Identification of the Na/K-ATPase Interacting Proteins

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Identification of the Na,K-ATPase Interacting Proteins

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DEDICATION

I dedicate this manuscript to my parents, Gongliang Jing and Xiuying Wang; my wife, Xing Li; and all of my family for their unconditional and loving support throughout my life.
ACKNOWLEDGEMENTS

It is an honor to acknowledge Dr. Zijian Xie, my major advisor, for his knowledge, patience, encouragement and assistance in designing, developing and monitoring this research study.

I would like to extend my sincere gratitude to my committee members, Dr. Jiang Liu and Dr. David R. Giovannucci for their advice and encouragement during my research.

Many thanks for Dr. Hongjuan Cui for her scientific expertise, sincere friendship, and supports and to Zhichuan Li for his help analysing positive library colonies.

I want to thank all of the people who work in Dr Xie’s lab for their friendship, patience, and understanding.

Finally, I would like to thank all of the faculty and staff at the Medical University of Ohio for making this possible.
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INTRODUCTION

The work of the past few years, built on the foundation of a number of excellent studies of the past 3 decades, has clearly shown that in addition to pumping Na\(^+\) and K\(^+\), the Na,K-ATPase also functions as a signal transducer to relay messages from the plasma membrane to the intracellular organelles through stimulus-induced protein-protein interactions involving the Na,K-ATPase, the neighboring plasma membrane proteins, and the organized cytoplasmic protein assemblies (Peng et al., 1996; Huang et al., 1997a, b; Xie et al., 1999; Hass et al., 2000; Liu et al., 2000). Specifically, binding of ouabain to the Na,K-ATPase activates Src, resulting in the transactivation of the EGFR and subsequent phosphorylation of Shc and activation of Ras/Raf/ERK\(_{1/2}\) cascade (Hass et al., 2000).

Recent studies (J. Tian and Z. Xie, unpublished results) showed that Src interacts with Na,K-ATPase directly, and serves as a direct effector of the signaling pump to transmit extracellular ouabain signal to different intracellular compartments. A GST-based pull down assay revealed that the nucleotide-binding domain 1 (ND1) of the sodium pump \(\alpha_1\) subunit is involved in interaction with the kinase domain of Src and plays an important role in regulation of Src kinase activity. Because there is evidence that the ND1 is highly exposed and may interact with other signaling proteins, I am interested in establishing an assay to identify the potential ND1-interacting candidates so that others can investigate the role of the ND1 in ouabain-activated signal transduction.
Sodium Pump (Na,K-ATPase)

The Na,K-ATPase (sodium pump) is a family II P-type ATPase found in the plasma membrane of most animal cells (Skou and Esmann, 1992; Lingrel and Kuntzweiler, 1994). It pumps three sodium ions out in exchange for two potassium ions inside by hydrolyzing one molecule of ATP. This transportation results in low intracellular Na\(^+\) and high intracellular K\(^+\) concentration (Skou and Esmann, 1992). The electrochemical gradient thus generated is critical in maintaining the osmotic balance of the cell, the resting membrane potential in most tissues, and the excitability of nerve and muscle cells (Lingrel and Kuntzweiler, 1994). The sodium gradient across the membrane also helps in the secondary transport of nutrients (e.g., glucose and amino acids), ions (e.g. H\(^+\), Ca\(^{2+}\), Cl\(^-\), O\(_4\)^{2-}, SO\(_4\)^{2-}) and neurotransmitters (Skou, 1990; Skou and Esmann, 1992).

The sodium pump is an oligomer made up of two non-covalently linked polypeptides: \(\alpha\) and \(\beta\) subunits. The \(\alpha\) subunit is a larger protein (112 kDa) that plays a major role in catalytic function of the enzyme. It contains an ATP binding site, phosphorylation site, and domains essential for binding of cations and cardiac glycosides. The \(\beta\) subunit is a glycosylated 55 kDa protein that is necessary for the correct assembly, proper conformation and normal activity of the sodium pump (Mercer, 1993). Lately, a third 8-14 kDa \(\gamma\) subunit also has been identified in kidney. This subunit is supposed to modify the function of the pump (Aystarkhova et al., 1999).
**Na,K-ATPase as a Signal Transducer**

In mid-1990s, our laboratories became interested in the possible role of Na,K-ATPase in the nonproliferative growth (hypertrophy) of the heart. Using the cultured cardiac myocyte as a model, our studies of the past few years (Peng et al., 1996; Huang et al., 1997a, b; Kometiani et al., 1998; Xie et al., 1999) have clearly indicated that the same nontoxic concentrations of ouabain that cause partial inhibition of Na,K-ATPase and an increase in cardiac contractility, also stimulate myocyte growth and protein synthesis, induce a number of early response proto-oncogenes, activate transcription factors activator protein 1 (AP-1) and NF-κB, and induce or repress the transcription of several late-response cardiac marker genes that are also regulated by other cardiac hypertrophic stimuli. These findings clearly establish that Na,K-ATPase indeed regulates the growth and the phenotype of the cardiac myocyte and opened a magnificent era of studying the cross-talk between signal transduction and pumping functions of Na,K-ATPase.

Significantly, recent studies have provided direct evidence that the sodium pump possesses an ion-pumping independent signaling function. While inhibiting the ion transporting function of Na,K-ATPase, paradoxically, ouabain activates the signal transduction function of the same enzyme. Upon ouabain binding, Na,K-ATPase interacts with membrane proteins in proximity as well as various cytosolic proteins to organize cascades of signaling complexes to transduce ouabain signal into various intracellular organelles including nucleus and mitochondria. In several different cell lines, the most proximal reaction after ouabain binds to the enzyme involves the activation and recruitment of Src to the Na,K-ATPase signaling complex. This results in the
transactivation of the epidermal growth factor receptor (EGFR). The down-stream events include the activation of p42/44, p38 MAP Kinases, PKC and increase in the intracellular calcium concentration and cardiac contractility, induction of some early response genes and activation of transcription factors AP-1 and NF-\(\kappa\)B. In cardiac myocytes, these changes eventually lead to enhanced expression of a variety of cardiac growth-related genes such as ANF and skACT, and the stimulation of protein synthesis resulting in myocyte hypertrophy.

Since many of the signaling events are activated by ouabain in the absence of changes in intracellular ion concentrations (Xie, 2001), we proposed that Na,K-ATPase, when inhibited by ouabain, recruits multiple proteins to form different signaling modules, such as intracellular calcium regulatory module in cardiac myocytes as well as in non-cardiac cells. These signal transduction functions of Na,K-ATPase are mediated by the interactions of Na,K-ATPase and other signaling proteins. There is a long history of the functional interactions of the Na,K-ATPase with intracellular soluble enzymes and other membrane proteins. Recent studies have documented that several functional domains are involved in the binding of the enzyme to PKC, PKA, PI-3 kinase, and cytoskeletal proteins such as ankyrin, actin, and spectrin.

There are multiple lines of evidence suggesting that the overall structure of the Na,K-ATPase is similar to that of SERCA. The crystal structure of SERCA 1a has been determined at 2.6 Å resolution in 2000 (Toyoshima et al., 2000). SERCA consists of four distinct functional domains. The A (actuator) domain contains the N-terminus and the
first cytoplasmic loop that connects to transmembrane helices M2 and M3. The central loop (CD3) of the α subunit contains the highly conserved P (phosphorylation) domain and the nucleotide-binding domain (ND) of the sodium pump α₁ subunit. The phosphorylation domain is buried close to the membrane whereas the N domain is highly exposed and has been shown to interact with other proteins (Zhang et al., 1998; Stoke et al., 1999; Toyoshima et al., 2000). Our lab has demonstrated that the signaling Na,K-ATPase forms a complex with Src. Binding of ouabain to this complex resulted in the activation of Src, which subsequently phosphorylates the effectors that either associate with or close to the signaling sodium pump. GST pull down assay showed that the interaction between the sodium pump and Src involves the kinase and SH2 domains of Src. We also found that the ND1 of the sodium pump α₁ subunit binds specifically to the kinase domain of Src and that ouabain binding to the sodium pump releases the kinase domain from the ND1 domain, resulting in Src activation and the subsequent signal amplification. These findings, taken together with the prior data from other laboratories, led us to propose that the ND1 plays an important role in the mediation of protein interaction, thus ouabain-provoked signal transduction. Because protein interaction is essential for the sodium pump to function as a signaling receptor, it is important to know which proteins interact with the ND1 of the sodium pump. Therefore, the aim of my research is to develop a functional assay so that we can identify potential interacting partners of the ND1. This information will help others in the lab to further deduce the signaling function of this important domain.
Tools for the Study of Protein-protein Interactions

Protein-protein interactions are an intrinsic part of nearly every cellular process. Thus, a practical strategy for studying the function of a protein of interest is to identify other proteins that interact with it. This may lead to the isolation of new components participating in the same pathway or the identification of previously characterized factors that can help elucidate the function of the protein under study. There are a number of methods available to study protein–protein interactions. Usually, a combination of techniques is necessary to validate, characterize and confirm protein interactions. Each technique has its particular strengths and weaknesses; these techniques are summarized briefly as listed in Table I.

Protein–protein interactions can be analyzed using traditional biochemical techniques like affinity and molecular size-based chromatography, affinity blotting, and immunoprecipitation (Phizicky and Fields, 1995). As the reagents used in these methods are very specific in nature, the results obtained are quite convincing in most cases. However, these traditional tools fail to analyze large-scale protein interaction networks efficiently, which is a necessity when one tries to analyze various types of protein–protein interactions occurring in an organism throughout the life cycle. In fact, the publication of the draft sequence of the human genome and proteomics-based protein profiling studies catalyzed resurgence in protein interaction analysis. Many relatively new technologies like yeast two-hybrid (Y2H) assay system, large-scale immunoprecipitation or glutathione S-transferase pull-down to purify the protein complexes followed by mass spectrometry to identify the interacting protein partners are becoming
Table I. Major Methods for Detecting Protein–protein Interactions

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Two-Hybrid System</td>
<td>The yeast two-hybrid system uses the transcription process to make predictions about protein interaction. This method is based on the ability of an interacting protein pair to bring together the DNA binding domain and activation domain of a transcription factor <em>in vivo</em> to produce a functional activator of transcription. The interaction can be detected by expression of the linked reporter genes.</td>
</tr>
<tr>
<td>Co-Immunoprecipitation (Co-IP)</td>
<td>This approach is designed to affinity purify a bait protein antigen together with its binding partner using a specific antibody against the bait. A common obstacle to perform co-immunoprecipitation studies is the availability of immunological reagents directed against the protein(s) of interest.</td>
</tr>
<tr>
<td>Pull-Down Assay</td>
<td>Pull-down assay is an affinity chromatography method that involves using a tagged or labeled bait to create a specific affinity matrix that will enable binding and purification of a prey protein form a lysate sample or other protein-containing mixture. Using this method one can determine whether the target protein co-precipitates with the purified GST-fusion protein bound to glutathione beads. This approach is an <em>in vitro</em> method and is semi-quantitative.</td>
</tr>
<tr>
<td>Surface Plasmon Resonance</td>
<td>Relate binding information to small changes in refractive indices of laser light reflected from gold surfaces to which a bait protein has been attached. Changes are proportional to the extent of binding. Special labels and sample purification are not necessary, and analysis occurs in real time. Surface Plasmon Resonance is a quantitative approach.</td>
</tr>
<tr>
<td>Mass Spectroscopy</td>
<td>Used in concert with affinity-based methods, such as co-IPs, to isolate binding partners and complexes and identify the component proteins using standard mass spectral methods. Mass Spectroscopy allows study of native complexes. This approach can be limited by the abundance of the proteins studied, and often requires that significant amounts of proteins be purified.</td>
</tr>
</tbody>
</table>
increasingly important to characterize and identify a huge number of protein interactions at a time. Among these techniques, Y2H is quite popular to detect pairwise interactions between proteins, since it is an inexpensive and in vivo technique.

**Yeast Two-Hybrid System**

The Y2H system was first described by Fields and Song in 1989. The basic concept emerged from previous experiments on transcription factors which usually contain separable DNA-binding domains (DBD) and transcriptional activation domains (AD). This property can be exploited to detect protein-protein interactions (Fields and Song, 1989; Chien et al., 1991).

The system we chose is based on the Gal promoters in yeast (Figure 1A). Biologically, the Gal promoters are activated by the Gal4 transcription factor, which is composed of a DNA activation domain (DNA-AD) and DNA binding domain (DNA-BD). The principle behind the Y2H technique is that the DNA-AD and DNA-BD can actually be part of separate proteins and still function to activate the Gal4 promoter if brought into the physical vicinity of one another, as with a protein-protein interaction. Traditionally in Y2H system, the gene of interest is expressed as a fusion to DNA-BD vector and a cDNA library is expressed as a fusion to DNA-AD vector. These two plasmids are then cotransformed into a yeast strain that is engineered with the Gal promoter upstream of lacZ and nutritional reporter genes. In yeast colonies where a protein from the library fused to the DNA-AD is interacting with the protein of interest fused to the DNA-BD, the DNA-AD and DNA-BD motifs come together and activate the reporter genes. In our
system, there are not only lacZ, but also ADE2 and HIS3 nutritional reporter genes. Rather than the traditional method of building a cDNA library for cotransformation with the DNA-BD/bait vector, we chose to use a human kidney cDNA library pretransformed into Y187, a MATα yeast strain. This strategy necessitated the development of not only a DNA-BD/bait vector containing our gene of interest, but also a DNA-BD/bait strain to be mated with the library DNA-AD strain (Figure 1B). Thus, unlike traditional systems, our system relies on the ability of the pretransformed DNA-AD/fish strain to mate with the DNA-BD/bait strain.

The advantage of Y2H screening is that it is a eukaryotic in vivo technique. Unlike most of the biochemical approaches where high quantities of purified proteins or good quality antibodies are required, only a bait protein and a prey cDNA library are needed to initiate a screening in Y2H assay. Weak and transient interactions, which are often the most interesting ones in signaling cascades can be detected by this system (Estojak et al., 1995). One of the most appealing features of the system is that the identification of an inter-acting protein implies the cloning of at least part of the corresponding gene, which can be utilized subsequently in many other studies.
Figure 1. Yeast Two Hybrid System

A. Traditional Yeast Two-hybrid Scheme

B. Scheme of Mating Based Yeast Two-hybrid System from Clontech

Construct and test DNA-BD/Bait Vector

Pretransformed Library DNA-AD Vector
MATERIALS

1. All the restriction enzymes used in the studies were purchased from Invitrogen or Promega.

2. All of chemicals were purchase from Sigma.

3. Bacto yeast extract, Bacto Tryptone, Bacto Peptone and granulate agar were obtained from Difco.

4. DH5α E. coli were from Invitrogen.

5. DNA primers used in the studies were obtained from Integrated DNA Technologies.

6. Electrophoresis grade agarose was from Invitrogen.

7. Low melting agarose was purchased from Invitrogen.

8. Match maker 3 yeast two hybrid screen kit, human kidney library, yeast minimal SD base were purchased from Clontech.

9. Mouse anti-Myc antibody was a gift from Dr. Hanfei Ding’s lab; Anti-mouse 2nd antibody was purchased from Sigma.

10. Pfu PCR polymerase was from Stratagene.

11. Pre-stained protein markers were obtained from Bio-Rad.

12. T4 DNA ligase was obtained from Invitrogen.

13. X-α-gal was from Clontech.
METHODS

Yeast Strains and General Yeast Protocols

Media: YPD medium supplemented with adenine (YPDA) was used to grow all yeast strains when nutritional selection was not needed. This media consisted of 20g/L peptone, 10g/L-yeast extract and 20g/L agar if plates were prepared. The pH was adjusted to 5.8 and the solution was autoclaved prior to the addition of 15ml of a sterile 0.2% adenine hemisulfate solution per liter. To test strains for phenotype transformation, nutritional selection was desired and accomplished with the use of synthetic dropout (SD) media supplemented with the appropriate amino acids. SD media consisted of 6.7g/L yeast nitrogen base without amino acids, plus the prescribed amount of dropout adenine supplement powder according to Clontech’s instructions (Table II). The solution pH was adjusted to 5.8 and autoclaved prior to the addition of sterile glucose solution to a final concentration of 2%. Synthetic dropout media included single dropout (Trp-, Leu- or Ura-) for phenotyping haploid transformants, double dropout (DDO= Trp-/Leu-) for selecting mated diploids, and quadruple dropout (QDO=Trp-/Leu-/Ade-/His-) for selecting diploid with a positive protein-protein interaction. All yeast strains were grown at 30 ºC (Table III).
Table II. Different Medias Used for Yeast Two Hybrid System

<table>
<thead>
<tr>
<th>Synthetic Dropout Media</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp-</td>
<td>Select for presence of DNA-BD</td>
</tr>
<tr>
<td>Leu-</td>
<td>Select for presence of DNA-AD</td>
</tr>
<tr>
<td>DDO</td>
<td>Select for mating of DNA-BD &amp; DNA-AD strains</td>
</tr>
<tr>
<td>TDO</td>
<td>Select for mating of DNA-BD &amp; DNA-AD strains &amp; positive yeast 2-hybrid reactions</td>
</tr>
<tr>
<td>QDO</td>
<td>Select for mating of DNA-BD &amp; DNA-AD strains &amp; positive yeast 2-hybrid reactions</td>
</tr>
</tbody>
</table>

Dropout supplements (Clontech) are added at different amounts per liter of synthetic dropout media (SD). Double Dropout = DDO = SD/-Trp/-Leu. Triple Dropout = TDO = SD/-Trp/-Leu/-His. Quadruple Dropout = QDO = SD/-Trp/-Leu/-His/-Ade.

Strains: The Y2H system used in these experiments was the BD Matchmaker Y2H System 3 with a human kidney Matchmaker Pretransformed Library (Clontech). See Table III for parent strains, their use and their nutritional selection markers.

Table III. Genotypes of Parent Strains Used in Yeast two-Hybrid

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reporters</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH109</td>
<td>MATa, Trp1- 901, Leu2- 3, 112, Ura3- 52, His3- 200, gal4Δ, gal80Δ, LYS2 :: GAL1UAS -GAL1TATA -HIS3, GAL2UAS -GAL2TATA -ADE2, URA3 :: MEL1UAS- MEL1TATA -lacZ MEL1</td>
<td>HIS3, ADE2, MEL1, LacZ</td>
<td>Parent strain for DNA-BD/bait strain</td>
</tr>
<tr>
<td>Y187</td>
<td>MATa, Ura3- 52, His3- 200, Ade2- 101, Trp1- 901, Leu2- 3, 112, gal4Δ, gal80Δ, met --, URA3 :: GAL1UAS -GAL1TATA -lacZ MEL1</td>
<td>MEL1, LacZ</td>
<td>Parent strain for DNA-AD/fish strain</td>
</tr>
</tbody>
</table>
Construction of Bait Vectors for Use in Yeast Two-Hybrid Screens

To generate a yeast fusion protein coding for the Gal4 DNA binding domain (DNA-BD) fused to the ND1 domains of sodium pump, pGBKT7 (Clontech) was used as the parent vector. The template used for PCR was pig Na,K-ATPase α1 subunit full length cDNA. A fragment containing 1135 bp to 1312 bp of pig Na,K-ATPase α1 subunit full length cDNA was generated by PCR using primers containing useful restriction site. The primers used in PCR were ND1 sense primer (CCG GAA TTC CTC ACC CAG AAC CGA ATG ACA GTG), and ND1 antisense primer (CGC AGG ATC CTT AGT TTT CCT GGT TGG CCT G). The gene fragment generated by PCR was purified by gel extraction kit (Qiagen). The pGBKT7 vector and ND1 gene fragment were digested by EcoRI and BamHI, and the ND1 insert was ligated into the pGBKT7 vector using T4 ligase (Invitrogen). The ligation mixture was transformed into E.coli. The insert-containing plasmid was identified by restriction analysis.

Generation of Bait Strain AH109[pGBKT7-ND1]

To make competent AH109 yeast cells, 2-3 large colonies from a stock plate were inoculated into 50 ml of YPDA media and grown overnight at 30°C with shaking at 250 rpm until OD600 > 1.5. 30 ml of this solution was diluted to an OD600 of 0.2-0.3 (300 ml) with YPDA and then allowed to grow at 30°C with shaking at 250 rpm until an OD600 of 0.4-0.6 was achieved. Cells were centrifuged at 1,000 x g for 5 min and washed once with 25ml of distilled water. The pellet then was resuspended in 1.5 ml of TE/LiAC (10 mM Tris-HCl, 1mM EDTA, 100 mM lithium acetate, pH 7.5), yielding competent cells. To transform the AH109 yeast strain, 100 μl of competent cells were
mixed with 100ng pGBK7-ND and 100 μg of herring testes carrier DNA. Additionally, 0.6 ml of PEG/LiAC (TE/LiAc with 40% PEG4000) were added, and the cells were incubated at 30°C, with 200 rpm shaking for 30 min. DMSO (70 μl) was added and the cells were gently inverted and then shocked at 42°C for 15 min. The cells were rested on ice for 2 min and then concentrated at 14,000 x g for 5 sec. Then they were resuspended in 500 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and plated on SD-Trp plates to select for transformants.

**Verification of DNA-BD/bait Protein Expression**

One single isolated fresh colony was used to prepare a 5-ml overnight culture in SD selection medium. At the same time a 10-ml culture of an untransformed yeast colony was prepared in YPD medium as a negative control. The overnight cultures were vortexed for 0.5-1 min to disperse cell clumps. For each clone to be assayed (and the negative control), the entire overnight culture was separately inoculated into 50-ml aliquots of YPD medium and incubated at 30°C with shaking (220-250 rpm) until the OD600 reaches 0.4-0.6. Then the culture was quickly chilled by pouring it into a prechilled 100 ml centrifuge tube. Immediately, the tube was placed in a prechilled rotor and was subjected to centrifugation at 1000 x g for 5 min at 4°C. The supernatant was poured off and the cell pellet was resuspended in 50 ml of ice-cold H2O. The pellet was recovered by centrifugation at 1,000 x g for 5 min at 4°C and the cell pellet was frozen by placing the tube on dry ice or in liquid nitrogen. 100 μl of prewarmed cracking buffer was added to per 7.5 OD600 units of cells (For example, 0.44 ml of cracking buffer for 33 OD600 units of cells). The cell pellets were quickly thawed by separately resuspending
each one in the prewarmed cracking buffer. Each cell suspension was transferred to a 1.5-
ml screw-cap microcentrifuge tube containing 80 μl of glass beads per 7.5 OD600 units
of cells. The samples were heated at 70°C for 10 min and then vortexed vigorously for 1
min. The debris and unbroken cells were pelletized in a microcentrifuge at 14,000 rpm
for 5 min, at 4°C. The supernatants were transferred to fresh 1.5-ml screw-cap tubes and
placed on ice (first supernatants). The tubes were placed in a 100°C (boiling) water bath
for 3-5 min and then vortexed vigorously for 1 min. The debris and unbroken cells were
pelletized in a microcentrifuge at 14,000 rpm for 5 min, at 4°C. Each supernatant (second
supernatant) was combined with the corresponding first supernatant.

The extracted yeast protein samples were boiled briefly and then were loaded on a 10%
SDS- PAGE gel. Protein samples were separated by SDS-PAGE and transferred to an
Optitran membrane; the membranes then were probed by mouse anti-c-myc antibody.

**Analysis of DNA-BD/bait Protein for Toxicity Effects**

Toxicity of the bait protein was analyzed for the host strain by comparing the growth rate
in liquid culture of cells transformed with the empty DNA-BD vectors and cells
transformed with the DNA-BD/bait plasmid.

**Analysis of DNA-BD/bait Protein for Transcriptional Activation**

AH109[pGBKT7-ND] strain was plated on SD- Trp, SD -Ade/-Trp, and SD -His/-Trp
plates. Growth on Trp- plates indicated the phenotype of the strains, whereas Ade-/Trp-
and His-/Trp- plates tested for the activation of the *ADE2* and *HIS3* reporters, respectively.

**Analysis of DNA-BD/Bait Protein for Effect on Mating Efficiency**

The AH109[pGBKT7-ND1] strain was mated with the Y187-pTD1-1 control strain. The mating mixtures were plated on SD/-Leu/X-α-Gal, SD/-Trp/X-α-Gal and SD/-Leu/-Trp/X-α-Gal. The mating efficiency was calculated and compared to the mating efficiency of Y187-pTD1 with AH109[pGBKT7-53] (positive control).

**Two-Hybrid Library Screening Using Yeast Mating**

To screen the human kidney library pretransformed into the Y187 fish strain, the following procedure was performed: One large (2-3 mm), fresh (<2 mo old) colony of AH109-pGBKT7-ND1 was inoculated into a 50 ml SD-Trp culture and grown overnight with shaking at 250 rpm, 30°C until the OD600 was greater than 0.8. The culture was centrifuged at 1,000 x g for 5 min and resuspended in the residual liquid (~5 ml) by vortexing. Just prior to use, one frozen aliquot (~1.0 ml) of the library culture was thawed in a water bath at room temperature and 10 μl was removed for later titering. The remainder of the 1ml aliquot of library stain was added to the bait strain and the volume was made up to 45 ml with 2xYPDA plus 60 μg/ml kanamycin. This culture was incubated in a 2 L Erlenmeyer flask for 24 h at 30°C, with shaking at 40 rpm. After 24 h of mating, the mating culture was centrifuged for 10 min at 1000g and resuspended in 10 ml of 2xYPDA/60μg/ml kanamycin. A small amount of this resuspension was diluted
1:10, 1:100, 1:1000 and 1:10,000 for plating onto control plates SD-Trp, SD-Leu, SD-Trp/-Leu. The remainder of the resuspension was plated onto 50 quadruple dropout (SD-Leu/-Trp/-His/-Ade) large plates using 200 μl of culture per plate. These plates were incubated at 30°C for about 2 wk until colonies were at least 1 mm in diameter.

**Retesting Phenotypes**

The initial colony may contain more than one AD/library plasmid, which could complicate the analysis of putative positive clones. So we restreaked the colonies and retested the phenotype of the colonies as listed below.

1. Positive colonies were restreaked on SD/–Leu/–Trp/X-α-Gal plates 2–3 times to allow loss of some of the interacting AD/library plasmids while maintaining selective pressure on both the DNA-BD and AD vectors. Plates were incubated at 30°C for 3 d. A mixture of white and blue colonies indicated segregation.

2. Well-isolated colonies were transferred to SD/–Ade/–His/–Leu/–Trp/X-α-Gal plates to verify that they maintain the correct phenotype.

3. The restreaked and retested Ade+/His+/Mel1+ colonies were collected on SD/–Ade/–His/–Leu/–Trp master plates in a grid fashion. Plates were incubated at 30°C for 4–6 d. After colonies were grown, plates were sealed with Parafilm and stored at 4°C for up to 4 wk.

**Yeast Plasmid Isolation**

Inserts in colonies identified by Y2H screenings were difficult to be amplified by whole cell PCR. For these cDNAs, yeast plasmid recovery was needed. To recover the DNA-
AD plasmid from clones, individual positive colony was picked and transferred to 1.5 ml of drop out medium (to maintain selection for the plasmid) in a 17 x 100 mm sterile, polystyrene round bottom tube. The liquid culture was grown overnight in QDO media and centrifuged for 3 min at 6,000 x g. After washing once with 1 ml dH2O, the cell pellet was resuspended in 100 µl of Spheroplast Buffer. The resuspended yeast cells were mixed well after addition of 4 µl Enzyme Solution, and incubated at 37°C for 1 h. The samples were then centrifuged at 6,000 x g for 4 min and the supernatant were removed carefully. 150 µl of Lysis Solution was added to the spheroplast pellet and the pellet was resuspended. After incubation at 95°C for 10 min the samples were chilled on ice. 50 µl of Precipitation Solution was added and the samples were mixed gently by inverting the tube 3-5 times followed by incubation on ice for 10-20 min. After centrifugation at 6,000 x g (7,500 rpm) for 4 min the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. The supernatant was re-centrifuged at 20,000 x g (14,000 rpm in a standard centrifuge) for 2 min to remove residual precipitate. An equal volume of chilled 100% isopropanol was added to the supernatant and mixed by inversion. Then the samples were incubated at -20°C for at least 1 h. After centrifugation at 20,000 x g (14,000 rpm) for 20 min at 4°C, the supernatant was removed. The pellet was washed once with an equal volume of 70% ethanol. After centrifugation at 20,000 x g (14,000 rpm) for 5 min, the pellet was dried and resuspended in 13 µl of dH2O.

**Sorting Colonies to Eliminate Duplicates**

The AD/library inserts were amplified by PCR and the PCR products were characterized by digesting with a frequent-cutter restriction enzyme *Hae* III. The fragment sizes were
analyzed by agarose gel electrophoresis; PCR products with varying DNA size were purified in low melting DNA agarose electrophoresis and gel slice containing the DNA band was cut. After electrophoresis the gel slice was thawed at 60°C and 15 µl was mixed with restriction reaction containing \textit{Hae} III enzyme to make up 30µl digest reaction and then was placed in 37°C water bath for 4 h. The digest reactions were thawed again at 60°C and then loaded into 2% DNA agarose gel for electrophoresis and photography. The DNA digestion pattern of colonies was compared and sorted. Assuming same DNA digestion pattern would generate same \textit{Hae} III cut pattern, restriction enzyme analysis of cDNA clones allowed classification of colonies into groups. Representative clones were transformed into \textit{E. coli}.

\textbf{Rescuing AD/Library Clones by Transformation of \textit{E. coli}}

The BD Matchmaker Two-Hybrid System 3 AD and DNA-BD cloning vectors carry the Amp\textsuperscript{r} and Kan\textsuperscript{r} markers, respectively. As a result, \textit{E. coli} host strains such as DH5\textalpha may be used to select for GAL4 AD or DNA-BD clones by their resistance to ampicillin or kanamycin. The DH5\textalpha max efficiency competent cells were thawed on wet ice and 30 µl of competent cells were aliquoted into chilled tubes. 1 µl of yeast plasmid was added to the cells (about 10 ng DNA), moving the pipette through the cells while dispensing. The tubes were gently tapped to mix. The cells were shocked at 42°C for 30 sec and allowed to recover in 1ml of SOC media at 37°C, with shaking at 250 rpm for 1 h. The transformation mixture was plated on LB/amp plates and incubated overnight at 37°C. Individual colonies were grown in LB/amp liquid culture, miniprepped (Qiagen).
Sequencing AD/Library Insert

The inserts in the positive AD/library clones were sequenced using the GAL4 AD Sequencing Primer (5’-TACCACTACAATGGATG-3’). The presence of an open reading frame (ORF) fused to the GAL4 AD sequence was verified, and the sequences were then compared to those in GenBank, EMBL, or other databases.
RESULTS

cDNA containing ND domain of Na,K-ATPase α₁ subunit and flanked by *Eco*RI and *Bam*HI site was PCR-Cloned from pig α₁ cDNA (Figure 2). PCR was performed as follows: 94°C for 5 min for one cycle; denaturation at 94°C for 1 min, annealing at 56°C for 1 min, elongation for 1 min at 72°C for 28 cycles; and a final extension at 72°C for 7 min.

Once amplified, the PCR product was digested by *Eco*RI and *Bam*HI and purified by gel extraction. Then the PCR fragment was subcloned into pGBK7 vector. The insert of ND1 was verified by restriction analysis (Figure 3, Lane 4 and Lane 5) and sequencing (data not shown).

Figure 2. PCR Amplification of ND Domain of Na,K-ATPase α₁ Subunit

Lane 1: 100 bp Ladder
After generation and verification, these DNA-BD vector were transformed into yeast strain AH109 to generate strain AH109[pGBK7-ND1]. AH109 strain carries the HIS3 and ADE2 nutritional reporters downstream of the Gal1 and Gal2 promoters, respectively. Once transformed with a DNA-BD vector, the resultant AH109[pGBK7-ND1] strain can be maintained and verified by growth on Synthetic Dropout minus tryptophan (SD-Trp).
Samples were prepared from the transformed and untransformed yeast, and then probed with antibodies to the C-Myc epitope tag. The expression of BD fusion protein in yeast strain AH109[pGBK77-ND1] was detected by western blot analysis (Figure 4).

Figure 4. Western Blot of BD-ND Expression in Yeast AH109 Detected by Anti Myc Antibody

Lane 1: Low range molecular weight marker; Lane 2: 10μl, Lane 3: 20μl, Lane 4:60μl, protein extraction from untransformed AH109 yeast; Lane 5: 10μl, Lane 6: 20μl, Lane 7: 60μl, protein extraction from AH109[pGBK77-ND1] strain; Lane 8: Positive control for c-myc Ab.

Prior the Y2H screening, all the strain phenotype were tested on various SD selection medium (Table IV) and a small-scale mating procedure was performed using the positive control strains.
Table IV. Phenotype Testing on Various SD Selection Media

<table>
<thead>
<tr>
<th>Reporter gene</th>
<th>Encodes</th>
<th>Positive Phenotype</th>
<th>Negative Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ or MEL 1</td>
<td>β-galactosidase</td>
<td>Blue colony</td>
<td>White colony</td>
</tr>
<tr>
<td></td>
<td>α-galactosidase</td>
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<tr>
<th>Phenotype Testing on Various SD selection Media</th>
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<table>
<thead>
<tr>
<th>SD/-Ade</th>
<th>SD/-His</th>
<th>SD/-Leu</th>
<th>SD/-Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH109</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Y187</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AH109[pGBK7-ND1]</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Y187[pACT2-cDNA]</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Control Diploid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

To verify the suitability of AH109[pGBK7-ND1] strain in the Y2H system, three parameters were evaluated for each strain: toxicity, autonomous activation and mating efficiency. AH109[pGBK7-ND1] strain was grown alongside with the AH109 strain transformed with empty DNA-BD vector. Both strains were found have a similar growth rate over 24 hours, indicating no toxicity. To test for autonomous activation, AH109[pGBK7-ND1] strain was plated on SD/-Trp, SD-Trp/-Ade and SD-Trp/-His plates. The strain expressed the phenotype correctly by growing on SD-Trp, but failed to
grow on SD-Trp/-Ade and SD-Trp/-His, indicating that ND1 did not contain an occult activation domain. In order to test the DNA-BD/Bait protein for effect on mating efficiency, AH109[pGBK7-ND1] strain was mated with Y187-PTD1 control strain. The mating efficiency (9%) was greater than 5%, the minimum considered acceptable by Clontech for screening their pretransformed library.

Once the AH109[pGBK7-ND1] had been verified for use in the Y2H system, they were mated to the human kidney cDNA library cloned into the pACT2 vector and pretransformed into Y187. These large-scale mating mixtures were plated on SD-Trp/-Leu/-His/-Ade (QDO) plates to identify positive colonies. A small amount of the mating mixture was plated to determine mating efficiency and the library was titered. The mating efficiency for this yeast hybrid screening was 6%, with 3.5x10^6 colonies screened (Table V). After 2-3 d incubation at 30°C, some colonies were visible on the library screening plates, but plates were incubated for more days to allow slower growing colonies to appear. Plates were moved out from the incubator until most of the colonies grew to about 2mm in diameter (Figure 5).

Table V. Summary of Yeast Two-Hybrid Screening

<table>
<thead>
<tr>
<th>Number of yeast to be examined</th>
<th>3.5X10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating efficiency</td>
<td>6%</td>
</tr>
<tr>
<td>Positive colonies</td>
<td>420</td>
</tr>
</tbody>
</table>
The initial library clones could contain more than one AD/library plasmid, which could complicate the analysis of putative positive colonies. So the positive diploid colonies isolated by nutritional selection on QDO plates, were restreaked on SD/-Leu/-Trp/X-α-gal plates three times to allow loss of some of the interaction of AD/library plasmids. A mixture of white and blue colonies indicates segregation (Figure 6). The well-isolated colonies were transferred to SD/-Ade/-His/-Trp/-Leu/ X-α-gal to verify that they maintain the correct phenotype (Figure 7). The restreaked and retested Ade⁺/His⁺/Mel1⁺ colonies were collected on SD/-Ade/-His/-Trp/-Leu/ X-α-gal plates in grid fashion. Plates were incubated at 30°C for 4-6 d and stored at 4°C as stock plates (Figure 8).

Yeast plasmid isolation kit from USB was used to prepare DNA of sufficient purity for use as PCR template or for transformation to E. Coli. After recovery of plasmid from positive colonies, the Matchmaker Amplimer Set (Clontech) was used to amplify AD-Library insert (Figure 9A). The PCR products of positive colonies were digested with Hae III restriction enzyme. After digestion, the samples were run on 2% DNA agarose gel. Colonies were sorted to eliminate duplicates by comparing digestion pattern of the PCR products (Figure 9B). Yeast plasmid rescued from the representative colonies were transformed into Max efficiency DH5α cells. DH5α cells were used to select for GAL-AD clones by their resistance to ampicillin.
Figure 5. Positive Colonies Appeared in SD/-Ade/-His/-Leu/-Trp Plates after 12 d Incubation at 30°C

Figure 6. Restreaking Positive Colonies on SD/-Leu/-Trp/ X-α-gal Plates
Figure 7. Transfer of Well-isolated Colonies to SD/-Ade/-His/-Trp/-Leu/ X-α-gal

Figure 8. Collection of the Restreaked and Retested Ade+/His+/Mel1+ Colonies on SD/-Ade/-His/-Trp/-Leu/ X-α-gal Plates
Figure 9. Sorting Colonies to Eliminate Duplicate

A. Amplification of AD/library insert by PCR

Lane 1: 100 bp Ladder, Lane 2: Colony 101, Lane 3: Colony 102, Lane 4: Colony 103, Lane 5: Colony 105, Lane 6: Colony 106, Lane 7: Colony 108, Lane 8: Colony 109, Lane 9: Colony 110, Lane 10: Colony 111, Lane 3: Colony 112, Lane 3: Colony 113

B. Characterization of PCR Products by Digestion with Hae III

Lane 1: 100 bp Ladder, Lane 2: Colony 101, Lane 3: Colony 102, Lane 4: Colony 103, Lane 5: Colony 105, Lane 6: Colony 106, Lane 7: Colony 108, Lane 8: Colony 109, Lane 9: Colony 110, Lane 10: Colony 111, Lane 3: Colony 112, Lane 3: Colony 113
The DNA plasmid was isolated from DH5α cells and purified by minipreparation kit (Qiagen). DNA-AD cDNA insert were sequenced by using the GAL AD sequencing primer. All the sequencing results were analyzed against Genebank Database for identification. The potential proteins interact with ND of Na,K-ATPase α₁ subunit were identified and summarized in Table VI.

Table VI. List of the Potential Na,K-ATPase Interacting Proteins

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>GeneBank ID</th>
<th>Colony Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAN binding protein 9</td>
<td>39812378</td>
<td>52, 53</td>
</tr>
<tr>
<td>UMP-CMP kinase</td>
<td>7706497</td>
<td>100,286,380</td>
</tr>
<tr>
<td>Uromodulin</td>
<td>4507833</td>
<td>299,460</td>
</tr>
</tbody>
</table>
DISCUSSION

Protein-protein interactions are essential virtually in all cellular processes ranging from DNA replication, transcription and translation to signal transduction, cell-cycle control and intermediary metabolism. In most cases, the interactions among proteins are dynamic and the knowledge of assembly and disassembly of them over time in response to complex signals helps one to perceive the process that makes life (Mukherjee et al., 2001). The characterization of Na,K-ATPase interacting proteins will also help us to identify the mechanisms and the pathways by which the enzyme acts as a signal transducer to relay messages, through protein-protein interactions, from the plasma membrane to the nucleus.

Since its advent, the two-hybrid system has proven its utility in the study of protein interactions. It has shown a tremendous success in the charting of genetic networks of proteins involved in processes from signal transduction to transcription regulation (Fields, 1997). Thus, we performed a Y2H screen using the ND1 domain of sodium pump α1 subunit as bait and identified several potential Na,K-ATPase interacting proteins.

One critical question in performing two-hybrid screens has been whether a positive isolate identified in the system actually represents a true \textit{in vivo} interaction. Therefore, some careful considerations such as false positives and false negatives should be given to the primary results of this Y2H screen.
False positive in the two-hybrid system is defined as the activation of reporters unrelated to the interaction of the bait with its specific prey proteins. Some proteins may contain regions with surfaces that have low affinities, for example, large hydrophobic surfaces, heat shock proteins, and ribosomal proteins. These proteins may form complexes with the Na,K-ATPase that are stable enough to result in a detectable positive phenotype. An association with a dissociation constant higher than $10^{-6}$ M will be detected in the Y2H system (Estojak et al., 1995). Therefore, any nonspecific protein-Na,K-ATPase interactions with a dissociation constant higher than $10^{-6}$ M can potentially lead to a positive signal. It is true that so many protein–protein interactions as well as protein-DNA interactions exist in the cell; however, any nonspecific interactions will cause a false signal in the Y2H system.

In general, false positives can be eliminated by verifying that reporter gene expression is specific for the presence of the target protein hybrid. Several methods have been designed to eliminate false positive isolated in the system, including: sway experiments (Bartel et al., 1993; Hong et al., 1996), combining plasmid (Legratin et al., 1994; Dagher et al., 1997), cotransformation (Ma and Ptashne, 1987), mating assay (Bendixen et al., 1994; Dagher et al., 1997) and using an inducible LexA system (Finley and Brent 1995). Some of these experiments are being performed to eliminate false positive isolated from the Y2H screening we performed.

Another consideration about this screening is the failure to detect known protein interactions. Recent studies in our lab showed Na,K-ATPase can interact with Src via the
nucleotide binding domain (ND1) of Na,K-ATPase $\alpha_1$ subunit. However, this tested protein-protein interaction was not detected in this Y2H screening. The failure to detect this expected interactions might be due to a number of reasons, such as (i) The expression of hybrid protein is toxic; (ii) the hybrid protein are not stably expressed in the yeast cell; (iii) the hybrid protein folds improperly; (iv) the hybrid protein cannot enter into the yeast nucleus; (vi) improper post-translational modification, such as phosphorylation, glycosylation, or acetylation (Estojak et al., 1995; Hong et al., 1996).

The Y2H system has been used to identify proteins that interact with the structurally defined cytoplasmic domains of the Na, K-ATPase $\alpha_1$ subunit. Dr. Caplan’s lab (Pagel et al., 2003) has completed the Y2H screens employing the large cytoplasmic loop containing the N and P domains as well as the A domains of the Na, K-ATPase $\alpha_1$ subunit as baits. Some proteins such as Protein Phosphatase 2A catalytic Subunit $\beta$, CD81, Snare-associated Protein have been identified as potential Na, K-ATPase interacting protein (Pagel et al., 2003). Another independent study performed by Lee et al. (2001) used the Y2H system to screen a rat skeletal muscle cDNA library and reported that cofilin interacts with the large cytoplasmic loop of Na, K-ATPase. However, our screen did not identify these proteins as interacting proteins with ND1 of Na, K-ATPase $\alpha_1$ subunit. It may because may be due to differences in the bait proteins that were used in the screens. Another reason is that the Y2H system may not be sensitive enough to detect all the protein-protein interactions at one screen.
Not all the protein-protein interactions detected in the yeast hybrid system will have a real function. Two-hybrid interactions can occur between proteins that normally do not interact (for example, some proteins are never expressed at the same time or in the same tissue or subcellular compartment). In order to show the interactions we identified are true \textit{in vivo} interactions, we need to verify the interactions by a different technique.

We have begun the process of characterizing the repertoire of interacting proteins detected in the screen and have selected several polypeptides listed below for further analysis.

**RAN Binding Protein 9**

RanBPM was originally cloned for its ability to bind to the small GTPase Ran, and was reported to localize to the centrosome, a microtubule organization center (Nakamura et al., 1998; Nishitani et al., 2001; Denti et al., 2004). Ectopically expressed RanBPM was recently shown to interact with different signaling proteins, including the Met receptor, and to cooperate in regulating intracellular signaling pathways (Wang et al., 2002; Zou et al., 2003; Bai et al., 2003). In particular, over expression of RanBPM was shown to induce activation of the Ras-Erk pathway and to activate transcription from the serum response element (Wang et al., 2002). The Ras pathway is a common and crucial component of the signaling of many other growth factors/receptor protein-tyrosine kinases (RPTKs). The activation and inactivation of Ras are regulated by guanine exchange proteins (GEPs) and GTPase-activating proteins (Olson and Marais, 2000). The major human GEP for Ras is Sos, which is constitutively associated with Grb2 (Egan et
al., 1993). For most RPTKs, in order to activate Ras, the Grb2-Sos complex is recruited by direct association with phosphorylated RPTKs to the plasma membrane where Ras is located (Skolnik et al., 1993). Ras downstream effectors include phosphatidylinositol 3-kinase, RalGDS, AF6, and Raf-1 (Spaargaren et al., 1994; Marshall, 1996; Steiner et al., 2000). Activated Raf-1 activates mitogen/extracellular signal protein kinase (MEK), which results in the phosphorylation and activation of Erk1/2 (Vojtek and Der, 1998).

One of the substrates for Erk is the transcription factor Elk-1, which upon activation, up-regulates the expression of immediate early response genes, such as c-Fos (Wasylyk et al., 1998; Zwartkruis et al., 1999). Based on previously published reports, we propose that RanBPM may interact with Na,K-ATPase α subunit and function as a scaffolding protein that coordinates signal inputs derived from cell surface receptors with intracellular signaling pathway.

Uromodulin (Tamm-Horsfall Protein)

Uromodulin is an 80 kDa glycoprotein synthesized exclusively in the thick ascending limb cells of Henle’s loop (TAL) with the exception of the macula densa (Bachmann et al., 1985, 1990; Serafini-Cessi et al., 2003). Uromodulin contains a zona pellucida domain (ZP), which is necessary for polymerization into the supramolecular structure of a filament, an elastase-sensitive fragment containing three calcium-binding epidermal growth factor (EGF)-like domains and a signal peptide, and a potential glycosyl-phosphatidylinositol (GPI)-anchor cleavage site (Jovine et al., 2002). Uromodulin is one member of the GPI-anchored proteins family. The GPI-anchored proteins on the surface of lymphocytes have been demonstrated to be signal transduction molecules. Many of
these proteins have been shown to stimulate lymphocyte proliferation, lymphokine production, calcium mobilization and tyrosine phosphorylation (Yeh et al., 1987; Stefanova et al., 1993). Recently, it has been recognized that GPI-anchored proteins expressed on lymphocytes coimmunoprecipitate with the Src family member tyrosine kinases lck, fyn (Thomas and Samelson, 1992; Shenoy-Scaria et al., 1993; Garnett et al., 1993), fgr, lyn, and hck (Stefanova, et al., 1993; Bohuslav et al., 1995). We already know that Src kinases are key proteins in the Na,K-ATPase mediated signal transduction (Xie and Askari, 2002). The importance of Uromodulin interacting with the ND1 of Na,K-ATPase needs further investigation.

**UMP-CMP Kinase**

UMP-CMP kinase belongs to the large family of eukaryotic nucleoside and NMP kinase for which AMP kinase is a prototype (Eriksson et al., 2002). The protein is made of two subdomains, each containing a nucleotide binding site. The site for the nucleotide donor of phosphate (in general ATP) presents a typical “P-loop” and a “lid domain” that closes down over the active site during the catalytic cycle (Vonrhein et al., 1995). UMP-CMP kinase is involved in both the *de novo* and the salvage pathway of nucleosides. It catalyses a compulsory step for the synthesis of UTP, CTP, and dCTP, that are incorporated into nucleic acids. UMP-CMP kinase also is involved in the phosphorylation of several anticancer and antiviral drugs that are given to patients suffering from AIDS and B Hepatitis (Liou et al., 2002).
CONCLUSIONS

1. The ND1 domain of Na,K-ATPase $\alpha_1$ subunit was subcloned to pGBK7 vector for the construction of DNA-BD/bait vector. The bait insert was verified by restriction analysis and sequencing.

2. The DNA-BD/bait vector was transformed to AH109. The expression of DNA-BD fusion protein was detected by western blot.

3. The properties of constructed AH109[pGBK7-ND1] strain were tested and this bait strain was suitable for yeast hybrid screening.

4. A two-hybrid screening using human kidney cDNA library was performed. Of $3.5 \times 10^6$ transformed colonies screened, 420 positive colonies grew in the SD/-Ade/-His/-Trp/-Leu plates and $\alpha$-galactosidase positive.

5. After phenotype retest and duplicates elimination, representative clones were sequenced and several potential proteins were identified by searching in NCBI database.
SUMMARY

Na,K-ATPase (or sodium pump) is a ubiquitous transmembrane enzyme that transports Na\(^+\) and K\(^+\) across the plasma membrane by hydrolysis. Our lab has proposed that the pump also act as a signal transducer for cardiotonic steroids by interacting with other proteins. Recent studies in our lab showed Na,K-ATPase can interact with Src via the nucleotide binding domain 1 (ND1) of Na,K-ATPase \(\alpha_1\) subunit. We have used the Y2H system to identify other proteins that interacts with the ND1 domain of sodium pump \(\alpha_1\) subunit. Our studies have made use of Gal4 based Y2H system from Clontech, in which interaction between the GAL4 activation and DNA binding domain triggers transcription of reporter genes. To ensure that the bait construct do not posses autonomous promoting activity, the single transformed yeast was tested for reporter gene expression.

A commercially available human kidney cDNA library was used in this yeast hybrid screening. Transformants expressing activation domain fusion proteins that interact with bait construct were selected on SD/-Ade/-His/-Trp plates and tested for lacZ expression. Of 3x10\(^6\) colonies screened, about 420 colonies grew in the selective media and were X-gal positive. Restriction enzyme analysis of cDNA clones allowed colony classification into groups. Representative clones were sequenced and several potential Na,K-ATPase \(\alpha_1\) subunit binding proteins were identified. These proteins may play a role in regulating pump distribution and function, further investigation about the nature and function of these interacting proteins need to be performed.
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Na,K-ATPase needs to interact with neighboring membrane proteins and organized cytosolic cascades of signaling complexes to function as a signal transducer. The identities of these interacting proteins and the nature of their association with the sodium pump have yet to be elucidated. Some studies have shown that the nucleotide binding domain 1 (ND1) of Na,K-ATPase α1 subunit play an important role when Na,K-ATPase interacts with other proteins. In an attempt to identify novel proteins that can interact with Na,K-ATPase, we used the ND1 domain of sodium pump α1 subunit as a bait to perform a yeast two hybrid screen in a human kidney cDNA library. We have obtained several cDNA clones encoding the potential Na,K-ATPase α1 subunit binding proteins. How these proteins interact with Na,K-ATPase and play a role in Na,K-ATPase mediated signal transduction needs further investigation.