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Possible Implication for an Indirect Interaction between Basic Fibroblast Growth Factor and (Na,K)ATPase

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The (Na,K)ATPase is responsible for generating the ionic gradients and membrane potentials by the exchange of intracellular Na^+ for K^+. It has been recently shown that (Na,K)ATPase is involved in the exocytic pathway of basic fibroblast growth factor (bFGF), although it is not known that bFGF is secreted to the outside of cell through direct interaction with (Na,K) ATPase. To understand the role for (Na,K)ATPase in the secretory pathway of bFGF, we have sought to identify the cytoplasmic domains of the α1 isoform of (Na,K)ATPase interacting with bFGF by yeast two-hybrid system. We have also investigated the interaction between the α2 isoform of (Na,K)ATPase and bFGF to find out whether the interaction is isoform-specific. We found that none of the cytoplasmic domains of (Na,K)ATPase isoforms interacted with bFGF. The result suggests that the interaction between bFGF and (Na,K)ATPase might be indirect, thus requiring other proteins which are involved in the formation of protein complexes for the interaction, although we cannot exclude the possibility that the interaction requires the element of the whole α subunit structure that was not present in the isolated α subunit cytoplasmic domains.

Key words : Yeast two-hybrid, bFGF, Na^+ pump, Cytoplasmic domain

INTRODUCTION

The (Na,K)ATPase, present in the plasma membrane of all animal cells, exchanges intracellular Na^+ for extracellular K^+ using the energy of ATP hydrolysis for the maintenance of an electrochemical gradients of Na^+ and K^+ ions across the plasma membrane (Jorgensen, 1982; Schwartz *et al.*, 1975). The ion gradients serve as energy source for the diverse cellular functions, such as regulation of cell volume, transport of certain solutes (sugars, amino acids, and other nutrients) and excitability of membrane (Lingrel and Kuntzweiler, 1994; Vasilets and Schwartz, 1993).

The minimal functional enzyme unit of (Na,K)ATPase is a heterodimer consisting of a catalytic α subunit and a glycosylated β subunit. Four isomeric forms of the α (α1, α2, α3, and α4) and three isomeric forms of β (β1, β2, and β3) have been identified and expressed in a tissue- and developemental-specific manner (Jorgensen and Andersen, 1986; Shamraj and Lingrel, 1994). The catalytic α subunit of ~100 kDa polypeptides contains phosphorylation site and binding sites for cations, ATP, and cardiac glycosides, such as digoxin and ouabain (Shull *et al.*, 1985).

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bFGF belongs to the fibroblast growth factor family consisting of nine structurally related members with various biological activities and is a prototype of acidic fibroblast growth factor (aFGF). bFGF is widely distributed and involved in important biological activities including angiogenesis, mitogenesis, cellular differentiation, wound-healing processes, and tumor neovascularization (Burgess and Maciag, 1989; Friesel and Maciag, 1995). A recent study demonstrated that bFGF inhibits the apoptosis of endothelial cells by *bcl*-2-dependent and independent mechanism (Karsan *et al.*, 1997). Although bFGF must be secreted from cell to function the biological activities as mentioned, it lacks conventional signal peptide sequence that most secretory proteins have (Burgess and Maciag, 1989; Muesch *et al.*, 1990). Therefore, main question for bFGF biology has been focused on the mechanisms that might account for the release.

It had been assumed that the efficient release of bFGF from a storage site required plasma membrane disruptions of epithelial cells (McNeil *et al.*, 1989; Muthukrishinan *et al.*, 1991). However, recent studies suggest that bFGF is exported through an alternative, energy-dependent, and non-endoplasmic reticulum (ER)/ Golgi pathway without compromising cell integrity or requiring cell death (Florkiewicz *et al.*, 1995; Mignatti *et al.*, 1992). Florkiewicz *et al.* (1998) demonstrated

that the bFGF export is inhibited by cardenolides, such as ouabain in Cos-1 cells, while the secretion of the protein containing signal sequence is not inhibited, suggesting that the α subunit of (Na,K)ATPase is involved in bFGF export. They also showed the functional interaction between bFGF and the α subunit of (Na,K) ATPase by co-immunoprecipitation. However, it was not known that bFGF is exported through direct interaction with the α subunit of (Na,K)ATPase since whole cell lysates were used for co-immunoprecipitation experiments (Florkiewicz *et al.,* 1998).

In this study, we investigated the possibility of direct interaction between bFGF and cytoplasmic domains of the α1 subunit and the β1 subunit of (Na,K)ATPase by yeast two-hybrid system. We also tested the interaction between the cytoplasmic domains of the α2 subunit of (Na,K)ATPase to see if the interaction is isoform-specific.

MATERIALS AND METHODS

Construction of plasmids

Materials for the yeast two-hybrid system were obtained from Dr. Roger Brent (Massachusetts General Hospital, Boston, MA) and cDNAs encoding the rat (Na,K)ATPase α1, α2, and β1 subunits were provided by Dr. Jerry Lingrel (University of Cincinnati, College of Medicine). We purchased mouse bFGF cDNA from ATCC (Rockvelli, MD). The cDNAs encoding the cytoplasmic domains of (Na,K)ATPase α1, α2, and β1, and the bFGF were amplified by polymerase chain reaction (PCR), using full-length (Na,K)ATPase cDNA as template and using primers which contain the sequences for the introduction of restriction sites for cloning into pEG202 or pJG4-5 vector (Table I).

The pEG202 constructs for the 5 cytoplasmic domains of the α2 and the one cytoplasmic domains of the β1, and the pJG4-5 construct for the cytoplasmic NH_2-terminal had been made in our previous study (Yoon and Lee, in press). Therefore, the pEG202 constructs for the cytoplasmic regions of the α1 and bFGF and the pJG4-5 construct for bFGF were made for this study. To amplify the α1L1 that encodes the cytoplasmic NH_2-terminal region (amino acids Met^1 to Cys^{93}), the primers 1 and 2 were used. The primers 3 and 4 were used to amplify α1L2 that encodes the cytoplasmic loop 1 (amino acids Glu^{151} to Ile^{292}). The primers 5 and 6 were used to amplify α1L3 that encodes the cytoplasmic loop 2 (amino acids Lys^{354} to Val^{759}). The primers 7 and 8 were used to amplify α1L4 that encodes the cytoplasmic loop 3 (amino acids Glu^{825} to Arg^{848}). The primers 9 and 10 were used to amplify α1L5 that encodes the cytoplasmic C-terminal (amino acids Ile^{935} to Tyr^{1023}). The primers 11 and 12 were used to amplify bFGF (amino acids Met^1 to Ser^{154}). PCR products encoding the desired cytoplasmic regions of the α1 subunit of (Na,K)ATPase and bFGF were digested with appropriate restriction enzyme (Table I). The α1L1, α1L3, and α 1L4 were ligated in-frame into EcoRI/XhoI digested pEG202. The α1L2 and α1L5 were ligated in-frame into BamHI/XhoI digested pEG202. The bFGF was ligated in-frame into EcoRI/XhoI digested pEG202 and pJG4-5. The ligation products were transformed into DH5α supercompetent cells and the cloned inserts were confirmed by restriction analysis of the plasmids.

The pEG202 to make LexA fusion protein contained the yeast His gene and LexA fusion protein is constitutively expressed from yeast ADH promoter. The pJG 4-5 contained the transcriptional activation domain under the control of the yeast GAL1 promoter.

Yeast culture

The EGY48 yeast strain (genotype: MATα, his3, trp 1, ura3-52, leu2 : : pleu2-LexAop6) was used as s host for pEG202 and pJG4-5 plasmid constructs. The pSH18-34 plasmid that contains GAL1 promoter was fused to lacZ and LexA operator.

By lithium acetate transformation method, yeast cells, EGY48/pSH18-34, were transformed simultaneously with LexA DNA binding fusion plasmids and transcriptional

Table I. Oligonucleotide primers for cloning of the cytoplasmic domains of (Na,K)ATPase α1 and β1 subunit, and bFGF

	Primer	5'→3'
1	α1L1 (sense)	GGAATTC(EcoRI)ATGGGGAAGGGGGTTGGA
2	α1L1 (antisense)	CCGCTCGAG(XhoI)GCAGAACTTGACCCACTC
3	α1L2 (sense)	CGGGATCC(BamHI)AGGAAGCCAAAAGCTCCAAG
4	α1L2 (antisense)	CCGCTCGAG(XhoI)GATGAAATGCTCGATCTC
5	α1L3 (sense)	GGAATTC(EcoRI)AAGAACTGCCTGGTGAAGAAC
6	α1L3 (antisense)	CCGCTCGAG(XhoI)CACAATGGAGGCAAAGTTGTC
7	α1L4 (sense)	GGAATTC(EcoRI)GAACAGGCTGAAAGTGAC
8	α1L4 (antisense)	CCGCTCGAG(XhoI)CCTCTCGTTCACCAGCTT
9	α1L5 (sense)	CGGGATCC(BamHI)AGGTCATCTGCAAGACCAGA
10	α1L5 (antisense)	CCGCTCGAG(XhoI)GTAGTACGTCTCCTTCTC
11	bFGF (sense)	GGAATTC(EcoRI)GCTGCCAGCGGCATCACC
12	bFGF (antisense)	CCGCTCGAG(XhoI)GCTCTTAGCAGACATTGG

activation domain fusion plasmids (Gietz *et al.*, 1992). Transformants were plated on solid medium lacking Ura, His, Trp, and Leu in the presence of glucose or galactose.

All yeast culture were grown in liquid or on solid medium composed of 0.67% yeast nitrogen base without amino acids (Difco, Detroit, U.S.A.), 0.087% dropout powder depending on the transforming plasmids, and either 2% glucose or 2% galactose as carbon source.

Immunoblotting of hybrid protein expression

The Western blotting of fusion proteins was performed to verify the appropriate size of the expressed proteins. The yeast cells expressing hybrid proteins were grown to an optical density of 0.8~1.0 at 600 nm. Protein extracts were obtained from cell pellets by vortexing six times each at 4°C for 30 sec, 200 μl yeast lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% TritonX-100, 0.5% SDS, 0.5 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM benzamidine) and 200 μl glass beads. Then, after the quantitation of protein extracts, they were separated by SDS-PAGE according to standard technique (Laemmli, 1970), transferred onto nitrocellulose and analyzed with ECL (Amersharm, Buckinghamshire, England). Fusion proteins were detected using anti-LexA polyclonal antibody and anti-HA monoclonal antibody.

RESULTS AND DISCUSSION

In this study, we have sought to identify the cytoplasmic domains of the α1 isoform of (Na,K)ATPase interacting with bFGF by yeast two-hybrid system to understand the role for (Na,K)ATPase in the secretory pathway of bFGF. We have also investigated the interaction between the α2 isoform of (Na,K)ATPase and bFGF to find out whether the interaction is isoform-specific. The results were that none of the cytoplasmic domains of (Na,K)ATPase isoforms interacted with bFGF, suggesting that the interaction between bFGF and (Na,K) ATPase might be indirect.

(Na,K)ATPase is known to have 8~10 topological arrangements. Until now, discrepancies exist among several current models in the transmembrane regions of the COOH-terminal third of the polypeptide in spite of substantial work (Yoon and Guido, 1994). The cytoplasmic regions of the α1 subunit of (Na,K)ATPase for this study were determined on the basis of the 8 transmembrane topology of (Na,K)ATPase depicted in Fig. 1. We fused 5 cytoplasmic domains of the α1 the subunit to the LexA DNA binding domain in pEG202 vector, yielding pEG202α1L1, pEG202α1L2, pEG202α 1L3, pEG202α1L4, and pEG202α1L5. We found in the previous study that the yeast transformed with pEGα1L1

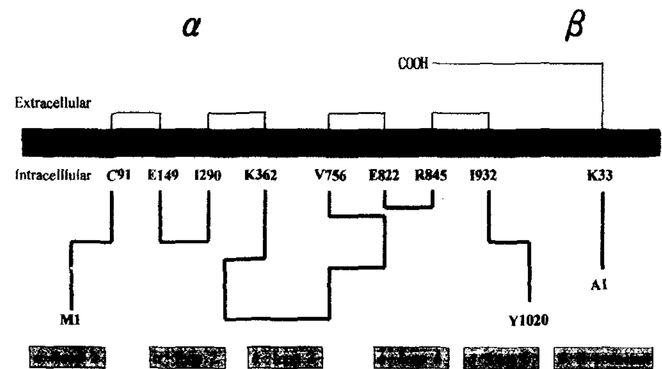

Fig. 1. Putative topological model for (Na,K)ATPase α and β subunits (modified from Yoon and Guidotti (1994)). Each cytoplasmic domain used in yeast two hybrid assay was amplified by PCR, cloned into either pEG202 for LexA fusions or pJG4-5 for B42 fusions.

and pEGα2L1 alone promoted growth on Leumedia without pJG4-5 fusion proteins, indicating that the NH_2-terminus of (Na,K)ATPase contain activation sequences (Yoon and Lee, in press). Therefore, we made the construct of pJG4-5α1L1 and pJG4-5α2L1 instead of pEG202 constructs. bFGF was fused to the binding domain of LexA in pEG202 and also fused to the B42 transcription activation domain in pJG4-5 to determine the interactions with α1L1 and α2L1 in pJG4-5 vector and other cytoplasmic domains in pEG202 vector.

The expression of these fusion proteins were confirmed by Western blotting if the fusion proteins are in correct reading frame. The protein sizes of the cytoplasmic domains of α2 and β1 subunits were determined using LexA antibody. In our previous study (Yoon and Lee, in press), LexA antibody detected 26.7 kDa of pEG202 alone, 42 kDa of pRFHM1 (negative control), 36.8 kDa of α2L1, 41.1 kDa of α2L2, 69 kDa of α2L3, 27.6 kDa of α2L4, 34 kDa of α2L5, 28.4 kDa of β1-N-terminus. In this study, we identified the correct protein sizes of the 5 cytoplasmic domains of α1 and bFGF by Western blotting with LexA antibody (Fig. 2A). In Fig. 2A, LexA antibody detected 26.7 kDa of pEG202 alone (lane 1), 42 kDa of pRFHM1 (negative control, lane 2), 36.8 kDa of α1L1 (lane 3), 41.1 kDa of α1L2 (lane 4), 69 kDa of α1L3 (lane 5), 27.6 kDa of α1L4 (lane 6), 34 kDa of α1L5 (lane 7), and 41 kDa of bFGF (lane 8), suggesting that all fusion proteins were expressed in right reading frame. We also identified expressed proteins for pJG4-5α2L1 and pJG4-5 bFGF, using HA antibody (Fig. 2B). Since in pJG4-5 vector activator gene was controlled by the yeast GAL1 promoter, the fusion proteins were detected galactose-dependently as shown in Fig. 2B. The size of each fusion protein was 38.5 kDa for bFGF (lane 2 in Fig. 2B) and 30.5 kDa for α2L1 (lane 4 in Fig. 2B).

We transformed EGY48 yeast cells containing pSH 18-34 reporter plasmid with appropriate recombinant

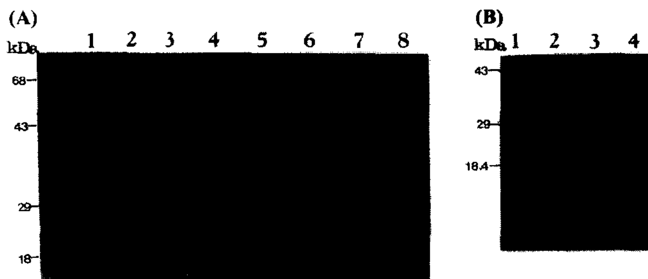

Fig. 2. Western blotting of the expressed fusion proteins. A) Cell extracts were obtained from yeast cells EGY48/pSH18-34 that had been transformed with one of following plasmids: pEG202/pJG4-5 bFGF (lane 1), pEG202pRFHM /pJG4-5 bFGF (lane 2), pEG202α1L1/pJG4-5bFGF (lane 3), pEG202α1L2/pJG4-5bFGF (lane4), pEG202α1L3/pJG4-5bFGF (lane 5), pEG 202α1L4/pJG4-5bFGF (lane 6), pEG202α1L5/pJG4-5 bFGF (lane 7), pEG202bFGF/pJG4-5α2L1 (lane 8). The extracts were seperated by 10% SDS-PAGE gel, transferred onto nitrocellulose, and analyzed with ECL using LexA antibody. B) Cell extracts were obtained from yeast cells EGY48/pSH18-34 that had been transformed with one of following plasmids in glucose and galactose media: pJG4-5bFGF in glucose (lane 1) and in galactose (lane 2) pJG4-5α2L1 in glucose (lane 3) and in galactose (lane 4). The extracts were seperated by 10% SDS-PAGE gel, transferred onto nitrocellulose, and analyzed with ECL using HA antibody.

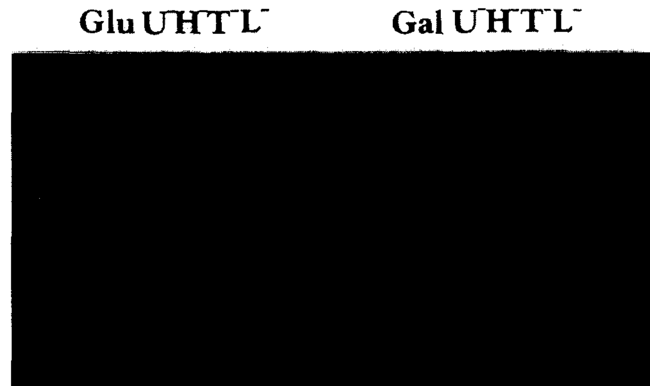

Fig. 3. Interaction between the cytoplasmic domains of (Na,K) ATPase and bFGF. The yeast cells EGY48/pSH18-34 were transformed with one of following plasmids: pEG202α1L3/pJG4-5 267(cofilin) (lane 1), pEG202pRFHM/pJG4-5bFGF (lane 2), pEG202/pJG4-5bFGF (lane 3), pEG202α1L1/pJG4-5bFGF (lane 4), pEG202α1L2/pJG4-5bFGF (lane 5), pEG202α1L3/pJG4-5bFGF (lane 6), pEG202α1L4/pJG4-5bFGF (lane 7), pEG202α1L5/pJG4-5bFGF (lane 8), pEG202β1-N-terminus/pJG4-5bFGF (lane 9), pEG202bFGF/pJG4-5α1L1 (lane 10). The transformants were streaked onto gucose $Ura^-His^-Trp^-Leu^-$, galactose $Ura^-His^-Trp^-Leu^-$ to test Leu2 expression.

plasmids of pEG202 and pJG4-5 constructs. The $Ura^-His^-Trp^-$ transformants were streaked onto glucose $Ura^-His^-Trp^-Leu^-$ and galactose $Ura^-His^-Trp^-Leu^-$ media (Fig. 3). The yeast cell EGY48/pSH18-34/pEG202α1L3/pJG 4-5cofilin was used as a positive control (lane1 in Fig. 3), and EGY48/pSH18-34/pEG202 pRFHM/pJG4-5bFGF and EGY48/pSH18-34/pEG202/pJG4-5bFGF were used as a negative control (lane 2, 3 in Fig. 3). As can be seen in Fig. 3, yeast cell EGY48/pSH18-34/pEG202α1L1/pJG4-5bFGF (lane 4), EGY48/pSH18-34/pEG202α1L2/pJG4-5bFGF (lane 5), EGY48/pSH18-34/pEG202α1L3/pJG4-5bFGF (lane 6), EGY48/pSH18-34/pEG202α1L4/pJG4-5bFGF (lane 7) and EGY48/pSH18-34/pEG202α1L5/pJG4-5bFGF (lane 8) did not grow on Leu^- media, suggesting that none of the cytoplasmic domains of the α1 subunit interact with bFGF. We also performed the same experiments using the 5 cytoplasmic domains of the α2 subunit instead of the α1 subunit to find out whether the interaction between bFGF and (Na,K)ATPase is isoform-specific. The results indicate that none of the cytoplasmic domains of α2 subunit interact with bFGF (data not shown). Therefore, the results from our study imply that the interaction between (Na,K)ATPase and bFGF is indirect, thus requires other proteins for the interaction, although we cannot exclude the possibility that the whole element of the α subunit structure is needed for the interaction with bFGF since we used the isolated cytosolic domains of the α subunit.

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