Molecular dissection of functional domains of the E1E2-ATPase using sodium and calcium pump chimeric molecules

D. B. Luckie,* V. Lemas,‡ K. L. Boyd,* D. M. Fambrough,‡ and K. Takeyasu*

*Department of Physiology, The University of Virginia, Charlottesville, Virginia 22908; ‡Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218; and §Department of Biological Chemistry, University of Maryland, Baltimore, Maryland 21218

ABSTRACT Proposed models for the catalytic subunit of the E_1E_2-ATPases (ion pumps) predict that the first four transmembrane domains ($M_1 - M_4$) reside in the NH_2 terminal one-third of the molecule, and the remainder ($M_5 - M_{10}$) in the COOH terminal one-third. The amino-acid sequences for the 5′-(*p*-fluorosulfonyl)-benzoyl-adenosine (FSBA) binding region residing just before M_5 segment are very well conserved among distinct ion pumps. Taking advantage of these models, we have constructed a set of chicken chimeric ion pumps between the ($Na^+ + K^+$)-ATPase α-subunit and the Ca^{2+}-ATPase using the FSBA-binding site as an exchange junction, thereby preserving overall topological structure as E_1E_2 ATPases. From various functional assays on these chimeric ion pumps, including ouabain-inhibitable ATPase activity, Ca^{2+} binding, Ca^{2+} uptake, and subunit assembly based on immuno-coprecipitation, the following conclusions were obtained: (*a*) A ($Na^+ + K^+$)-ATPase inhibitor, ouabain, binds to the regions before M_4 in the α-subunit and excerts its inhibitory effect. (*b*) The regions after M_5 of the ($Na^+ + K^+$)-ATPase α-subunit bind the β-subunit, even when these regions are incorporated into the corresponding domains in the Ca^{2+}-ATPase. (*c*) The corresponding domains of the Ca^{2+}-ATPase, the regions after M_5, bind ^{45}Ca even when it is incorporated into the corresponding position of the ($Na^+ + K^+$)-ATPase α-subunit.

INTRODUCTION

Recent advances in recombinant DNA technology have permitted the dissection of distinct molecular domains of functional proteins. One approach to identify the critical domains that determine specificity of distinct molecules for particular ions, inhibitors, and subunits is to construct chimeric molecules between distinct functional proteins that have very similar structures. So far, this type of approach has been successful in studying structure-function relationships between (*a*) the α- and the β-adrenergic receptors (1), (*b*) muscarinic acetylcholine receptor subtypes (2), (*c*) the chicken and human EGF-receptors that have different affinity for EGF (3), and (*d*) the skeletal and cardiac isoforms of the dihydropyridine-sensitive Ca^{2+} channel (4). In these studies, the entire higher order structures are likely to be preserved as functional proteins with some kind of common fundamental function. We have been taking this type of approach to investigate the structure-function relationship of ion-translocating ATPases (E_1E_2-type ATPases or ion pumps).

Each ion pump has a strict specificity for ions and is blocked by specific inhibitors; the ($Na^+ + K^+$)-ATPase is blocked by a plant alkaloid, ouabain, the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase by thapsigargin (5), and the ($H^+ + K^+$)-ATPase by omeprasol (6, 7). Some ion pumps (e.g., the Ca^{2+}-ATPase) have a single catalytic subunit with a molecular weight of ~100 kD, and the others (e.g., the [$Na^+ + K^+$]-ATPase and the [$H^+ + K^+$]-ATPase) consist of two subunits; a catalytic α-subunit and a smaller glycoprotein β-subunit (8–10). Approaches employing construction and expression of chimeric ion pumps will be able to identify the critical domains that determine specificity for particular ions, inhibitor-binding sites, and/or subunit assembly, complementing the information obtained by the other approaches such as chemical modification (11).

Address correspondence to Dr. Takeyasu.

EXPERIMENTAL PROCEDURES

Strategy

Fig. 1 illustrates a general model for the catalytic subunit of a variety of E_1E_2-type ATPases including the ($Na^+ + K^+$)- and the Ca^{2+}-ATPase. This model is consistent with many biochemical investigations (8, 11) and recent cDNA cloning and sequencing studies (12, 13). The key features of this model are: (*a*) The catalytic subunits of the E_1E_2-ATPases form very similar higher order structures with 7–10 membrane spanning domains even though the primary amino acid sequences of these ATPases are significantly different (~30% homology). (*b*) There are four evolutionarily conserved regions; the region between M_2 and M_3, phosphorylation-domain, fluorescein-isothio-cyanate (FITC) binding-domain, and

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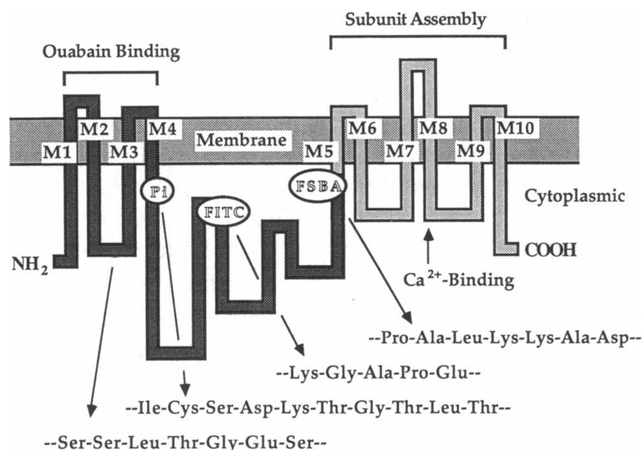

FIGURE 1 General model for topological orientation of the E_1E_2-ATPase catalytic subunit across the membrane. Amino acid sequences for four highly conserved regions between different ATPases are shown; region between M_1 and M_2, phosphorylation site (Pi), and FITC- and FSBA-binding domains. Potential transmembrane segments are numbered, and critical domains identified in this studies are also indicated; ouabain-binding segment, Ca^{2+}-binding region, and assembly domain with the β-subunit.

the 5′-(*p*-fluorosulfonyl)-benzoyl-adenosine (FSBA) binding region. The amino acid sequences for these regions are also shown in Fig. 1. (*c*) The first four transmembrane segments are localized just before the conserved phosphorylation domain, and the remainder after the FSBA-binding region. (*d*) The middle of the molecule is located on the cytoplasm face of the membrane and contains three conserved regions, the phosphorylation site, the FITC-binding site and the FSBA-binding site.

Using the general model, one might speculate that different types of ion pumps may be formed by just combining four different portions (the NH_2-terminal fragment before the phosphorylation site, the fragment between the phosphorylation- and FITC-sites, the fragment between the FITC and FSBA-site, and the COOH-terminal region after the FSBA-site) at three common junctions, the phosphorylation site, the FITC-binding domain and the FSBA-binding region. Indeed, this is our strategy for dissection of functional domains of the E_1E_2-ATPase. Utilizing a heterologous expression system allows us to identify the avian proteins in transfected mammalian cells using monoclonal antibodies specific to the avian ion pumps. Additionally, it is possible to monitor enzymatic functions of the relatively ouabain-sensitive chicken molecules expressed in mouse L cells, of which the $(Na^+ + K^+)$-ATPase is relatively ouabain-resistant.

Chimeric mutant cDNAs encoding chicken Na-pump/Ca-pump chimeric molecules

Construction of the chimeric cDNAs has been described previously (14). Briefly, a unique restriction site, EcoNI, which is endogenous to both the SR calcium pump cDNA (15) and the PM sodium pump α1-subunit cDNA (16), was used as an exchange point for recombination. This exchange occurred at nucleotide 2134 (from the translation initiation site) on the α1-subunit cDNA and at nucleotide 2170 on the calcium pump cDNA, both of which encode lysine (Lys*) residues within the evolutionarily conserved FSBA-binding domain (17, 11) (-Pro-Ala-Leu-Lys*-Lys-Ala-). These chimeric cDNAs and wild type cDNAs were cloned into an expression plasmid, DLSRaα (18), which contains the SV40 late promoter and a retrovirus enhancer for high level of mRNA production suitable for transient expression. For stable expression, a plasmid, pSVDF (19) derived from pSV_2CAT (20), was used.

Transfection and cell culture

COS-1 cells growing in a 150-mm culture dish were cotransfected with 8 μg of DLSRaα DNA containing either chimeric or wild-type cDNAs and 8 μg of DL-SRaα/cDNA encoding the chicken $(Na^+ + K^+)$-ATPase β-subunit (19). This was done by using a DEAE-dextran method. Mouse L cells (in 100-mm culture dish) expressing the β1-subunit of the chicken $(Na^+ + K^+)$-ATPase (19) were cotransfected by the calcium phosphate precipitation method with 0.1 μg of pSVneo DNA and 1 μg of pSVDF/chimeric or wild-type cDNA. The transfected cells were selected in a media containing G418 sulfate (Gibco Laboratories, Lawrence, MA) (~500 μg/ml), and then screened for expression of the chicken chimeric molecules using chicken specific monoclonal antibodies (see below).

ATPase assay

The rate of ATP hydrolysis was determined as described previously (21); for each ADP produced in the assay, a molecule of NADH was oxidized to NAD^+ and this was monitored spectroscopically by a decrease in absorbance at 340 nm. The ATPase assay medium (1 ml) contained 5 μl of cell homogenate (300–500 μg total protein), 120 mM NaCl, 15 mM KCl, 30 mM triethanolamine pH 7.4, 4 mM $MgCl_2$, 3 mM Na_2ATP, 0.5 mM EGTA, 2 mM NaN_3, 2.5 mM phosphoenolpyruvate, 0.5 mM NADH, 10 U/ml pyruvate kinase, 30 U/ml lactate dehydrogenase, and 0.2% Triton X-100. Ouabain-sensitive nATPase activity was obtained by calculating the differ-

ence in activity in the presence and absence of 1 mM ouabain.

Immunoprecipitation and monoclonal antibodies

Monoclonal antibodies 7C (specific for the chicken (Na^+ + K^+)-ATPase α1-subunit [16]) and 5D2 (specific for the chicken SR Ca^{2+}-ATPase (22), and TRITC-labeled goat-anti-mouse IgG were used to identify the chicken proteins in transfected cells. Metabolic labeling procedure followed by immunoprecipitation (23, 24) were employed to characterize the ability of chimeric molecules to assemble with the chicken (Na^+ + K^+)-ATPase β-subunit. Transfected cells grown in 2 ml DMEM containing fetal calf serum/35 mm dish were subjected to incubation in methionine-free DMEM containing 50 μCi/ml [^{35}S]methionine to label cellular proteins metabolically. Following the labeling, the cells were solubilized with 3 ml extraction buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM benzamidine, 5 mM *N*-ethylmaleimide, and 1 mg/ml bacitracin). After cell debris was removed by sedimentation at 15,000 *g* for 15 min, 50 μl of packed Sepharose 4B beads coupled to monoclonal antibody 24, specific to the chicken (Na^+ + K^+)-ATPase β-subunit (23, 25), were added to the supernatant and incubated for 18 h at 4°C on a rocking platform. The immunobeads were washed thoroughly, and the bound antigen was eluted from the beads with 2 × 100 μl elution solution containing 0.5% deoxycholate and 0.1 M triethylamine (pH 11.5). Metabolically labeled and immunobead-purified materials were analyzed on SDS-PAGE followed by fluorography.

^{45}Ca-transport and ^{45}Ca-binding assays using microsomal preparation from cultured cells

$^{45}Ca^{2+}$-binding and -transport assays were done in microsomal fractions of transfected or untransfected COS-1 cells, as described previously (26, 27). A typical microsomal preparation was prepared from twenty 150-mm culture dishes containing COS-1 cells 72 h after transfection. The final microsomal fraction containing chicken ATPases was stored at −70°C, for at longest two weeks, until used. The assay reactions for $^{45}Ca^{2+}$ uptake were started by adding microsomal preparation into the assay medium (20 mM MOPS [pH 7.0], 80 mM KCl, 5 mM $MgCl_2$, 5 mM Na-oxalate, 200 μM EDTA, 2.5 mM ATP, 200 μM $CaCl_2$, and ^{45}Ca [0.4 mCi/ml]) at 25°C. At various time points after the addition of the microsomal preparation, 1 ml aliquots of the reaction mixture (10 μg protein/ml) were filtered through a Millipore filter and washed with 10 mM MOPS (pH 7.0) and 2 mM $LaCl_3$, and the radioactivity remaining on the filter was counted. For $^{45}Ca^{2+}$-binding assays, microsomal fractions (200 μg/ml) were incubated at 37°C for 90 min in the assay medium (100 mM MOPS [pH 6.8], 80 mM KCl, 100 μM $CaCl_2$, 2.6 μCi $^{45}Ca^{2+}$, 200 μM EGTA, 4 μM A23187), quenched with 2 mM $LaCl_3$ and 10 mM MOPS (pH 6.8) and then, filtered through a Millipore filter for counting. Specific $^{45}Ca^{2+}$ binding was defined as the difference between the binding of $^{45}Ca^{2+}$ in the presence and absence of 500 μM chromium ATP (Cr-ATP).

RESULTS AND DISCUSSION

Ouabain-binding domains in the (Na^+ + K^+)-ATPase

Several gene-transfer experiments have indicated that the (Na^+ + K^+)-ATPase α-subunit is responsible for affinity for ouabain (16, 28–31). Recently, using site-directed mutagenesis, Price and Lingrel (31) have demonstrated that the extracellular domain between M_1 and M_2 consisting of 11 amino acids (especially Arg116 and Asp127) is the major ouabain binding domain. However, whether ouabain binds only to these sites or to additional site(s) to exert its action remains to be determined; the (Na^+ + K^+)-ATPase α-subunit may posses multiple ouabain-binding sites. To address this question, we have transfected mouse L cells with cDNAs encoding chicken Ca^{2+}/(Na^+ + K^+)-ATPase chimeric molecules. Because the (Na^+ + K^+)-ATPase of mouse L cells is relatively ouabain-resistant, this avian/mouse hybrid system allows us to monitor enzymatic functions of the relatively ouabain-sensitive chimeric molecules.

Mouse L cells that express the chicken sodium-pump β-subunit (19) were transfected with these chimeric cDNAs, and selected for expression of the encoded chicken proteins using monoclonal antibodies 7C (specific to the chicken [Na^+ + K^+]-ATPase α-subunit [16]) and 5D2 (specific to the chicken SR Ca^{2+}-ATPase [22]). The cloned cells were then subjected to ATPase activity assays in the presence and absence of ouabain. Fig. 2 shows effect of various concentrations of ouabain on the ATPase activity measured in the buffer (in the presence of Na^+ and K^+, and in the absence of Ca^{2+}) optimized for the (Na^+ + K^+)-ATPase. The ouabain-inhibition of the ATPase activity of cells, transfected with DNA encoding either the wild-type (Na^+ + K^+)-ATPase α-subunit or Chimera I, is biphasic; ~60% shows IC_{50} of <10 μM and the rest IC_{50} of ~300 μM. On the other hand, ouabain has a monophasic inhibitory effect (IC_{50}: ~300 μM) in untransfected mouse Ltk^- cells. Because the

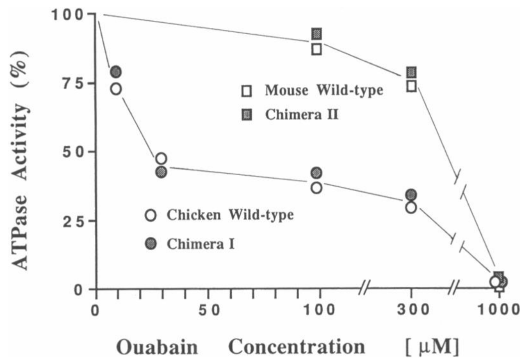

FIGURE 2 Ouabain-inhibitable ATPase activity of sodium- and calcium-pump chimeric molecules. Enzyme linked assay system was used to measure ($Na^+ + K^+$)-ATPase activity of transfected (Chimera I, Chimera II, and wild-type α1-subunit) and untransfected (mouse wild-type) cell homogenates, in the presence and absence of ouabain. ATPase activities of cell homogenate were measured in the presence of 0.2% Triton X-100 which partially solubilizes and clears turbid sample and allows chemicals access to both sides of the membrane. Mitochondorial ATPase and Ca^{2+}-ATPase activities were blocked by addition of 2 mM NaN_3 and 10 mM EGTA into the reaction mixture, respectively. The ATPase activity (60–80 nmol ATP cleaved/mg protein/min) that can be inhibited by 1 mM ouabain was defined as 100%.

specific activity of cell homogenate varies from 50 to 100 nmol ADP cleaved/mg protein/min depending upon the condition of cell growth, it is difficult to make a direct comparison of the specific activity between the endogenous and transfected ATPases. Therefore the data were expressed as percentage of control so that the affinity for ouabain can be easily compared. At present, functions of Chimera II distinct from those of the endogenous mouse ATPase cannot be detected in our system (see also below).

It has been known that the calcium pump does not bind ouabain, and that the endogenous mouse sodium pump does not have high affinity sites for ouabain. One of the chimeric molecules, Chimera I, consisting of the NH_2 terminal two-thirds of the α1-subunit of the ouabain-sensitive chicken ($Na^+ + K^+$)-ATPase and the COOH terminal one-third of the sarcoplasmic reticulum Ca^{2+}-ATPase exhibited ouabain-sensitive ATPase activity very similar to that of the wild-type chicken ($Na^+ + K^+$)-ATPase when assayed in cell homogenate. These results that show an inhibitory effect of ouabain on the wild-type and chimeric molecules suggest that ouabain does not have to bind to the ectodomains after M_5, because this high affinity site for ouabain newly appeared in transfected cells must come from the chicken wild-type α1-subunit and Chimera I.

Calcium transport activity and calcium-binding domains in the Ca^{2+}-ATPase

The ATPase activity of Chimera I could be stimulated by adding Ca^{2+} to the assay system (Fig. 3), indicating that the COOH terminal one-third of the Ca^{2+}-ATPase possess the ability to interact with Ca^{2+} ions. This effect of Ca^{2+} ions could be detected neither in untransfected mouse L cells nor in cells transfected with wild-type α1-subunit DNA. To characterize the ability of this region of the Ca^{2+}-ATPase to bind Ca^{2+} ions further, we have established many mouse L cell lines stably transfected with the chicken wild-type Ca^{2+}-ATPase cDNA as well as Chimera II cDNA. However, we have not been able to detect Ca^{2+}-stimulated ATPase activity for unknown reasons. Therefore, to detect Ca^{2+}-dependent function of the chicken ATPase molecules, we expressed these cDNAs in COS-1 cells according to the methods described by Clarke et al. (26, 27).

The binding of ^{45}Ca to the microsomal fraction obtained from transfected COS-1 cells was measured in the presence of Cr-ATP. Fig. 4 shows the Ca^{2+}-binding ability of wild-type and chimeric ion pumps transiently expressed in COS-1 cells; Chimera I exhibited Ca^{2+}-binding activity as well as the wild-type Ca^{2+}-ATPase, while Chimera II and the wild-type ($Na^+ + K^+$)-ATPase

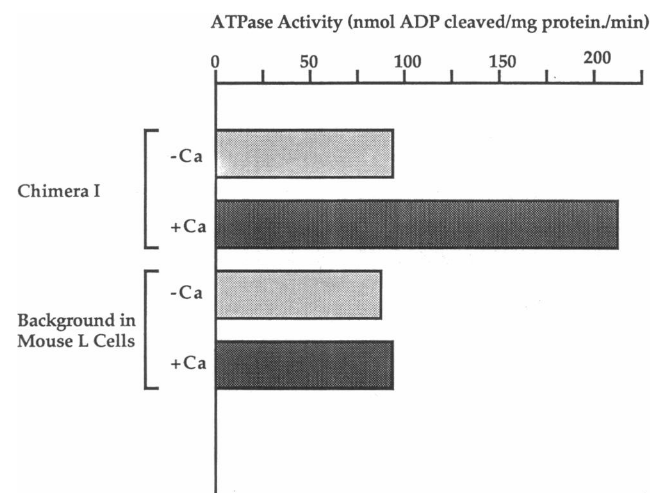

FIGURE 3 Effect of calcium ion on the ATPase activity of homogenate from transfected cells. The same ATPase assay system as described in Fig. 2 was used, and the ATPase activity was compared before and after addition of 300 μM $CaCl_2$ (resulting in ~4 μM free Ca^{2+}) into the reaction system. The ATPase activity of transfected cells before and after Ca^{2+}-addition was 92.6 and 221 nmol ATP cleaved/mg protein/min, whereas the activity of control cells before and after Ca^{2+}-addition was 86.7 and 94.1 nmol ATP cleaved/mg protein/min.

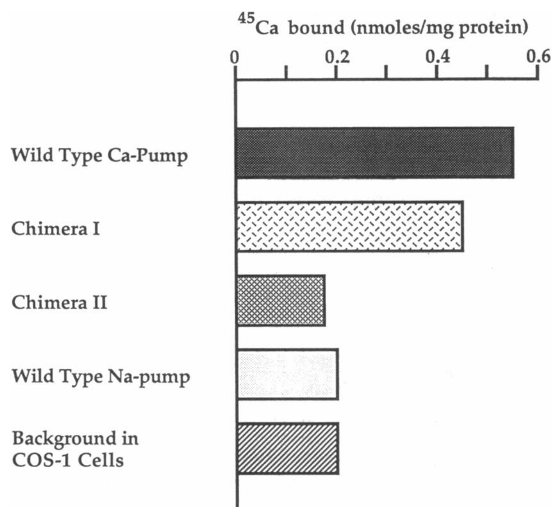

FIGURE 4 ^{45}Ca-binding to the microsomal fractions from COS-1 cells cotransfected with DNA encoding (*a*) wild-type Ca^{2+}-ATPase, (*b*) Chimera I, (*c*) Chimera II, and (*d*) wild-type ($Na^+ + K^+$)-ATPase α1-subunit, together with DNA encoding chicken ($Na^+ + K^+$)-ATPase β-subunit. Background was measured in cells transfected with expression vector, DLRsα, without any insert.

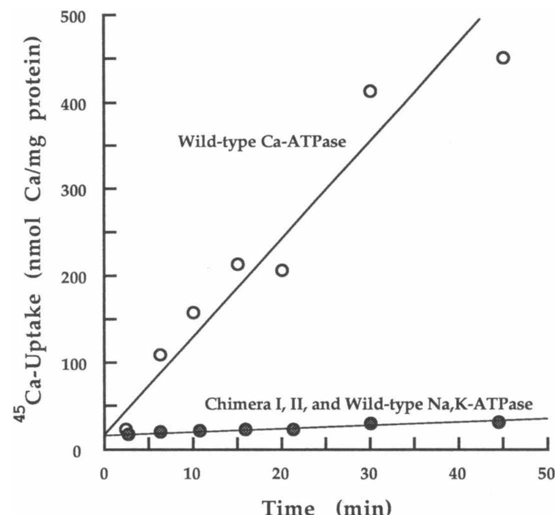

FIGURE 5 ^{45}Ca-uptake by the microsomal fractions from COS-1 cells cotransfected with DNA encoding (*a*) wild-type Ca^{2+}-ATPase, (*b*) Chimera I, (*c*) Chimera II, and (*d*) wild-type ($Na^+ + K^+$)-ATPase α1-subunit, together with DNA encoding chicken ($Na^+ + K^+$)-ATPase β-subunit.

α-subunit showed no Ca^{2+}-binding activity over the background. These results indicate that the COOH terminal one-third of the SR Ca^{2+}-ATPase binds Ca^{2+} ion with high affinity. To correlate the effect of this Ca^{2+}-binding to the Ca^{2+}-transporting activity, we have measured ATP-dependent ^{45}Ca-uptake in the microsomal fractions of transfected COS-1 cells. However, Ca^{2+}-transport activity was found only in the microsomal fraction obtained from cells transfected with wild-type Ca^{2+}-ATPase DNA (Fig. 5). It might be that the critical sites for Ca^{2+}-binding and for ion-translocation are different. Further effort to construct additional chimeric molecules with Ca^{2+}-transport activity will be necessary. In this sense, it is noteworthy that Clarke et al. (26) has proposed that the transmembrane segments, M_4–M_7, contain calcium-binding sites on the basis of their demonstration that substitution of charged amino acids in these regions resulted in loss of ion-pump activity.

Domains responsible for assembly with the β-subunit

It has been difficult to study functions of individual subunits of multisubunit ion pumps, such as the ($Na^+ + K^+$) ATPase. However, two strategies have been successful: (*a*) expression of DNAs encoding ouabain-sensitive sodium pump in cells of an ouabain-resistant organism, and vice versa; and (*b*) over expression of sodium-pump DNAs in organisms, such as *Xenopus* oocytes and yeast. By using these expression systems, extensive studies have been carried out to explore roles of the β-subunit (20, 30, 32–34). The consensus idea deduced from these studies is that the β-subunit is critically involved in the stabilization and transport of the α-subunit to the plasma membrane. Transfected and cloned mouse L cells expressing chicken chimeric ion-pump molecules have offered a good opportunity in which to identify the domains of the α-subunit critical for assembly with the β-subunit.

The criterion for the assembling ability is the immunoprecipitation of the chimeric molecules by use of a monoclonal antibody specific to the β-subunit. We have cloned a set of transfected mouse L cell lines expressing either one of the chimeric molecules together with the chicken β-subunit, and subjected them to the immunoprecipitation analysis. The cells were labeled with (^{35}S)methionine, solubilized in 1% Triton-X100, and the solubilized and labeled materials were purified by immunoaffinity column. The purified products were analyzed by SDS-PAGE and fluorography. The final autoradiograms showed that Chimera II, consisting of the NH_2 terminal two-thirds of the Ca^{2+} ATPase and the COOH terminal one-third of the ($Na^+ + K^+$)-ATPase α-subunit, was assembled with the chicken β-subunit (35). This suggests that the COOH terminal one-third of the Na pump contains the domain(s) required for assembly with the β-subunit, and is consistent with a recent study of expression of sodium/calcium pump chimeric molecules in *Xenopus* oocytes, (36).

CONCLUSIONS

Some of the critical domains for E_1E_2-ATPase function identified in this study are summarized in Fig. 1. First, the ouabain-binding domains will be localized within the NH_2 terminal one-third of the $(Na^+ + K^+)$-ATPase α-subunit, whereas the domain responsible for assembly with the β-subunit will reside within the COOH terminal one-third of the $(Na^+ + K^+)$-ATPase α-subunit. Second, the COOH terminal one-third of the SR Ca^{2+}-ATPase contains the Ca^{2+}-binding site(s). Our studies on the structure-function relationship of E_1E_2-ATPases using chimeric approaches have been qualitative and still incomplete in terms of quantitative analysis. In the future, it is interesting and important to further define inhibitor- and ion-binding regions, particularly ^{86}Rb-occlusion domains, Na-sensitive sites, thapsigargin-binding regions, aiming at identifying minimal structural requirements for specific ion-transport activity.

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DISCUSSION

Session Chairman: Ira Levin *Scribes:* Fen Zhang and Yinong Zhang

ROBERT FARLEY: By sequence homology, there appear to be several regions of possible structural similarity among the different ion pumps. I think it is reasonable to expect ions to bind to comparable regions of different pumps. You can demonstrate Ca-stimulated ATP hydrolysis consistent with Ca binding at a stimulatory site in Chimera I.

Have you done any experiment to show that Na and K stimulate ATPase activity in Chimera I? This the first part of my question.

Related to this, have you excluded the possibility that the Chimera I is in fact not a Na-K ATPase, but rather an ouabain-sensitive Ca ATPase in which ATP hydrolysis is uncoupled from transport? If so it would be the first example of an uncoupled ion pump to my knowledge.

DOUGLAS LUCKIE: As to your second question, we completely agree with you. This Chimera I is highly likely to be an uncoupled ATPase.

As to your first question, we are currently characterizing the properties of Chimera I. As far as we know, Chimera I does not pump Ca, while it binds Ca. We think this might mean that ion binding and ion pumping functions are independent of one another in terms of the structures concerned.

FARLEY: Regarding Ca binding, have you estimated the stoichiometry of Ca binding to this Chimera?

LUCKIE: No, I haven't done such quantification. But we hope to estimate the stoichiometry by comparing the amounts of Ca bound and the P_i incorporated.

RAJINI RAO: Chimera I appears not to interact with the β-subunit, yet it has ouabain-sensitive ATPase activity. What are the implications for the role of the β-subunit? My understanding is that so far the β-subunit is required for ouabain binding, ouabain-sensitive ATPase activity, and also for the targeting of the pump to the plasma membrane?

KUNIO TAKEYASU: It is an interesting point. It seems that Chimera I does not need the β-subunit but still appears to have some kind of function. It has been supposed that the Na, K-ATPase α-subunit needs the β-subunit for its function. By the way, what do we mean by "function of the α-subunit"? We probably mean its ouabain sensitive ATPase activity on the plasma membrane. Regarding this, we know now that (*a*) the β-subunit does not affect the affinity for ouabain and (*b*) the β-subunit facilitates the appearance of the α-subunit on the cell surface. In the latter case, the β-subunit may be related to stabilization of the α–β-complex.

FARLEY: Related to the question on the requirement for the β-subunit, your assay for assembly is based on the immunoprecipitation of the complex by the anti-β-antibody. Can you exclude the possibility that the antibody binding site on β is blocked by the carboxy-terminal domain of the Ca pump in Chimera I?

LUCKIE: I don't think we can, but we think that it is very unlikely, considering that Chimera II assembles with the β-subunit very well.

RAO: Ca stimulated the ATPase activity of Chimera I but did not appear to be transported. Are you considering adding back segments of the Ca pump to try to restore Ca transport?

LUCKIE: Yes. We have made a construct consisting of the NH_2