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A marine algal Na^+-activated ATPase possesses an immunologically identical epitope to Na^+,K^+-ATPase

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Immunological homology was investigated between *Heterosigma akashiwo* (a marine alga) Na^+-activated ATPase and animal Na^+,K^+-ATPase. The former polypeptide [(1989) Plant Cell Physiol. 30, 923–928] reacted with anti-serum raised against the amino-terminal half of the pig kidney Na^+,K^+-ATPase α subunit. It is suggested that the Na^+,K^+-ATPase epitope within the amino-terminal region is conserved in the plant Na^+-activated ATPase, and the region containing the epitope may be important for Na ion transport.

Akashiwo Na^+-ATPase; Na^+,K^+-ATPase; Epitope mapping; Antibody

1. INTRODUCTION

The plasma membrane of the marine alga raphidophycean biflagellate *Heterosigma akashiwo* contains an Na^+-activated ATPase which plays an important role in maintaining the low intracellular sodium concentration of the organism in sea water. In our previous paper [1], the Na^+-activated ATPase was shown to be sensitive to vanadate, to form a phosphorylated intermediate in the presence of Na^+ and to be dephosphorylated in the presence of K^+, suggesting a similar reaction mechanism to that of Na^+,K^+-ATPase, which exists exclusively in animal cells [2,3], even though the molecular weights of these ATPases are not the same, i.e., 140 kDa for *H. akashiwo* ATPase [1] and 110 kDa for Na^+,K^+-ATPase α subunit [4]. To see whether there is any structural homology between them, we used polyclonal antibodies against the amino-terminal half of the pig Na^+, K^+-ATPase α1 polypeptide. It is suggested that Na^+, K^+-ATPase and *H. akashiwo* Na^+-activated ATPase have a common epitope.

2. MATERIALS AND METHODS

2.1. *Cell culture and cell fractionation*

An axenic clone of *H. akashiwo* (strain No. OHE-1) was purchased from the National Institute for Environmental Studies in Japan. Culture conditions and medium components were the same as described in the previous paper [1]. Plasma membrane was obtained according to the silica microbeads method as described before [1].

2.2. *Serum preparation*

Rabbit anti-serum (Antibody PK) was raised against pig kidney Na^+,K^+-ATPase (specific activity of 1,180 μmol P_i/mg/h). Pre-immune serum was obtained from an unimmunized rabbit.

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2.3. *Immunoblotting*

SDS-PAGE was performed by the method of Laemmli [4]. Proteins on the gel were electrophoretically transferred to a nitrocellulose filter [5]. After blocking with 0.1% (w/v) polyoxyethylene sorbitan monolaurate (Tween-20) and 0.1% (w/v) gelatin in phosphate-buffered saline (PBS) for 1 h, the filter was incubated with the same buffer containing 1,000-fold-diluted Antibody PK for 1 h with gentle agitation. After rinsing with PBS, the filter was incubated in 1,000-fold-diluted horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Laboratories, Inc.) in PBS containing 0.1% gelatin for 1 h. After rinsing with PBS, the antibody binding was visualized by reaction with diaminobenzidine and hydrogen peroxide.

2.4. *Affinity purification of antibody*

The α and β subunits of sheep kidney Na^+,K^+-ATPase were separated by SDS-PAGE and blotted onto a nitrocellulose filter [6]. After rinsing with PBS containing 0.1% gelatin, the filter was treated with Antibody PK at room temperature for 12 h, and washed with PBS containing 0.1% Tween-20 three times and then with PBS three times. The antibody-adsorbed filter was incubated in 0.1 M glycine-HCl (pH 2.5) buffer for 2 min to dissociate each mono-specific antibody and the eluates were neutralized with 1 M Tris-HCl (pH 9.0) and used as α and β-monospecific antibody, respectively.

2.5. *Identification of phosphorylated intermediate*

The phosphorylated intermediate of Na^+-activated ATPase was identified by acid SDS-PAGE as described previously [1].

2.6. *Proteolysis of the Na^+,K^+-ATPase α1 subunit*

Selective tryptic cleavage of the α1 subunit of sheep kidney Na^+, K^+-ATPase was carried out as described before [7].

3. RESULTS AND DISCUSSION

3.1. *Antibody binding sites*

Anti-pig Na^+,K^+-ATPase rabbit serum (Antibody PK) consists of anti-α and anti-β subunit antibodies

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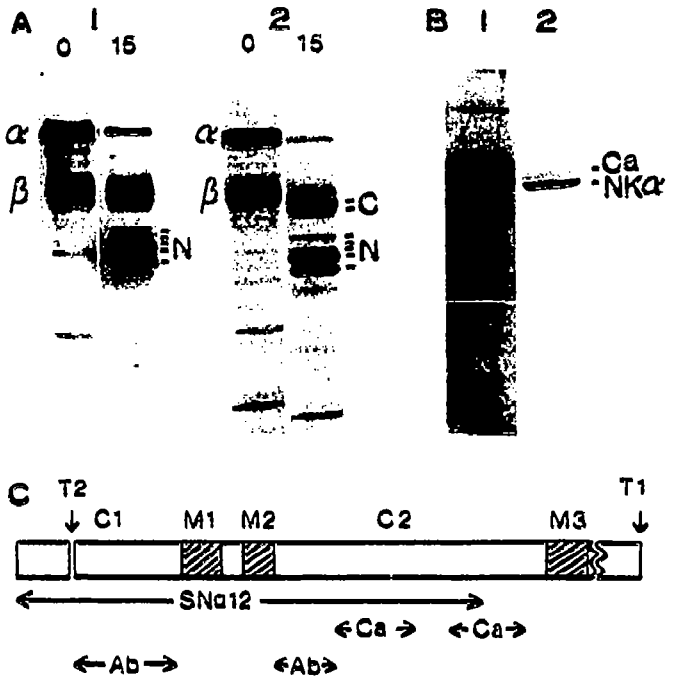

Fig. 1. Identification of antibody binding sites. (A) Reaction of Antibody PK with tryptic fragments of sheep kidney Na^+, K^+-ATPase. Na^+,K^+-ATPase was treated with trypsin for the indicated number of minutes as previously described [7]. Samples of the digested Na^+, K^+-ATPase (0.8 μg protein for immunoblotting) were submitted to SDS-PAGE and transferred to a nitrocellulose filter. The blot was incubated with Antibody PK and the bound antibodies were detected as described in section 2 (A1). (A2) Coomassie brilliant blue staining of the gel after SDS-PAGE. α, α subunit; β, β subunit; N, amino-terminal fragment and subfragments of the α subunit; C, carboxy-terminal fragments. (B) Reaction of Antibody PK with Ca^{2+}-ATPase. Sarcoplasmic reticulum (9.6 μg) (kindly provided by Dr. M. Kawakita, Tokyo University) from rabbit skeletal muscle was submitted to SDS-PAGE and the proteins were analyzed by immunoblotting with Antibody PK: antibody staining (B2) and Coomassie brilliant blue staining (B1). Ca, Ca^{2+}-ATPase; NKα, contaminating Na^+, K^+-ATPase α subunit. (C) Location of Antibody PK binding sites on amino-terminal fragment of the α1 subunit (amino-terminus to the left). T1, primary trypsin cleavage site; T2, secondary cleavage site; M1, M2 and M3, transmembrane segments 1, 2 and 3, respectively; C1 and C2, cytoplasmic domain 1 and 2, respectively; SNα12, SNα-1,2 peptide (9); Ca, homologous regions to sarcoplasmic reticulum Ca^{2+}-ATPase (11); Ab, presumed binding sites of Antibody PK.

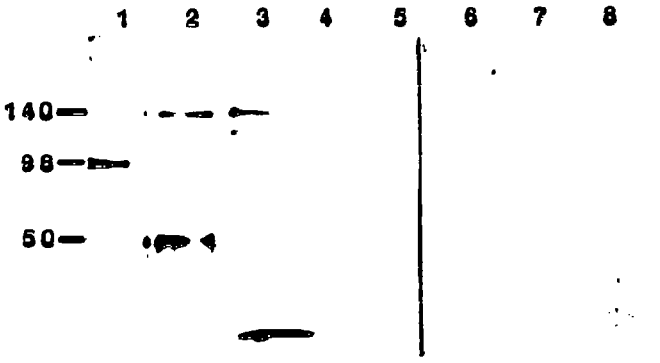

Fig. 2. Distribution of 140 kDa antigen of *H. akashiwo* plasma membrane fraction. Each subcellular fraction obtained by the silica microbeads method was analyzed by immunoblot analysis. Lane 1, sheep kidney Na^+,K^+-ATPase (100 ng). Lanes 2 and 5, plasma membrane fraction of *H. akashiwo* (1 μg). Lanes 3 and 6, bead-unbound membrane fraction (10 μg). Lanes 4 and 7, supernatant fraction (10 μg). After SDS-PAGE and blotting, nitrocellulose filters were incubated with Antibody PK (lanes 1, 2, 3 and 4) or non-immune serum (lanes 5, 6 and 7) and the bound antibodies were detected as described in section 2.

(see 0 min in A1 of Fig. 1). To examine the major binding sites of the anti-α antibodies on the sheep α1 subunit, we used immunoblot analysis on ligand-dependent proteolytic fragments of the sheep α1 subunit. In the presence of K^+, trypsin initially cleaved a peptide bond (the site T1 between Arg-438 and Ala-439 of sheep α1) near the center of the α1 peptide, followed by a secondary cleavage in the amino-terminal region (close to the site T2 between Lys-30 and Glu-31) (A2 in Fig. 1), in agreement with previous observations [7,8]. Antibody PK strongly stained the amino-terminal fragment and its further digested subfragments, but did not stain the carboxy-terminal fragment (see 15 min in A1 of Fig. 1). This result is consistent with the observation that the antibody-binding sites on the α1 subunit of the Na^+, K^+-ATPase are located mainly on the amino-terminal half (Gly-1 to Arg-438) [8]. Antibody PK immunoprecipitated an in vitro translocation product of SNα-1,2 [9], the amino-terminal fragment (31 kDa, Fig. 1C) of the human Na^+,K^+-ATPase α1 subunit, but did not immunoprecipitate that of SNα-3,4 [9], the fragment containing the 3rd and 4th transmembrane regions (Dr. H. Homareda, unpublished result). The antibody did not cross-react with rabbit sarcoplasmic reticulum Ca^{2+}-ATPase (Fig. 1B). As the amino acid sequences of the phosphorylation site and the ATP binding site were conserved in both Na^+,K^+-ATPase α subunit and Ca^{2+}-ATPase, these conserved regions cannot contain the epitope to the antibody. The antibody stained three α isoforms, i.e., α1, α2 and α3, of Na^+,K^+-ATPase [10]. Thus, the Antibody PK seems to have been raised against a well-conserved region within the amino-terminal halves of the three α-isoforms, i.e. the cytoplasmic domain 1 (Glu 31-Gln 88 in α1 subunit) and/or the domain 2 (Ser 140-Ser 172 in α1 subunit) (Fig. 1C).

3.2. *Immunological detection and evaluation of antigen*

Fig. 2 shows the immunological cross-reactivity of Antibody PK with proteins of the plasma membrane fractions obtained from *H. akashiwo* cells by the silica microbeads method. In the previous paper [1], we reported that highly purified plasma membrane was collected into the bead-bound fraction (M+B) with more than 60% recovery, and the residual plasma membrane escaped into the bead-unbound fraction (M-B). Anti-

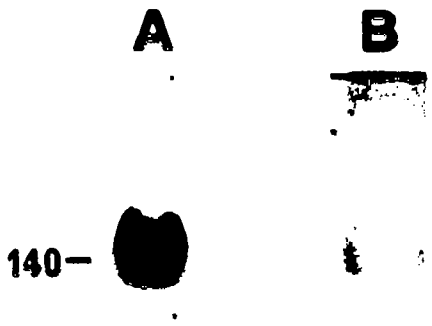

Fig. 3. Immunoblot analysis of the phosphorylated intermediate. The phosphorylated Na^+-activated ATPase in the bead-bound plasma membrane fraction (20 μg) was subjected to acid SDS-PAGE as described before [1]. Lane A shows the autoradiogram of the phosphorylated intermediate and lane B shows the immunoblot of the phosphorylated intermediate.

body PK cross-reacted with 140 kDa polypeptide of both M+B (lane 2 in Fig. 2) and M-B (lane 3) fractions, but not with the proteins of the supernatant fraction (lane 4). The distribution of the cross-reacted 140 kDa polypeptide corresponded to that of plasma membrane and to that of Na^+-activated ATPase activity (data not shown). No cross-reactivity was observed with pre-immune serum used as a control (lanes 5, 6 and 7). The phosphorylated intermediate of the Na^+-activated ATPase was investigated with Antibody PK. A polypeptide that cross-reacted with Antibody PK was detected at the same position (Fig. 3B) as the phosphorylated intermediate, suggesting the cross-reactive peptide is a P-type ATPase molecule.

In order to confirm that the α subunit of the animal Na^+,K^+-ATPase had homology to the 140 kDa polypeptide of *H. akashiwo* cells, monospecific antibodies to the α and β subunits of sheep kidney Na^+,K^+-ATPase were purified and allowed to react with the *H. akashiwo* plasma membrane. Anti-α monospecific antibody stained the 140 kDa polypeptide, but anti-β monospecific antibody did not (data not shown).

These results show that the catalytic polypeptide of the *H. akashiwo* Na^+-activated ATPase has Na^+,K^+-ATPase-specific epitopes, which are located on the amino-terminal half of the Na^+,K^+-ATPase α subunit. The immunologically homologous sites between these ATPases were different from other conserved sites of P-type ATPases such as ATP-binding and phosphorylation sites [2,3,10]. These results suggest that the *H. akashiwo* Na^+-activated ATPase belongs to the P-type transporting ATPase family, which includes Na^+,K^+-ATPase [10,12], Ca^{2+}-ATPase [13] and gastric H^+,K^+-ATPase [14]. The homologous region of Na^+,K^+-ATPase α subunit with *H. akashiwo* Na^+-activated ATPase may contribute to a crucial aspect of the sodium-transport mechanism, such as ion-discrimination or ion-gating.

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