile water to prevent nutrient carry-over from the glucose medium and then transferred to 50 mL of galactose/raffinose dropout medium and incubated at 30 °C with shaking (~250 rpm) for 2 days to induce expression of the protein. The reaction tube was set up with a known volume ( $v$ ) of cell suspension diluted to 1 mL with Z buffer (60 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 40 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 10 mM KCl, 1 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 50 mM 2-mercaptoethanol). A drop of 0.1% of SDS and two drops of chloroform were added to each reaction mixture, which was then heated at 30 °C in a water bath for 15 min before adding 0.2 mL of 4 mg  $\text{mL}^{-1}$  ONPG. Beta-galactosidase activity was detected with *o*-nitrophenol at 420 nm (Miller, 1972).

## Results

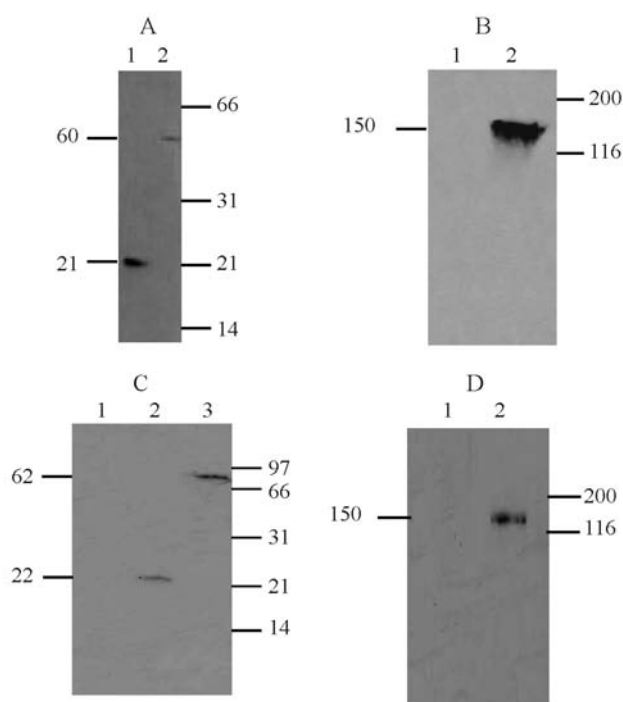
### Expression of the fusion proteins in yeast

In the Gal4 system, the expression of Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins in yeasts were tested by Western blot analysis using polyclonal anti-Gal4DBD and anti-human kanadaptin as primary antibodies, respectively. As expected, the Gal4DBD-kAE1 fusion protein was 60 kDa (Figure 3A). The Gal4AD-kanadaptin fusion protein was expressed and detected by anti-human kanadaptin (Figure 3B) and was found to have a molecular weight of 150 kDa which was greater than the expected 119 kDa, possibly due to post-translational modification such as glycosylation of the fusion protein in the yeast.

In the LexA system, the B42AD-kAE1 fusion protein was found to be 62 kDa as expected (Figure 3C) after detection using polyclonal anti-HA. The LexA-kanadaptin fusion protein was found to be expressed after detection by either polyclonal anti-human kanadaptin (Figure 3D) or monoclonal anti-LexA as primary antibody (data not shown). The fusion protein had a molecular weight of 150 kDa, which was greater than the expected 110 kDa, possibly due to the same reasons as suggested above for the Gal4AD-kanadaptin fusion product.

### Interaction between human kanadaptin and kAE1 in the Gal4 system

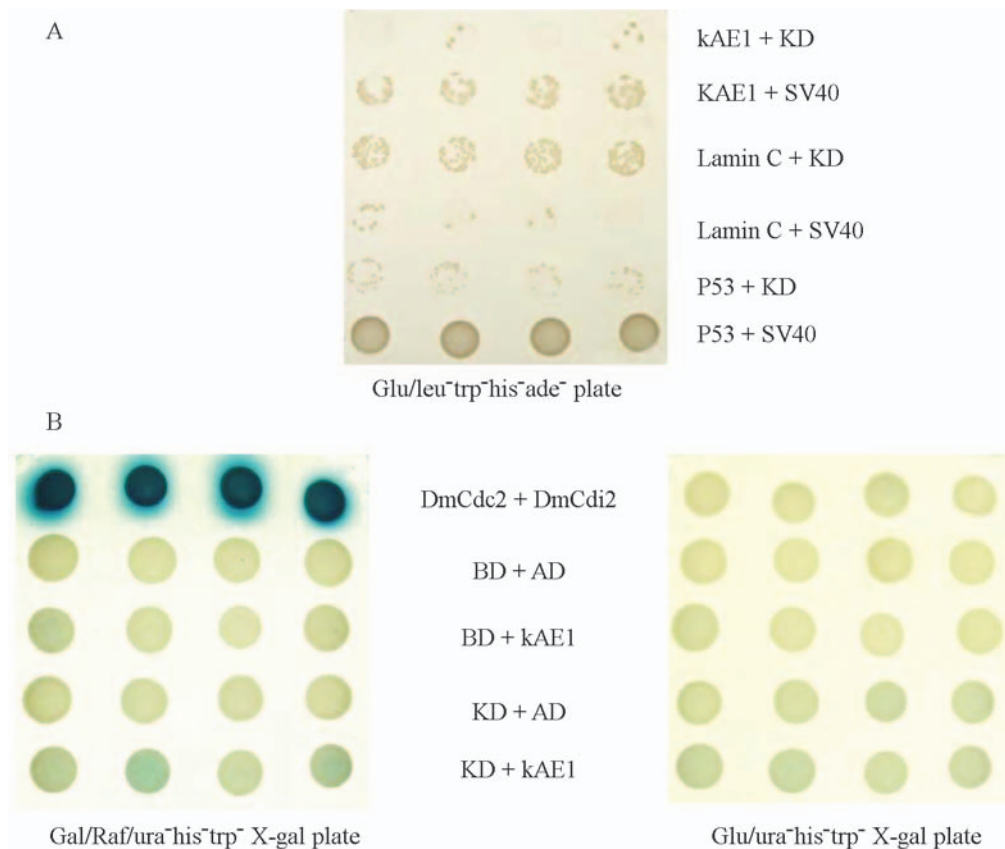
To make sure that both kAE1 and kanadaptin fusion did not activate the reporter gene by themselves, we tested for auto-activation and found that neither Gal4DBD-kAE1 nor Gal4AD-kanadaptin auto-activated the reporter genes (data not shown). The interaction was then examined by mating yeast cells expressing Gal4DBD-kAE1 with yeast cells expressing Gal4AD-kanadaptin. These yeast cells could not grow on dropout plates lacking histidine and adenine (Figure 4A), indicating that the Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins could not interact to activate *HIS3* and *ADE2* genes in the yeast cells. Further-



**Figure 3** - Western blot analysis of fusion protein expression in yeasts. (A) Gal4DBD-kAE1 expression was detected with mouse monoclonal antibody against Gal4DBD. Lane 1 is the crude extract of AH109 containing pGBKT7 and lane 2 the crude extract of AH109 containing pGalkAE1. The Gal4DBD and Gal4DBD-kAE1 fusion proteins are expressed at 21 kDa (lane 1) and 60 kDa (lane 2), respectively. (B) Gal4AD-kanadaptin expression was detected with mouse polyclonal anti-human kanadaptin antibody. Lane 1 is a crude extract of Y187 and lane 2 is a crude extract of Y187 transformed with pGalKd. The size of the Gal4AD-kanadaptin fusion protein is about 150 kDa. (C) The expression of B42AD-kAE1 was detected with rabbit polyclonal anti-HA antibody. Lane 1 is a crude extract of RFY231, lane 2 is a crude extract of RFY231 containing pJZ4 and lane 3 is a crude extract of RFY231 containing pADkAE1. The B42AD-kAE1 fusion protein is 62 kDa in size. (D) The expression of LexA-kanadaptin was detected with mouse polyclonal anti-human kanadaptin antibody. Lane 1 is a crude extract of RFY206-*lacZ* and lane 2 is a crude extract of RFY206-*lacZ* transformed with pLexKd. The LexA-kanadaptin fusion protein is expressed at the size of about 150 kDa.

more, the result of the enzyme assay showed that  $\beta$ -galactosidase activity in the transformants expressing Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins were not significantly different from that of the negative controls (Figure 5A, Table 1). This finding indicates that the Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins expressed in yeast might not interact with one another and thereby not activating the *lacZ* reporter gene.

The interaction between the Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins was also investigated by co-transformation in yeast cells. The fusion proteins showed no auto-activation of the two reporters *HIS3* and *ADE2*. Yeast cells expressing both the Gal4DBD-kAE1 and Gal4AD-kanadaptin fusion proteins could not grow on dropout plates lacking histidine and adenine (data not shown), indicating that no interaction between these two fusion proteins occurred.



**Figure 4** - Analyses of fusion protein interactions after mating two yeast transformants. (A) Gal4 yeast two-hybrid. Yeast cells AH109 containing pGalkAE1 were mated with yeast cells Y187 containing pGalKd. Mating transformants expressing p53 and SV40 large T antigen were used as positive controls and mating transformants expressing lamin C and SV40 large T antigen were used as negative controls. Each spot represents an individual diploid after mating. Yeast transformants grew at 30 °C for 4 days on glu/leu<sup>-</sup> trp<sup>-</sup> his<sup>-</sup> ade<sup>-</sup> dropout plates. (B) LexA yeast two-hybrid. Yeast cells RFY231 containing pADkAE1 were mated with yeast cells RFY206-*lacZ* containing pLexKd. Yeast cells expressing DmCdc2 and DmCdi2 were used as positive controls and yeast cells expressing BD and AD were used as negative controls. Each spot represents an individual diploid after mating. Yeast transformants grew at 30 °C for 4 days on the dropout plates as indicated.

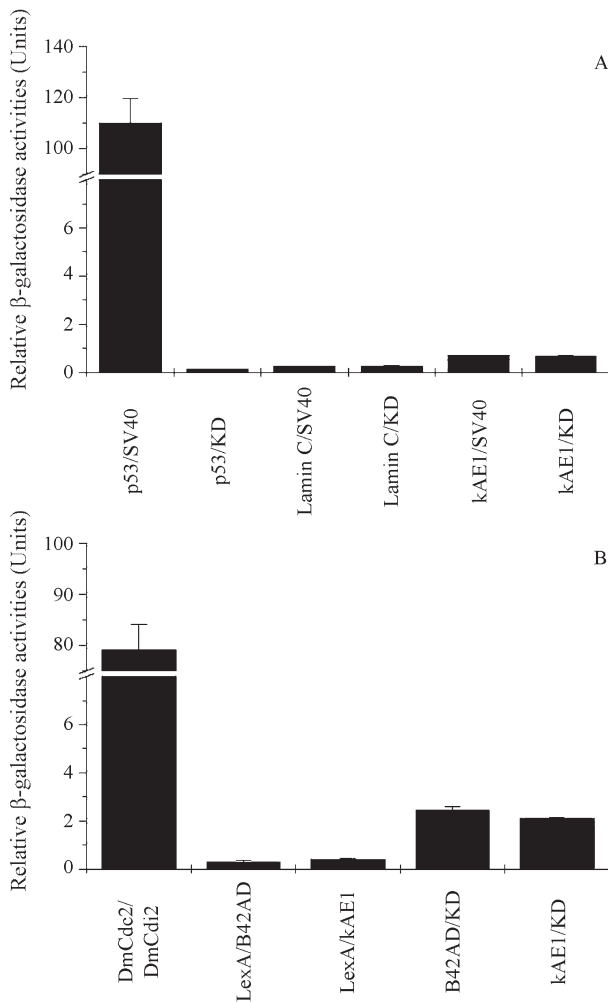
### Interaction between human kanadaplin and kAE1 in the LexA system

The B42AD-kAE1 and LexA-kanadaplin fusion proteins were tested for auto-activation of the two reporter genes *LEU2* and *lacZ* before performing the mating assay. The result showed that B42AD-kAE1 did not activate the *lacZ* reporter gene but LexA-kanadaplin weakly activated transcription of this gene (data not shown). To test the interaction by mating assay, yeast cells expressing B42AD-kAE1 fusion protein were mated with yeast cells expressing LexA-kanadaplin fusion protein. The result showed that these yeast cells gave faint blue colonies on the plates containing X-gal in both gal/raf and glu dropout media (Figure 4B). This might have been the result of weak auto-activation of the reporter gene by LexA-kanadaplin. The activity of  $\beta$ -galactosidase for the transformants expressing B42AD-kAE1 and LexA-kanadaplin fusion proteins were not significantly different from that of the clones expressing LexA and B42AD-kAE1, and LexA-kanadaplin and B42AD, respectively (Figure 5B, Table 1)

**Table 1** - Gal4 and LexA yeast two-hybrid  $\beta$ -galactosidase activity assays. Two separate colonies from each clone were assayed and each colony was independently assayed in triplicate (N = 6).

System	Yeast transformants	Units of $\beta$ -galactosidase activity $\pm$ SEM <sup>1</sup>
Gal4 yeast two-hybrid	p53 + SV40	109.96 $\pm$ 9.51
	p53 + KD	0.14 $\pm$ 0.00
	Lamin C + SV40	0.23 $\pm$ 0.01
	Lamin C + KD	0.25 $\pm$ 0.02
	kAE1 + SV40	0.69 $\pm$ 0.02
	kAE1 + KD	0.68 $\pm$ 0.01
LexA yeast two-hybrid	DmCdc2 + DmCdi2	79.18 $\pm$ 4.90
	LexA+ B42AD	0.30 $\pm$ 0.04
	LexA + kAE1	0.41 $\pm$ 0.04
	B42AD+ KD	2.45 $\pm$ 0.14
	kAE1+ KD	2.10 $\pm$ 0.05

<sup>1</sup>Standard error of the mean.



**Figure 5** - Beta-galactosidase activity for determination of fusion protein interactions in yeast-two hybrid studies. The bar graphs illustrate the relative  $\beta$ -galactosidase activities (units) in cell lysates from mating yeast-transformants expressing the different pairs of proteins or fusion proteins as indicated. Two separate colonies from each clone were assayed and each colony was independently assayed in triplicate (N = 6). There was no obvious difference in activity from six independent experiments so no error bar could be shown in the experiments that showed minimal or absent activities. (A) The  $\beta$ -galactosidase assay in the Gal4 yeast two-hybrid system. Transformants expressing p53 and SV40 large T antigen were used as positive controls and transformants expressing lamin C and SV40 large T antigen as negative controls. Transformants expressing different combinations of fusion proteins were also used as additional controls. (B) The  $\beta$ -galactosidase assay in the LexA yeast two-hybrid system. Transformants expressing DmCdc2 and DmCdi2 were used as positive controls and transformants expressing BD and AD as negative controls. Transformants expressing different combinations of fusion proteins were also used as additional controls.

In the co-transformation assay, B42AD-kAE1 and LexA-kanadaptn fusion proteins were also tested for auto-activation with the two reporters *LEU2* and *lacZ* before performing the interaction. The result showed that transformants containing B42AD-kAE1 showed no auto-activation of the *LEU2* and *lacZ* reporter genes while the transformants containing LexA-kanadaptn could grow on media lacking leucine and gave faint blue colonies on X-gal

plates as compared to a negative control (data not shown). Yeast cells expressing B42AD-kAE1 and LexA-kanadaptn grew on dropout plates lacking leucine and gave faint blue colonies on the X-gal plates. However, the  $\beta$ -galactosidase assay showed that the enzyme activity of the transformants expressing B42AD-kAE1 and LexA-kanadaptn were not significantly different from those of negative controls (data not shown).

## Discussion

In this study, we used the Gal4 and LexA yeast two-hybrid systems to investigate whether the cytoplasmic N-terminal domain of human kAE1 can physically interact with human kanadaptn. By using these yeast two-hybrid systems in conjunction with mating and co-transformation procedures, we found that the cytoplasmic N-terminal domain of human kAE1 could not interact with human kanadaptn because none of the reporter genes were activated. In a different study by our group using cultured human embryonic kidney (HEK293) cells, we have also observed that human kAE1 and kanadaptn co-expressed in these cells were located at different sites when detected by immunofluorescence staining (Kittanakom S. *et al.*, 2004b). kAE1 was present at the plasma membrane and cytoplasm whereas kanadaptn was localized predominantly in the nucleus. In addition, no interaction between kAE1 (or eAE1) and kanadaptn expressed in HEK293 cells could be demonstrated either by co-immunoprecipitation or 6xHis-tagged co-purification methods. Thus, in contrast to data from mouse kanadaptn, human kanadaptn may not interact with kAE1 in human kidney cells.

Different kAE1 and kanadaptn structures occur in humans and mice and these may affect their ability to bind to other proteins. Comparison between human and mouse cytoplasmic N-terminal domains of kAE1 showed about 25% difference in their amino acid sequences. As compared to mouse kanadaptn, human kanadaptn contains an additional 262 N-terminal amino acids and a 28 amino acid insertion near the C-terminus. Since most of the human kanadaptn appears to be highly homologous to murine kanadaptn, it is possible that the additional 262 amino acids found at the N-terminus of human kanadaptn that are absent from mouse kanadaptn may occlude or interfere with binding to the human kAE1 N-terminus. The detailed analysis of amino acids 1 to 262 of human kanadaptn using the simple modular architecture research tool (SMART, Schultz *et al.*, 1998; Schultz *et al.*, 2000) showed that this region contains a forkhead-associated (FHA) domain between amino acid residues 188 and 249 (Figure 1). The FHA domain is a multifunctional phosphopeptide-binding module that has been found in a wide variety of proteins from both prokaryotes and eukaryotes (Hofmann and Bucher, 1995; Sun *et al.*, 1998; Durocher *et al.*, 1999; Li *et al.*, 1999; Liao *et al.*, 1999; Hammet *et al.*, 2000) and was

first identified in a group of forkhead transcription factors (Hofmann and Bucher, 1995) and has since been identified in many signaling proteins, including protein kinases, protein phosphatases, proteases, kinesins and zinc finger proteins in both yeasts and animals (Schultz *et al.*, 2000). The Chk2-like cell cycle checkpoint protein kinases play crucial roles in cellular damage and replication blocks and are characterized by the presence of FHA domain (Matsuoka *et al.*, 1998). The existence of FHA domain suggests that human kanadaplin may be involved in other, different, cellular processes.

Kanadaplin is widely expressed in many mouse tissues such as kidney, lung, liver, brain, testis, heart, and skeletal muscle (Chen *et al.*, 1998) but not in the  $\alpha$ -intercalated cells of rat and rabbit kidney (Hubner *et al.*, 2003), while kAE1 only expresses in kidney  $\alpha$ -intercalated cells. Kanadaplin is a multi-domain structure containing three nuclear localizing sequences (NLS) involved in translocation of this protein into the nucleus of mammalian cells (Hubner *et al.*, 2003), indicating that this protein may have a signaling role in the nucleus. Furthermore, immunofluorescence staining has revealed strong kanadaplin signals in association with the mitochondria of several cell types such as proximal and distal tubule cells, cerebellar cortex cells, enterocytes, hepatocytes and pancreatic acinar cells (Hubner *et al.*, 2003). From these findings and the results of our yeast two-hybrid and eukaryotic expression studies, it is therefore unlikely that human kanadaplin is involved in targeting kAE1 to the plasma membrane and is also unlikely that defects in kanadaplin and its interaction with kAE1 results in distal renal tubular acidosis (dRTA).

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