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

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Abstract

Kidney anion exchanger adaptor protein (Kanadaptin) 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 α-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 kanadaptin may cause this trafficking impairment and be the basis for dRTA pathogenesis. However, it is unknown whether kAE1 can really interact with kanadaptin in humans. We have thus investigated the interaction between human kAE1 and human kanadaptin 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 kanadaptin could not activate the transcription of the AD2, 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 kanadaptin 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 kanadaptin in human cells. Considerably different structures of both kAE1 and kanadaptin in mice and humans may lead to different binding properties of the proteins in these two species.

Key words: kidney anion exchanger 1, kanadaptin, 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 Cl-/HCO3- across the plasma membrane and cytoskeletal anchorage (Showe et al., 1987). Kidney AE1 is located on the basolateral membrane of acid-secreting α-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 μ1B subunit have indicated that the μ1B 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 (kanadaptin) was first isolated from mouse 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 kanadaptin was identified (Chen et al., 1998). Mouse kanadaptin 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 kanadaptin 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 α-intercalated cells (Chen et al., 1998). This suggests that kanadaptin might play a role in the targeting of kAE1-containing vesicles to basolateral membrane of α-intercalated cells. However, immunofluorescence and subcellular fractionation studies have demonstrated that kanadaptin is localized within the nuclei of various epithelial and non-epithelial cultured cell types (Hubner et al., 2002) and immuno-staining revealed strong kanadaptin 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 kanadaptin cDNA (AY028435) was cloned and characterized by our group. The human kanadaptin protein contains 796 amino acids, has 262 amino acids longer than mouse kanadaptin 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 kanadaptin in human cells has not yet been demonstrated but the evidence from mouse and rabbit cells indicate that human kanadaptin 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 kanadaptin may lead to impaired trafficking of kAE1 to the basolateral membrane of the α-intercalated cells where it mediates Cl-/HCO3− 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 produces different results because the different fusion proteins may affect protein folding and interfere with the protein-protein interaction.

**Figure 1** - Differences in structure between human and mouse kanadaptin. The upper picture shows human kanadaptin and the lower picture mouse kanadaptin. Human kanadaptin has 262 amino acids more than mouse kanadaptin 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 kanadaptin. DSRM = double-stranded RNA-binding motif, E motif = glutamic acid motif and SH3 = Src homology domain 3.
interaction (Crickinge 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 kanadaptin. In the Gal4 system, Gal4DBD was fused to kAE1 and Gal4AD to kanadaptin, while in the LexA system, B42AD was fused to kAE1 and LexA to kanadaptin. In both systems, the interactions were studied by mating and co-transformation procedures.

Materials and Methods

Materials

The yeast, *Saccharomyces cerevisiae*, strains AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1US::GAL1TATA-HIS3, GAL2US::GAL2TATA::ADE2 URA3::MEL1US::MEL1TATA -lacZ MEL1), and Y187 (MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1US::GAL1TATA-lacZ MEL1), the plasmid pGBK7, 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 (MATa ura3-1 his3 trp1Δ:: hisG 3LexAop-LEU2:::leu2), and RFY206 (MATa his3Δ200 leu2-3 lys2Δ201 ura3-52 trp1ΔhisG) and the plasmid pNLex and pIZ4 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 DH5α (supE44 ΔlacZΔM15 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA46 thi-1 relA1) from a stock culture maintained in our laboratory. Affinity-purification used ProBond™ Ni²⁺ beads from Invitrogen (Calsbad, USA). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from Roth (Karlsruhe, Germany) and O-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma Chemical Co. (St. Louis, USA).

Cloning of human kanadaptin cDNA

Human kanadaptin 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® reagent (Life Technologies, Gaithersburg, MD, USA). The full-length human cDNAs were synthesized using the oligo(dt) 12-18 primer and SUPERSCRIBTM Pre-amplification System for First Strand cDNA Synthesis (Life Technologies). Human kanadaptin primers for two-step (nested) PCRs were designed from human expressed sequence tags (ESTs) searched by using the mouse kanadaptin mRNA sequence. The PCR was performed in a GeneAmp 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 kanadaptin sequence was deposited in GenBank (AY028435). The isolated human kanadaptin cDNA was cloned into the Xhol and EcoRI sites of prokaryotic (pTrcHisA) vector and transformed into *E. coli* cells strain DH5α. The recombinant plasmids were screened by plasmid mini-preparation and restriction endonuclease digestion. Human kanadaptin protein was expressed in *E. coli* and purified for polyclonal antibody production.

Production of mouse polyclonal anti-human kanadaptin antibody

Mouse polyclonal anti-sera directed against human kanadaptin was generated using 6xHis-kanadaptin fusion protein as an immunogen. The recombinant human kanadaptin 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-kanadaptin fusion protein was induced by adding 0.25 mM isopropyl-1-thio-β-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™ Ni²⁺ beads was used to purify 6xHis-kanadaptin eluted with 100 mM imidazole. To generate polyclonal antibody against human kanadaptin, we immunized mice with affinity-purified 6xHis-kanadaptin. In the first immunization, 6xHis-kanadaptin (20-60 μg protein) was combined with equal volume of complete Freund’s adjuvant and then subcutaneously injected into mice. After 2 weeks, anti-kanadaptin antibody was raised and the antibody titer was checked by dot-blot enzyme immunoassay. Mice were boosted for two weeks with 6xHis-kanadaptin 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-
trose 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 pGBK7 plasmid. Plasmid pLexKd containing full-length human kanadaptin cDNA (2.4 kb) in pNLex (expressing the fusion protein, nuclear localizing signal and LexA protein) was constructed by amplifying kanadaptin cDNA and digesting the purified PCR product with EcoRI and SalI. After digestion the purified fragment was ligated with EcoRI/SalI-digested pNLex. Plasmid pGalkKd was constructed by digesting pLexKd with EcoRI and SalI. The kanadaptin 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 was detected using monoclonal anti-Gal4DBD as primary antibody and HRP-conjugated anti-mouse IgG as secondary antibody. The expression of the Gal4AD-kanadaptin fusion protein was detected using polyclonal anti-human kanadaptin 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-kanadaptin fusion protein was detected using either polyclonal anti-human kanadaptin 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 kanadaptin interaction, yeast strain AH109 containing pkAE1 was mated with yeast strain Y187 containing pGalkKd or the plasmids pGalkKd and pGalkAE1 were co-transformed into yeast strain AH109. Transformants were selected on glu/tp'r'-leu' dropout plates. The interaction between the two proteins was determined by activation of three reporter genes, HIS3, ADE2 and lacZ on glu/tp'r'-leu'his'ade' dropout plates and by assaying the activity of β-galactosidase in the transformant yeasts.

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

The interaction between kAE1 and kanadaptin was also examined by mating yeast strain RFY231 containing pKAEM0 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/his'tp'r' dropout plates. The interaction between the two proteins was determined by activation of two reporter genes, LEU2 and lacZ on gal/raf'/ura'his'tp'r'leu', glu/ura'his'tp'r'leu', gal/raf'/ura'his'tp'r' X-gal and glu/ura'his'tp'r' X-gal plates. The activity of β-galactosidase in the transformant yeasts containing both recombinant plasmids was also determined.

Assay of β-galactosidase activity

To measure β-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 Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄·H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 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 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. Furthermore, the result of the enzyme assay showed that β-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.
Interaction between human kanadaptin and kAE1 in the LexA system

The B42AD-kAE1 and LexA-kanadaptin 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-kanadaptin 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-kanadaptin 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-kanadaptin.

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 ± SEM$^1$ |
|-------------------------------|--------------------|-----------------------------------------------|
| Gal4 yeast two-hybrid         |                    |                                               |
| p53 + SV40                    | 109.96 ± 9.51      |                                               |
| p53 + KD                      | 0.14 ± 0.00        |                                               |
| Lamin C + SV40                | 0.23 ± 0.01        |                                               |
| Lamin C + KD                  | 0.25 ± 0.02        |                                               |
| kAE1 + SV40                   | 0.69 ± 0.02        |                                               |
| kAE1 + KD                     | 0.68 ± 0.01        |                                               |
| LexA yeast two-hybrid         |                    |                                               |
| DmCdc2 + DmCdi2               | 79.18 ± 4.90       |                                               |
| LexA + B42AD                  | 0.30 ± 0.04        |                                               |
| LexA + kAE1                   | 0.41 ± 0.04        |                                               |
| B42AD + KD                    | 2.45 ± 0.14        |                                               |
| kAE1 + KD                     | 2.10 ± 0.05        |                                               |

$^1$Standard error of the mean.
In the co-transformation assay, B42AD-kAE1 and LexA-kanadaptin fusion proteins were also tested for auto-activation with the two reporters \( \text{LEU2} \) and \( \text{lacZ} \) before performing the interaction. The result showed that transformants containing B42AD-kAE1 showed no auto-activation of the \( \text{LEU2} \) and \( \text{lacZ} \) reporter genes while the transformants containing LexA-kanadaptin 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-kanadaptin 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-kanadaptin 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 kanadaptin. 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 kanadaptin 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 kanadaptin 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 kanadaptin was localized predominantly in the nucleus. In addition, no interaction between kAE1 (or eAE1) and kanadaptin expressed in HEK293 cells could be demonstrated either by co-immunoprecipitation or 6xHis-tagged co-purification methods. Thus, in contrast to data from mouse kanadaptin, human kanadaptin may not interact with kAE1 in human kidney cells.

Different kAE1 and kanadaptin 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 kanadaptin, human kanadaptin contains an additional 262 N-terminal amino acids and a 28 amino acid insertion near the C-terminus. Since most of the human kanadaptin appears to be highly homologous to murine kanadaptin, it is possible that the additional 262 amino acids found at the N-terminus of human kanadaptin that are absent from mouse kanadaptin may occlude or interfere with binding to the human kAE1 N-terminus. The detailed analysis of amino acids 1 to 262 of human kanadaptin 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 kanadaptin may be involved in other, different, cellular processes.

Kanadaptin 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 α-intercalated cells of rat and rabbit kidney (Hubner et al., 2003), while kAE1 only expresses in kidney α-intercalated cells. Kanadaptin 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 kanadaptin 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 kanadaptin is involved in targeting kAE1 to the plasma membrane and is also unlikely that defects in kanadaptin and its interaction with kAE1 results in distal renal tubular acidosis (dRTA).

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