cDNA Cloning of Na,K-ATPase α-Subunit from Embryos of the Sea Urchin, Hemicentrotus pulcherrimus

Authors: Mitsunaga-Nakatsubo, Keiko, Yamazaki, Ken, Hatoh-Okazaki, Midori, Kawashita, Hiroto, Okamura, Chisato, et. al.

Source: Zoological Science, 13(6) : 833-841

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.833
cDNA Cloning of Na⁺, K⁺-ATPase α-Subunit from Embryos of the Sea Urchin, Hemicentrotus pulcherrimus

Keiko Mitsunaga-Nakatsubo1,*, Ken Yamazaki1,*, Midori Hatoh-Okazaki1, Hiroto Kawashita1, Chisato Okamura1, Koji Akasaka2, Hiraku Shimada2 and Ikuo Yasumasu1,***

1Department of Biology, School of Education, Waseda University, 1-6-1, Nishiwaseda, Shinjuku-ku, Tokyo 169-50, Japan
2Graduate Department of Gene Science, Faculty of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashi-Hiroshima 739, Japan

ABSTRACT—Na⁺, K⁺-ATPase α-subunit cDNA of the sea urchin, Hemicentrotus pulcherrimus, was obtained by twice screening prism and gastrula λgt10 cDNA libraries using an oligonucleotide probe derived from a mostly conserved region, FSBA (5'-p-(fluorosulfonyl)-benzoyladenosine) binding site of cation transport ATPases. The 5'-end of the non-coding region was determined by primer extension and the region was amplified by 5'-RACE method. The sea urchin α-subunit cDNA consists of 4401 nucleotides and encodes 1038 amino acid residues (MW, 114 kDa). The predicted primary structure, except N-terminal region, has similar degree of high homology to various metazoan Na⁺, K⁺-ATPase α-subunits. Alignment of amino acid sequence and a hydropathy profile also predicts eight putative transmembrane segments at least. The phylogenetic tree suspected from alignment of amino acid sequences of 21 species suggests that sea urchin and vertebrate Na⁺, K⁺-ATPase α-subunits seem to have evolved from a common origin, before vertebrate α-subunit divided into three isoforms.

INTRODUCTION
Na⁺, K⁺-ATPase is a membrane-bound enzyme responsible for active transport of Na⁺ and K⁺ across the plasma membrane in most animal cells. The protein is composed of two subunits; a large catalytic α-subunit and a smaller glycosylated β-subunit (Lingrel and Kuntzveiler, 1994). The α-subunit contains an intracellular ATP-binding site (Farley et al., 1984), a phosphorylation site (Post et al., 1973; Walderhaug et al., 1985), and an extracellular binding site for cardiac glycosides such as digoxigenin and ouabain (Schwartz et al., 1975; Lingrel et al., 1990). Three α-isofoms, α1, α2 and α3, in mammals (Shull et al., 1986; Hara et al., 1987; Herrera et al., 1987) and in chicken (Takeyasu et al., 1988, 1990), two in Artemia (Baxter-Lowe et al., 1989; Macías et al., 1991), and only one in Drosophila melanogaster (Lebovitz et al., 1989), as well as three β-isoforms, β1, β2 and β3 (β3 is only found in Xenopus laevis (Good et al., 1990)) have been identified so far (Sweedner et al., 1989). Each subunit isoform of vertebrates shows different tissue-specific and developmentally regulated expressions (Herrera et al., 1987; Orlowski and Lingrel, 1988).

Enzymatic studies revealed that the plasma membrane of sea urchin eggs and embryos contains an ouabain-sensitive Na⁺, K⁺-ATPase activity (Kinsey et al., 1980; Mitsunaga et al., 1986, 1989), which is stimulated just after fertilization (Ciapa et al., 1984). We have shown the change in the activity of Na⁺, K⁺-ATPase during early development of the sea urchin Hemicentrotus pulcherrimus (Mitsunaga-Nakatsubo et al., 1992a). The activity begins to increase at the mesenchyme blastula stage and reaches the maximum at the gastrula stage. The increase at the gastrula stage is actinomycin D-sensitive and is probably due to the increase in ectoderm cells. Northern blot analysis, using a fragment of the Hemicentrotus Na⁺, K⁺-ATPase α-subunit cDNA, reveals that the mRNA is about 4.6 Kb long and its maximum expression is at the mesenchyme blastula and the gastrula stages (Mitsunaga-Nakatsubo et al., 1992b).

In this study, we have isolated the sea urchin cDNA clone coding Na⁺, K⁺-ATPase α-subunit, deduced its primary structure from the nucleotide sequence and compared it with those of various metazoans.
MATERIALS AND METHODS

Culture of embryos
Games of the sea urchin, Hemicentrotus pulcherrimus, were obtained by an intracoelomic injection of 0.5 M KCl. Eggs were washed with artificial sea water (ASW), inseminated and allowed to develop at 20°C.

Extraction of total RNA from embryos
Total embryonic RNA was extracted from embryos by the guanidium/hot phenol method (Feramisco et al., 1982). Poly(A)+ RNA was fractionated using oligo-(dT) cellulose spin columns (Clontech Lab., Inc.) according to the manufacturer's procedure.

Screening
First screening: 10⁶ clones of the cDNA library constructed from the poly(A)+ rich RNA of prism stage embryos (Akasaka et al., 1987) were screened with a 47-mer oligonucleotide probe. The probe (5'GGGAGTGATCGCTGGTGATGACCTGGTGCCAGGCG3') corresponds to a FSBA binding site (GAIVAVTGVDVPISPHHKH) at the amino terminal region of rat Na+, K+-ATPase α-subunit (Shull et al., 1986). This is a highly conserved region among eukaryotes. The oligonucleotide was 5'-end ³²P-labeled with T4-polynucleotide kinase and used as the probe for plaque hybridization as described previously (Mitsunaga-Nakatsubo et al., 1992b). Ten positive clones not containing the amino terminal region were obtained.

Second screening: To obtain longer clones carrying the amino terminal region for Na+, K+-ATPase α-subunit, another cDNA library of the gastrula stage was constructed using a 20-base synthetic primer (5'GCIGGGGAGTCATTGACACCGTCICCIGTTACAGCCACAATGGCACC3') synthesized based on its amino acid sequence (K. Mitsunaga-Nakatsubo, K. Yamazaki et al., 1986). Nucleotide sequences of Na+, K+-ATPase α-subunit: phosphorylation site, FITC-binding site and two FSBA-binding sites. For a probe to screen the insert for sequencing.

Extraction of total RNA from embryos
Total embryonic RNA was extracted from embryos by the guanidium/hot phenol method (Feramisco et al., 1982). Poly(A)+ RNA was fractionated using oligo-(dT) cellulose spin columns (Clontech Lab., Inc.) according to the manufacturer's procedure.

Screening
First screening: 10⁶ clones of the cDNA library constructed from the poly(A)+ rich RNA of prism stage embryos (Akasaka et al., 1987) were screened with a 47-mer oligonucleotide probe. The probe (5'GGGAGTGATCGCTGGTGATGACCTGGTGCCAGGCG3') corresponds to a FSBA binding site (GAIVAVTGVDVPISPHHKH) at the amino terminal region of rat Na+, K+-ATPase α-subunit (Shull et al., 1986). This is a highly conserved region among eukaryotes. The oligonucleotide was 5'-end ³²P-labeled with T4-polynucleotide kinase and used as the probe for plaque hybridization as described previously (Mitsunaga-Nakatsubo et al., 1992b). Ten positive clones not containing the amino terminal region were obtained.

Second screening: To obtain longer clones carrying the amino terminal region for Na+, K+-ATPase α-subunit, another cDNA library of the gastrula stage was constructed using a 20-base synthetic primer (5'GCIGGGGAGTCATTGACACCGTCICCIGTTACAGCCACAATGGCACC3') synthesized based on its amino acid sequence (K. Mitsunaga-Nakatsubo, K. Yamazaki et al., 1986). Nucleotide sequences of Na+, K+-ATPase α-subunit: phosphorylation site, FITC-binding site and two FSBA-binding sites. For a probe to screen the insert for sequencing.

RESULTS AND DISCUSSION

Several conserved regions have been reported in active sites of Na+, K+-ATPase α-subunit: phosphorylation site, FITC-binding site and two FSBA-binding sites. The FSBA-binding sites located at the C-terminal region was selected and an antisense oligonucleotide (47 mer) was chemically synthesized based on its amino acid sequence reported in mammals (Shull et al., 1986). 10⁶ recombinant phages from a λgt10 cDNA library of the prism stage embryos were screened, as described previously (Mitsunaga-Nakatsubo et al., 1992b). Ten positive signals were obtained. They included the nucleotide sequences encoding amino acid sequence of the probe region, whereas none of them had the amino terminal region. Clone SUA5 was selected and its nucleotide sequence was determined (Fig. 1).

To obtain cDNA clones carrying the N-terminal region, a cDNA library of the gastrula stage was constructed using oligonucleotide primer corresponding to the antisense strand sequence near the 5'-end of clone SUA5. 10⁶ recombinant phages were screened with the 5' region of SUA5 (268 bp fragment digested with EcoRI and HindIII as shown in Fig. 1). A 779 bp cDNA clone with a 42-bp overlap at the 5'-end of clone SUA5 (SUA51) was obtained (as shown in Fig. 1). cDNA fragment including this overlapping region could be amplified by RT-PCR method using two pairs of primers, which locate opposite sides of the region (HNKUP, 689–710 and HNKDOW, 1498–1518 or ISOCCH2, 1578–1597; nucleotide numbers correspond to Fig. 2). In addition, Northern blot analysis with probes of specific fragment from each cDNA clone revealed identical size and expression patterns of mRNA during development (data not shown). These results suggest that SUA5 and SUA51 are the cDNA clones coding the same mRNA of Na+, K+-ATPase α-subunit.

The 5'-noncoding region of cDNA was amplified by the RACE method of Frohman et al. (1988) to determine its sequence. First strand cDNA was synthesized using the antisense primer (ISOCHK2; 20 mer from the position 1578 to 1597 in Fig. 2). Deoxycytidine was polymerized to the 3'-end of the first strand, using dCTP and terminal deoxynucleotidyl transferase. PCR was performed with an oligo(dG)₁₅₋₁₈ primer and Primer4 (at positions 334-353 in Fig. 2) located upstream to the ISOCHK2, which was used for first strand cDNA synthesis. PCR-amplified DNA was extracted and ligated with cosmid vector Charmid 9-42 (Nippon Gene, Toyama), to analyze the insert for sequencing.

Nucleotide sequence of Na+, K+-ATPase α-subunit
The EcoRI fragments of the cDNAs (SUA5 and SUA51) were inserted into pUC118 or pUC119 and the overlapping subclones were generated using the stepwise deletion method (Heinkoff et al., 1984). DNA sequencing was performed using the Sequenase version 2.0 7-deaza-dGTP kit (United State Biochemical Corp., USA) and both strands of the cDNA were sequenced.

Sequence alignment
Sequence alignment and numbers of nucleotide substitutions per site were estimated using in algorithm method described by Gotoh (1993). Construction of phylogenetic tree was performed by the neighbor-joining method (Saitou and Nei, 1987).

Chemicals
[³²P]ATP and [α-³²P]dCTP were purchased from Radiochemical Centre, Amersham, Bucks, UK. Restriction endonucleases and the other enzymes were obtained from Takara Shuzo Co., Ltd., Kyoto, Toyobo Co., Ltd., Japan and Nippon Gene Co., Ltd., Japan. Sequenase version 2.0 7-deaza-dGTP kit and random primer labeling kit were the products of the United States Biochem. Co., USA and Nippon Gene Co. Ltd., Japan, respectively. Artificial sea water was obtained from Jamarin laboratory, Osaka, Japan.

RESULTS AND DISCUSSION

Several conserved regions have been reported in active sites of Na+, K+-ATPase α-subunit: phosphorylation site, FITC-binding site and two FSBA-binding sites. For a probe to screen the cDNA of sea urchin Na+, K+-ATPase α-subunit, one of the FSBA-binding sites located at the C-terminal region was selected and an antisense oligonucleotide (47 mer) was chemically synthesized based on its amino acid sequence reported in mammals (Shull et al., 1986). 10⁶ recombinant phages from a λgt10 cDNA library of the prism stage embryos were screened, as described previously (Mitsunaga-Nakatsubo et al., 1992b). Ten positive signals were obtained. They included the nucleotide sequences encoding amino acid sequence of the probe region, whereas none of them had the amino terminal region. Clone SUA5 was selected and its nucleotide sequence was determined (Fig. 1).

To obtain cDNA clones carrying the N-terminal region, a cDNA library of the gastrula stage was constructed using oligonucleotide primer corresponding to the antisense strand sequence near the 5'-end of clone SUA5. 10⁶ recombinant phages were screened with the 5' region of SUA5 (268 bp fragment digested with EcoRI and HindIII as shown in Fig. 1). A 779 bp cDNA clone with a 42-bp overlap at the 5'-end of clone SUA5 (SUA51) was obtained (as shown in Fig. 1). cDNA fragment including this overlapping region could be amplified by RT-PCR method using two pairs of primers, which locate opposite sides of the region (HNKUP, 689–710 and HNKDOW, 1498–1518 or ISOCCH2, 1578–1597; nucleotide numbers correspond to Fig. 2). In addition, Northern blot analysis with probes of specific fragment from each cDNA clone revealed identical size and expression patterns of mRNA during development (data not shown). These results suggest that SUA5 and SUA51 are the cDNA clones coding the same mRNA of Na+, K+-ATPase α-subunit.

The 5'-noncoding region of cDNA was amplified by the 5'-rapid amplification of cDNA ends (RACE) method. One of clones, R4 had a 346 bp overlapping region of SUA51 and extended 7 bp upstream. The extended length of the clone R4 nucleotide sequence corresponded closely to the major transcription start site determined by primer extension (data not shown). Therefore, the nucleotide sequence combining SUA5, SUA51 and R4 together is shown as a single sea urchin Na+, K+-ATPase α-subunit.
Sea Urchin Na\(^+\), K\(^+\)-ATPase \(\alpha\)-Subunit cDNA

K\(^+\)-ATPase \(\alpha\)-subunit sequence (Fig. 2). The total number of nucleotides is 4401. The cDNA has a long untranslated sequence in the 3' region with a poly(A) additional signal AATAAA approximately 20 nucleotides upstream from the start of the 3'-poly(A) tail. The first methionine codon within the open reading frame is located upstream of a lysine-rich cluster (at positions 115–168, amino acid residues 37–54), which is reported to be a putative ion-selectable domain of Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit. A termination codon is found next to Tyr-1038. The molecular weight of the protein is calculated to be 114 kDa.

As shown in Fig. 3, the overall hydropathy profile of the \(\alpha\)-subunit is similar to that of sheep kidney (Shull et al., 1985). Eight major hydrophobic regions are indicated as H1-H8 in the figure. Alignment of amino acid sequences of sea urchin \(\alpha\)-subunit with those of diverse species also showed high degrees of sequence similarity in these hydrophobic regions (Fig. 4). Therefore, it is suggested that at least eight putative transmembrane domains also exist in sea urchin Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit. The regions between H1-H2, H3-H4, H5-H6 and H7-H8, respectively, are putative short extracellular loops, whereas the domains between H2-H3 and H4-H5, respectively, are to be longer cytoplasmic loops. The amino acid sequence of the most highly conserved region, H4, matches almost perfectly between sea urchin and other species, whereas the average identity of the entire length is about 76%. Signal peptide could not be identified in the amino terminal region. Like other Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit reported in diverse species (Kawakami et al., 1985, 1986; Ovchinnikov et al., 1986; Shull et al., 1985), the signal peptide is not included in the sea urchin ATPase.

The catalytic sites, reported to be present in the cytoplasmic regions (Lingrel et al., 1990; Lingrel and Kuntzweiler, 1994), were also identified in the region between H4 and H5 transmembrane sites, and are all highly conserved in the amino acid sequences (Fig. 4). Phosphorylation site around the aspartyl residue (no. 393) almost perfectly matches with those of other species. ATP binding-site around the Lys (at 523) probed with fluorescein 5'-isothiocyanate (FITC) is also highly homologous. Binding sites of FSBA, another ATP analogue (AX;VHVGSDDLK (I) and QGAIVAVGDVNS-PALX;K (II)) (Ohta et al., 1986) are also identified in the sea urchin Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit. Especially, the latter FSBA-binding site FSBA II, used as a probe for screening, matches almost perfectly except one amino acid residue at the N-terminal end of this region. On the contrary, the deduced N-terminal amino acid sequence for sea urchin differs from the sequences reported in vertebrates (Fig. 4) and is comparatively long like that for Drosophila melanogaster.

The activity of sea urchin embryonic Na\(^+\), K\(^+\)-ATPase is ouabain-sensitive (Kinsey et al., 1980; Ciapa et al., 1984; Mitsunaga et al., 1986, 1989). It has been reported that uncharged amino acids are present at the terminus of H1-H2 extracellular domain of every ouabain-sensitive Na\(^+\), K\(^+\)-ATPase, and the substitutions of the amino acids into charged amino acids invariably produce a resistant enzyme (Price et al., 1989; Horisberger et al., 1991; Lingrel and Kuntzweiler, 1994). In sea urchin, one of the termini of H1-H2 is negatively charged (Glu at 135), though the other is not (Asn at 146). Relatively high concentration of ouabain necessary for inhibition of sea urchin Na\(^+\), K\(^+\)-ATPase activity, may be due to the amino acid residues at the termini of the H1-H2 extracellular domain. In Na\(^+\), K\(^+\)-ATPase of Artemia, which have low affinity for ouabain, the same amino acids as in sea urchin are present at the termini of H1-H2.

Three kinds of Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit isoforms, which are products of different genes, were demonstrated in chicken (Takeyasu et al., 1988, 1990), rat (Shull et al., 1986) and...
Fig. 2. Nucleotide sequence of cDNA for sea urchin Na\(+\), K\(+\)-ATPase \(\alpha\)-subunit and its deduced amino acid sequence. The nucleotide sequence is a combination of SUA5, SUA51 and R4. The number of the nucleotide residues is given at the right end of each line. The deduced amino-acid sequence is shown below the nucleotide sequence. Eight major hydrophobic regions (H1-H8), likely to be transmembrane domains, are boxed. Catalytic sites in the cytoplasmic region were identified by homology with diverse ATPases shown by underlines. P: phosphorylation site, FITC: fluorescein isothiocyanate (FITC) binding region, FSBA I, II: 5'-fluorosulfonyl)-benzoyl-adenosine binding regions.

human (Kawakami et al., 1986; Ovchinnikov et al., 1988; Shull et al., 1989). Two kinds of isoforms were also reported in Artemia (Baxter-Lowe et al., 1989; Macías et al., 1991). However, only a single gene for \(\alpha\)-subunit was found in Drosophila melanogaster (Lebovitz et al., 1989). A region of significantly low homology between the isoforms of vertebrate was reported to be locating 11 amino acid upstream from the lysine residue in the FITC-binding site (Lingrel et al., 1990). This region deduced from the sea urchin cDNA resembles that of \(\alpha_3\).

A possible evolutionary relationship of Na\(+\), K\(+\)-ATPase \(\alpha\)-subunit is proposed in Fig. 5. The tree suggests that the sea urchin Na\(+\), K\(+\)-ATPase \(\alpha\)-subunit has evolved from a common ancestor of vertebrates and has diverged before the three vertebrate isoforms (\(\alpha_1\), 2 and 3) appeared. Iwabe et al. (1996) also reported a phylogenic tree of ion transport ATPases, and also proposed that evolution of these tissue-specific isoforms occurred after the separation of invertebrate and vertebrate. Two isoforms of Artemia were suggested to occur after vertebrate-invertebrate divergence (Macías et al., 1991).
Fig. 3. Kyte-Doolittle hydropathy plot of Na\(^{+}\), K\(^{+}\)-ATPase \(\alpha\)-subunit (Kyte and Doolittle, 1982). The window was set at 20 amino acids. On the abscissa, the position in the primary amino acid sequence is indicated. The numbers on the ordinate are the relative hydrophobicity values, hydrophobic being positive and hydrophilic being negative. The hydrophobic peaks corresponding to the proposed transmembrane regions are indicated (H1-H8).

1991). However, biochemical analysis of Hydra Na\(^{+}\), K\(^{+}\)-ATPase reveals the existence of two types of ouabain-sensitive ATPase (Canfield et al., 1992). Analysis of the isoforms in sea urchin embryos is now under investigation.

ACKNOWLEDGMENTS

The authors wish to express their hearty thanks to Dr. O. Gotoh of Saitama Cancer Center for his kind help of constructing the phylogenetic tree and alignment of Na\(^{+}\), K\(^{+}\)-ATPase. We are also grateful to Dr. H. Yonekawa of the Tokyo Metropolitan Institute of Medical Science for his helpful advice on the construction of phylogenetic tree and evolution.

REFERENCES

Fig. 4. Alignment of amino acid sequences of \(\alpha\)-subunits of the Na\(^+\), K\(^+\)-ATPase from the sea urchin *Hemicentrotus pulcherrimus* (SU), *Hydra vulgaris* (HD; Canfield et al., 1992), the brine shrimp *Artemia franciscana* (AR; Macías et al., 1991), *Drosophila melanogaster* (DM; Levobitz et al., 1990), the electric ray *Torpedo californica* (TC; Kawakami et al., 1985), chicken (C1, C2 and C3; \(\alpha_1\), \(\alpha_2\) and \(\alpha_3\) isoforms respectively, Takeyasu et al., 1988, 1990) and sheep (SP; Shull et al., 1985). Sequences are shown in one-letter amino acid code. Gaps (-) are inserted in the sequences to achieve maximum alignment. Identical amino acids, relative to the *H. pulcherrimus* sequence, are indicated by dots. The eight putative transmembrane segments proposed by Shull et al. (1985) for the sheep kidney \(\alpha\)-subunit are underlined and labeled with H1-H8. The active sites identified by homology with other ATPases are indicated by underlines (P: phosphorylation site, FITC: FITC-binding region, FSBAI, II: FSBA-binding regions).
Fig. 5. Phylogenetic analysis of Na⁺, K⁺-ATPase α-subunit amino acid sequences. The tree was built using the neighbor-joining method applied to a categories distance matrix on the basis of the deduced amino acid sequences coded by sea urchin, *Hydra vulgaris*, *Drosophila melanogaster* (Levobitz et al., 1991), chicken (*Gallus domesticus*), white sucker (*Catostomus commersoni*), giant toad (*Bufo marinus*), rat α1-3 (*Shull et al., 1988; Ovchinnikov et al., 1986), sheep (*Ovis aries*), and human α1-3 (Kawakami et al., 1986; Shull et al., 1989; Ovchinnikov et al., 1988). The horizontal bar represents 1 amino acid replacement per 100 sites.


(Received June 24, 1996 / Accepted August 12, 1996)