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Cell Surface Expression of a Specific Antigenic Site on the Catalytic Subunit of $(Na^+ + K^+)$-ATPase

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Structural localization of a peptide region, KRQPRNPKTDKLVNE, in the catalytic subunit of $(Na^+ + K^+)$-ATPase was investigated using a specific antibody directed against this peptide in cultured African green monkey kidney CV-1 cells. Immunofluorescence staining of frozen cell sections shows that an anti-KRQPRNPKTDKLVNE antibody (SSA95) interacts with its antigenic site and binds to the extracellular side of the cell membrane. Indirect immunofluorescence and flow cytometric analyses confirmed the presence of this epitope on intact cell surfaces. These results suggest that the KRQPRNPKTDKLVNE region of the $(Na^+ + K^+)$-ATPase is expressed on the cellular membrane surface.

Key Words: **$(Na^+ + K^+)$-ATPase; site-specific antibody; flow cytometry; immunofluorescence staining; positive inotropy.**

$(Na^+ + K^+)$-ATPase (1) is an integral membrane protein that transports three Na^+ out of the cell and two K^+ into the cell for each ATP hydrolyzed (2–5). Purified $(Na^+ + K^+)$-ATPase is composed of two polypeptides referred to as α and β subunits (6–11). The α-subunit, consisting of 1016 amino acids (12, 13), is the catalytic subunit (14) responsible for the active transport of the Na^+ and K^+ ions across the plasma membrane of all animal cells (15). The β-subunit is a glycoprotein (16) 302 amino acids in length, whose physiological role in cation transport remains obscure (17, 18). Despite extensive study, the membrane folding structure of the α-subunit of the $(Na^+ + K^+)$-ATPase enzyme remains controversial. Functional studies have shown that antibodies raised to the epitope KRQPRNPKTDKLVNE, in the catalytic subunit of $(Na^+ + K^+)$-ATPase of cardiac myocytes competes with [^{3}H]ouabain labeling and induces a positive inotropic effect (19). To date, there are two major conflicting molecular models regarding the localization of KRQPRNPKTDKLVNE motif in the α-subunit of the enzyme. Model I suggests that this epitope is located on extracellular side of the membrane (12, 18, 20). Model II suggests that it is in the intracellular side (21). The functional studies are consistent with Model I (19). To further examine the membrane location of this region, a polyclonal antibody (SSA95) raised to the epitope KRQPRNPKTDKLVNE, was used to map this antigenic site in the endogenous $(Na^+ + K^+)$-ATPase in CV-1 cells. Three complementary approaches, immunofluorescent staining of section cell suspensions, flow cytometric analysis, and Victor-2 multilabel counter measurement were employed, and the results convincingly demonstrate that the KRQPRNPKTDKLVNE region of the $(Na^+ + K^+)$-ATPase is expressed on the extracellular surface and is accessible to interact with SSA95 to generate a biological effect.

MATERIALS AND METHODS

CV-1 cells and site-specific antibody. African green monkey kidney CV-1 cells were obtained from the American Type Culture Collection (Rockville, MD). A peptide with the amino acid sequence, KRQPRNPKTDKLVNE, was synthesized and site-specific polyclonal antibody (SSA95) against this peptide sequence was generated in New Zealand white rabbits using KLH as a peptide carrier (Genemed, South San Francisco, CA). SSA95 was further purified through an affinity column directed against the same synthetic peptide of the $(Na^+ + K^+)$-ATPase. Western blots analyses indicate that SSA95 specifically recognizes $(Na^+ + K^+)$-ATPase and does not cross-react with Ca^{2+}-ATPase (19). The synthetic peptide was also utilized as a specific peptide blocker (PB95).

Immunofluorescent staining. Cultured African green monkey CV-1 cells were dissociated from the cell-growth container by 0.2% EDTA (Sigma Chemical Co., St. Louis, MO) solution, covered with

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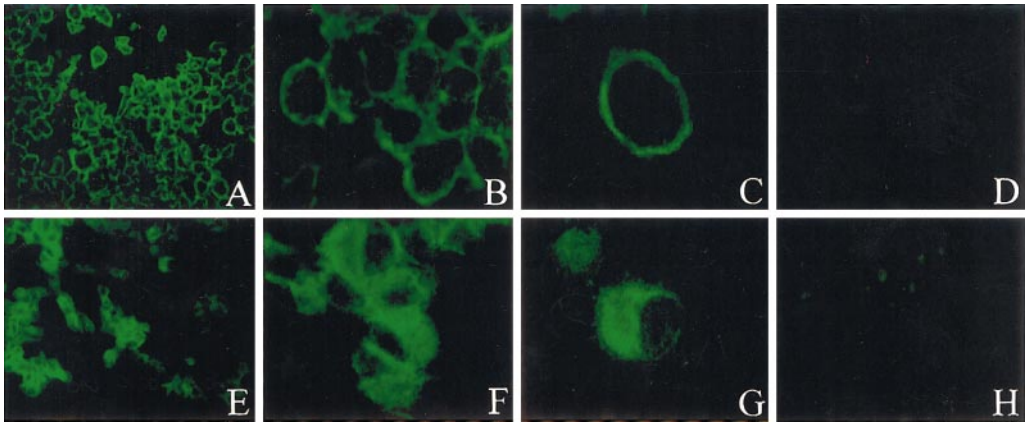

FIG. 1. Visualization of SSA95 bound to CV-1 cell membrane by immunofluorescence. Nonpermeabilized African green monkey CV-1 cells were embedded in Tissue-Tek OCT medium, frozen, and sectioned at 8 μm. The sections were blocked with 10% goat serum and 1% BSA in PBS and incubated with or without SS95 as described in the methods. (Column 1) CV-1 cell sections staining at a magnification of 400× (A and E). (Column 2) A group of fluorescent-labeled CV-1 cell image at 1500× (B and F). (Column 3) Single CV-1 cell image at 2000× (C and G). (Column 4) Population of cells in the presence of secondary antibody (D) or specific peptide blocker PB95 (H) at 400×. (Top row) Cross-section of CV-1 cells. (Bottom row) Sections retained large cell membranes. Similar results were obtained in four separate experiments. The results indicate that SSA95 antibody binds to its antigenic site of the $(Na^+ + K^+)$-ATPase on the surface of the cell membrane.

OCT mounting medium, and frozen in liquid nitrogen as described previously (22). Sections (8 μm) of each tissue type were cut on a cryostat, collected on silane-coated slides, and air dried for 30 min. The sections were incubated with blocking solution containing 1% bovine serum albumin (BSA) and 10% normal goat serum in phosphate buffered saline (PBS, pH 7.4) for 60 min at room temperature. Following incubation with SSA95 (1:100) for 60 min, the slides were washed in PBS three times prior to being incubated with a fluorescein-isothiocyanate (FITC) conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA; diluted 1:75) for 1 h at room temperature. Washed slides were treated with antifade glycerol (Molecular Probes, Inc., Eugene OR). The slides were evaluated and photographed with an epifluorescence microscope (Olympus BX60) equipped with appropriate filter sets to allow visualization of fluorescein.

Flow cytometric analysis. Membrane surface expression of the KRQPRNPKTDKLVNE region of $(Na^+ + K^+)$-ATPase was analyzed using indirect immunofluorescence and flow cytometry as previously described (23) with modifications. All experimental steps were performed at room temperature. Briefly, native CV-1 cells (approximately 60,000 cells/sample) were first incubated with purified Fc fragments of rabbit IgG (28 μg/ml, Jackson Laboratories) for 60 min, then in another blocking solution containing 0.2% BSA and 10% normal goat serum in PBS for an additional 60 min. The cells were washed and then incubated with SSA95 (28 μg/ml) or with SSA95 + PB95 (1:500) for 2 h. The control CV-1 cell sample was incubated with purified rabbit IgG (28 μg/ml, Sigma). Washed cells were then incubated with FITC-conjugated goat anti-rabbit IgG (Jackson Laboratories) for 1 h. Labeled cells were washed and analyzed using a Becton–Dickinson FACSCalibur flow cytometer after excitation at 488 nm, and for each sample at least 55,000 events were collected.

Victor-2 multilabel counter measurement. CV-1 cells were freshly prepared as described for the flow cytometry experiment. Fluorescent data was acquired using a Victor-2 multilabel counter (E. G. Wallac, Inc., Perkin–Elmer Life Sciences). Samples of 200 μl were loaded into 96-well microtiter black plates and excitation was carried out at 485 nm and emission spectra at 535 nm were collected with 1 second integration. The different conditions were read in triplicate. Experimental data are presented as mean of standard deviation. Paired Student's *t* test was used to determine significance.

RESULTS

Extracellular Immunofluorescent Staining of a Specific Antigenic Site

To investigate the membrane expression of the KRQPRNPKTDKLVNE region of $(Na^+ + K^+)$-ATPase, we first visualized the membrane location of this region of the enzyme in CV-1 cell frozen sections by immunofluorescent staining with SSA95. Figure 1 demonstrates that SSA95 interacts with its antigen on the cell surface membrane. Fluorescence is concentrated at the cell membrane in populations of cells viewed at 400× and 1500× (Figs. 1A and 1B) and is clearly evidence in a higher power view (2000×) of a single cell (Fig. 1C). Moreover, the fluorescence only stained CV-1 cell membrane surface when cut-sections retained large cellular membrane (Figs. 1E–1G), not other portions of the cell (i.e., cytoplasmic or nuclear immunostaining). The specific peptide blocker PB95 significantly eliminated CV-1 cell immunofluorescent staining indicating the specificity of SSA95 binding to the cell membrane. To identify background staining or non-specific staining, control experiments, using secondary antibody only, were performed; only very weak fluorescence was observed as shown in Fig. 1D.

Expression of SSA95 Binding Site

Extracellular surface membrane expression of the antigenic site for SSA95 was further demonstrated by flow cytometry. Incubation with either FITC-labeled secondary antibody or rabbit IgG alone did not cause a shift in fluorescence intensity (Figs. 2A and 2B). In

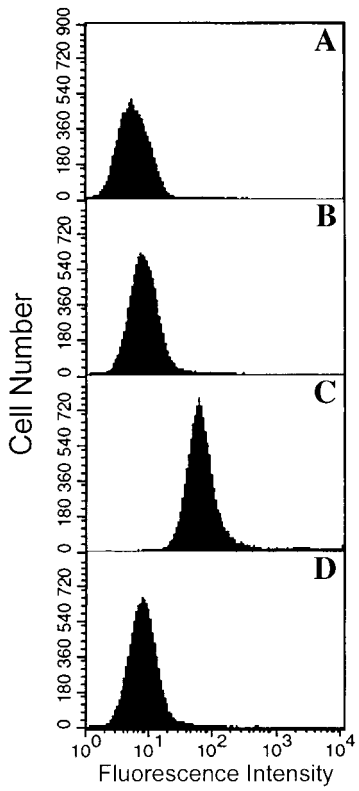

FIG. 2. Flow cytometric analysis of SSA95 binding sites on intact CV-1 cells. Histograms were generated by flow cytometry after labeling with or without SSA95. (A) Untreated CV-1 cells + secondary antibody. (B) cells incubated with rabbit IgG alone + secondary antibody. (C) cells incubated with SSA95 + secondary antibody. (D) Same as C, except in the presence of PB95. Results are from a single experiment representative of six independent experiments.

contrast, incubation with SSA95 and secondary antibody produced an increase in fluorescence labeling (Fig. 2C) and led to a marked shift in the fluorescence intensity. Pre-incubation with PB 95 significantly abolished the fluorescent labeling as further evidence of the specificity of the antibody-antigen interaction (Fig. 2D). Our experimental data were further confirmed by an additional analysis using a Victor-2 multilabel counter. Native CV-1 cells were indirectly labeled with fluorescein conjugated goat anti-rabbit antibody under the same conditions as for flow cytometry. Figure 3 shows that the relative fluorescence intensity was 4-fold higher in the presence of SSA95 than the background labeling with rabbit IgG. PB95 largely inhibited immunoactivity of SSA95, thus confirming the specific binding of SSA95 to the extracellular membrane surface.

DISCUSSION

The use of specific antibodies to analyze cell-surface expression of particular antigenic sites has been widely applied in the study of the transmembrane topology of integral membrane proteins. In the present study, we have utilized a site-specific antibody, SSA95, to detect its antigenic site in CV-1 cells. Immunofluorescent staining (Fig. 1) shows that the $_{833}$KRQPRNPKTD-KLVNE$_{847}$ region (rat numbering) of the α-subunit of (Na^+ + K^+)-ATPase is located on the extracellular membrane surface. The results from both flow cytometric analysis (Fig. 3) and Victor-2 multilabel counter measurements (Fig. 2) reveal that this epitope directly interacts with SSA95 to form an antibody-antigen complex on intact CV-1 cells. Results from three independent experiments consistently suggest that this epitope, a specific antigenic site for SSA95, is expressed on the outside membrane surface of the CV-1 cells.

As a critical control in the present study, native CV-1 cells were preincubated with purified Fc fragments of rabbit IgG for 60 min in order to completely saturate the Fc receptor binding sites on cell surface to avoid Fc receptor-mediated crosslinking reaction during interaction between SSA95 and its specific epitope (Figs. 2 and 3). We purposely selected F(ab′)$_2$ fragments of fluorescein-conjugated anti-rabbit secondary antibodies in an attempt to reduce nonspecific background fluorescent staining. SSA95 recognized its specific antigenic site on cell membrane (Figs. 1–3) under these carefully controlled experimental conditions, suggesting specific binding of SSA95 to the (Na^+ + K^+)-ATPase. Further, a specific peptide blocker significantly reduced the cell surface localization of SSA95,

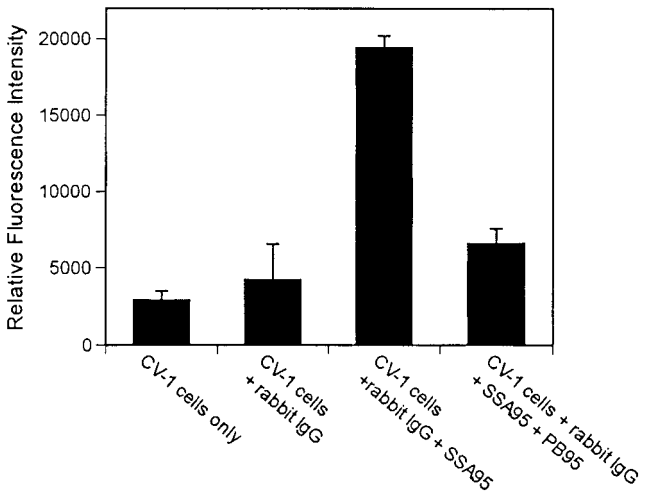

FIG. 3. Measurement of the accessibility of KRQPRNPKTD-KLVNE region of the (Na^+ + K^+)-ATPase on cell membranes. Native CV-1 cells were indirectly labeled with fluorescence-conjugated antibody in the presence or absence of SSA95 under the same conditions as described in the legend to Fig. 2 and indicated in the figure. Data represent means ± SD from eight experiments. The results consistently suggest that this particular region, as an antigenic site for SSA95, is expressed on the cell surface and accessible to interact with SSA95.

additional confirmation of the specificity of SSA95 binding.

It has been suggested that the primary amino acid composition of the $_{833}$KRQPRNPKTDKLVNE$_{847}$ region (rat numbering) of the α-subunit of $(Na^+ + K^+)$-ATPase is on the extracellular side based on hydropathy plots (24) of membrane-spanning segments of the enzyme (12, 13). Several laboratories have also reported that this particular region of $(Na^+ + K^+)$-ATPase is folded in the extracellular localization (25–29). Our data are consistent with those reports and further support the assumption that this region of the $(Na^+ + K^+)$-ATPase is located on the cell surface. Recently we have also found that SSA95 acts from the outside of intact cardiac myocytes to increase intracellular Ca^{2+} concentration and inotropy (19), further supporting the concept that the antigenic determinant, KRQPRNPKTDKLVNE epitope of the $(Na^+ + K^+)$-ATPase, is located on the extracellular side of the enzyme.

Our results are consistent with topologic model I of the $(Na^+ + K^+)$-ATPase rather than model II. The extracellular location of this domain and the functional consequences of antibody binding at this site (19), make this domain an attractive target for pharmacological regulation of cardiac contractility.

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