

26 Amino Acids of an Extracellular Domain of the Na,K-ATPase α -Subunit Are Sufficient for Assembly with the Na,K-ATPase β -Subunit*

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Chimeric cDNAs encoding a sarcoplasmic/endoplasmic reticulum Ca-ATPase (SERCA1) and regions of the Na,K-ATPase α -subunit were constructed to seek the minimal region of the α -subunit sufficient for assembly with the Na,K-ATPase β -subunit. cDNAs encoding a chimera and the chicken β -subunit were coexpressed in mammalian cells and assembly was assayed by immune precipitation of the chimeric subunit with a monoclonal antibody to the chicken β -subunit. A chimera containing 26 amino acyl residues of the Na,K-ATPase α 1-subunit (NDVEDSYGQQWTFEQRKIVEFTCHTA) (Asn⁸⁹⁴ to Ala⁹¹⁹) that replaced the corresponding avian SERCA1 Ca-ATPase amino acyl residues (Thr⁸⁷¹ to Thr⁸⁹⁸) was able to assemble with the chicken β -subunit. This α -subunit region is predicted to be extracellular, located between membrane-spanning domains 7 and 8 (H7-H8). Chimeras that assembled with full-length β -subunit also assembled with a β -subunit chimera that retained only the ectodomain of the chicken β 1-subunit. These results suggest that the Na,K-ATPase α -subunit has the same topology in the membrane as the sarcoplasmic reticulum Ca-ATPase, probably with 10 membrane-spanning domains, and that the aminoacyl residues between membrane domains H7 and H8 are involved in assembly with the β -subunit in the extracellular/luminal space.

Identification of domains involved in assembly of multisubunit membrane proteins can lead to an understanding of the mechanisms controlling assembly and may provide information about three-dimensional structure. Expression of chimeric subunits and/or subunits lacking portions of their polypeptide chains has been a successful approach in identifying domains involved in subunit assembly (1-4).

The Na,K-ATPase is a heterodimer comprised of an ~100-kDa α -subunit and a ~40-60-kDa glycoprotein β -subunit. Both subunits are required for ion transport (5, 6). The α -subunit bears the ATP and cation binding sites and is therefore considered the catalytic subunit. The β -subunit appears to be involved in the structural and functional maturation of the holoenzyme (7, 8) and subsequent transport to the plasma membrane (5, 9, 10). Assembly occurs during or soon after biosynthesis (11) and is required for exit from the endoplasmic

reticulum (ER)¹ (12). In our continuing study of Na,K-ATPase subunit assembly, we have measured the ability of Na,K-ATPase β -subunits to assemble with chimeras between the sarcoplasmic/endoplasmic reticulum Ca-ATPase and the Na,K-ATPase α -subunits.

The sarcoplasmic/endoplasmic reticulum Ca-ATPases are P-type ATPases of ~100 kDa that lack a β -like subunit. Some regions of the Na,K-ATPase and Ca-ATPase show a high level of amino acid sequence similarity, and hydrophobicity plots suggest that they have similar topology in the membrane (13). Our earlier work with the Na,K-ATPase/Ca-ATPase chimeras added further support to the view that the two catalytic subunits have the same topology in the membrane (1). Despite their similarities, the Ca-ATPase and the Na,K-ATPase α -subunit have little amino acid sequence similarity in the carboxyl-terminal third, the region that contains the specific Na,K-ATPase α -subunit aminoacyl residues necessary for assembly with the β -subunit. Therefore, in making chimeras to define the assembly region more closely, we could rely only on the patterns of hydrophobicity. Using this guide, we constructed chimeric cDNAs encoding the Ca-ATPase substituted by portions of Na,K-ATPase α -subunit in the carboxyl-terminal region. Expression of these chimeric cDNAs together with chicken Na,K-ATPase β -subunit cDNA in HeLa cells followed by precipitation of chimera- β complexes with a monoclonal antibody to the chicken β -subunit allowed us to seek the minimal Na,K-ATPase α -subunit sequence that would still mediate subunit assembly. This work and parallel work on the β -subunit (14, 15) show the importance of the α - and β -subunit ectodomains in the assembly process.

EXPERIMENTAL PROCEDURES

cDNAs Used for Transfection—cDNAs encoding the α 1-subunit of the chicken Na,K-ATPase (16) and the chicken SERCA1 Ca-ATPase (17) were used for construction of chimeric cDNAs in pBluescript (Stratagene, La Jolla, CA). A cDNA encoding the β 1-isoform of the chicken β -subunit in pBluescript (1) was used throughout the study. A chimeric cDNA (DPP β) encoding the rat dipeptidylpeptidase IV cytoplasmic and transmembrane domains fused to the extracellular domain of the chicken β 1-subunit has been described (15). In making chimeras, some segments of cDNA were synthesized by polymerase chain reaction methods. Briefly, 50 ng each of the appropriate oligonucleotides, together with 25 ng of either Na,K-ATPase or Ca-ATPase cDNA, were used to generate a cDNA fragment following the protocol supplied by Perkin-Elmer. These cDNA fragments were either digested with the appropriate restriction enzymes to generate fragments that were cloned directly into a chimeric cDNA construct or first cloned into the pCRTM vector (Invitrogen, San Diego, CA), digested, and subcloned into a chi-

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¹ The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic reticulum Ca-ATPase; C₁₂E₈, octaethylene-glycol dodecyl ether; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody

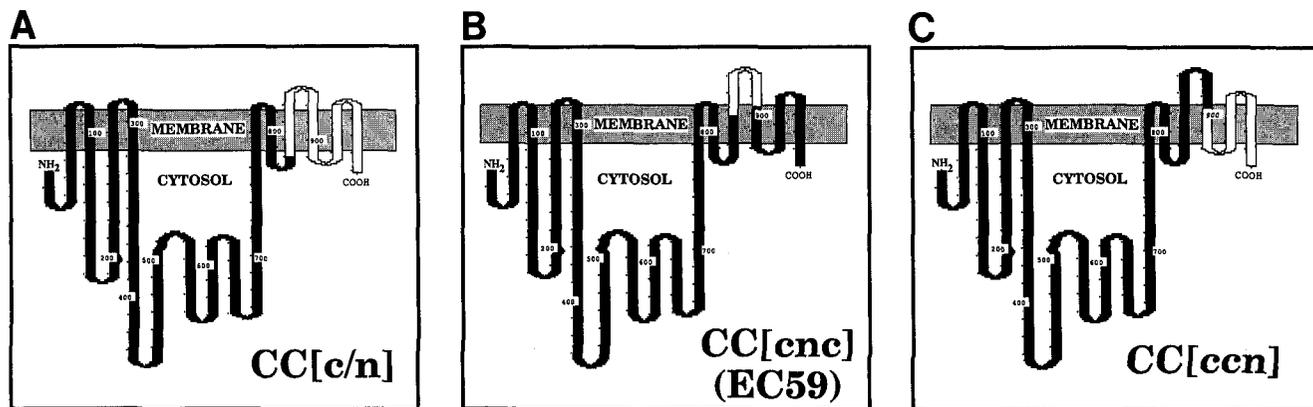


FIG. 1. Structure and predicted topology of chimeric proteins. A cartoon representation of CC[c/n], CC[c/n/c] (also referred to as EC59), and CC[c/c/n] are shown. Nomenclature for these constructs is set by three uppercase letters which represent amino-terminal, central, and carboxyl-terminal domains of the Na,K-ATPase (*N*) and Ca²⁺-ATPase (*C*) proteins. Bracketed lowercase letters replace an uppercase letter when junctions between the Na,K-ATPase or Ca²⁺-ATPase occur within the carboxyl-terminal domain. All the constructs are fit to the topology model proposed for the Ca²⁺-ATPase (26). Portions of the chimeric protein representing Ca²⁺-ATPase (black) and Na,K-ATPase (open) are shown. Small dots outside rectangles mark every 10th residue, and numbers identify locations of residues bearing those position numbers. Amino acid numbering within the carboxyl terminus does not refer to corresponding residue numbers of the actual chicken Na,K-ATPase α_1 -subunit or the Ca²⁺-ATPase. This occurs because the two catalytic proteins differ slightly in their length. Residues within transmembrane domains were fitted from hydrophobicity plots and are considered approximate.

meric cDNA construct. Nucleotide sequences of all the polymerase chain reaction-generated fragments were confirmed by DNA sequencing.

The CC[c/n] cDNA, described earlier (1), was modified in this study to extend the sequence encoding Ca-ATPase by 48 nucleotides (16 amino acids). The other chimeras used in this study consisted of the chicken SERCA1 Ca-ATPase with short segments replaced by corresponding regions of the Na,K-ATPase. The Na,K-ATPase component and flanking SERCA1 aminoacyl sequences of these chimeras are shown in Fig. 4.

Cell Culture and Transfections—HeLa cells were grown in 35-mm tissue culture dishes in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 250 μ g/ml gentamicin (medium, serum, and gentamicin from Life Technologies, Inc.). Transient expression was based on the recombinant vaccinia virus system (18) with the modifications as described earlier (1), except that 10 μ g (10 μ l) LipofectACE™ cationic liposomes (Life Technologies, Inc.) were used for transfection experiments. Transfection efficiencies and relative levels of expression of transiently expressed proteins were determined as before (1).

Monoclonal Antibodies—Two monoclonal antibodies were used. Chicken-specific monoclonal antibody CaF1-5C3-IgG (mAb-5C3) recognizes a cytosolic epitope of the Ca-ATPase (19). Chicken-specific monoclonal antibody β 24 (mAb- β 24) recognizes an epitope in the extracellular domain of the β 1-subunit (11, 20). These antibodies and the hybridomas that secrete them are available from the Developmental Studies Hybridoma Bank, Department of Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, MD.

Metabolic Labeling and Immune Precipitation—Cells were labeled in methionine-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 100–150 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine (Tran³⁵S-label; ICN Pharmaceuticals) for 3 h beginning 2 h or more after transfection. Detergent extracts were prepared from these cells, and immune precipitations were performed as described in Ref. 1 except that cell were solubilized with 75 μ g/ml C₁₂E₈ detergent (Calbiochem) in buffer (10 mM Tris, pH 7.5, 150 mM NaCl), and antigen bound to immunobeads was washed four times with the same detergent buffer. mAb- β 24 was coupled to activated beads (oxidized cross-linked Sepharose CL-6B) by reductive amination reactions (21) as described earlier (1).

RESULTS

Assembly of Chimeric Proteins with Chicken β -Subunit—We have shown that the carboxyl-terminal 161 amino acids of the Na,K-ATPase α -subunit are sufficient for assembly with the β -subunit (1). In that study we used mAb- β 24 that recognizes an epitope in the extracellular domain of the β 1-subunit that is exposed in native Na,K-ATPase. The immune precipitation of α -subunits or chimeric catalytic subunits with mAb- β 24 immunobeads therefore serves as an operational definition of the assembled state. The relatively low yields of the chimeric proteins coprecipitated with the β -subunit in our previous study were found to be due to dissociation of complexes during solu-

bilization and immune precipitation, rather than the loss of a domain critical for assembly. The yields of chimeric proteins CCN and CC[c/n] coprecipitated with the β -subunit were approximately the same as the yield of Na,K-ATPase α -subunit when the detergent C₁₂E₈ was used (data not shown).

The structures of CC[c/n] (161 amino acids of the α -subunit), CC[c/n/c] (59 amino acids of the α -subunit), and CC[c/c/n] (103 amino acids of the α -subunit) chimeric proteins are depicted in Fig. 1, with their predicted topology in cell membranes. Each chimera or wild-type Ca-ATPase was coexpressed in HeLa cells with the chicken β 1-subunit, and assays for subunit assembly were performed (Fig. 2). As expected, CC[c/n] chimera assembled with the chicken β -subunit (Fig. 2, lane 2), whereas the CC[c/c/n] chimera (Fig. 2, lane 4) and wild-type Ca-ATPase (CCC; Fig. 2, lane 5) did not. However, CC[c/n/c] (Fig. 2, lane 3), containing the proposed 59 amino acid H7-H8 domain of the α -subunit, did coprecipitate with the chicken β -subunit, although the yield was much less than for the CC[c/n] chimera. This indicated that the 59 aminoacyl residues of the Na,K-ATPase α -subunit in chimera CC[c/n/c] were sufficient for assembly with the β -subunit.

To verify expression and to identify the apparent molecular weight of each of the chimeras (defined by electrophoretic mobility), we recovered chimeras and CCC by immune precipitation from supernatants that had previously been incubated with mAb- β 24 immunobeads. The mAb-5C3 (chicken-specific anti-Ca-ATPase antibody) immunobeads precipitated CC[c/n], CC[c/n/c], CC[c/c/n], and CCC (Fig. 3, lanes 2–5). Therefore, CC[c/c/n] (Fig. 2, lane 4) was available for interactions with the β -subunit but did not assemble.

Because the yield of CC[c/n/c], from here on referred to as chimera EC59, coprecipitating with the β -subunit was much less than for the CC[c/n] chimera, we wondered whether replacing the predicted extracellular H9-H10 domain of the Ca-ATPase with the corresponding α -subunit domain might increase the stability of the chimera- β complex. EC5911, a chimera with the predicted extracellular H7-H8 domain (59 residues) and H9-H10 domain (11 residues) of the Na,K-ATPase α -subunit (Fig. 4), did coprecipitate with greater yield than the EC59 chimera (Fig. 5, lane 4). This suggests that although α - β assembly interactions predominantly occur with the single Na,K-ATPase H7-H8 domain (EC59), the regions that associate during the assembly with the β -subunit possibly

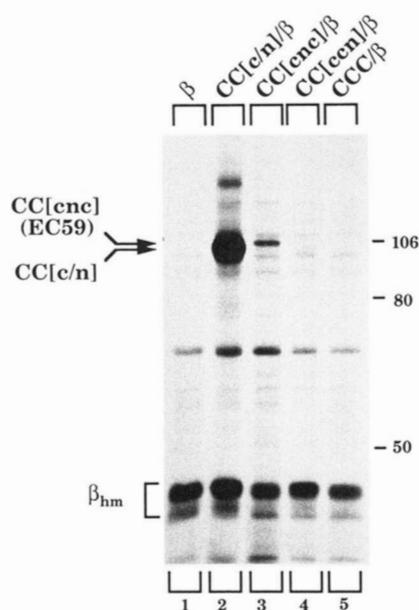


FIG. 2. Assembly of the CC[c/n] and CC[c/n/c] (also referred to as EC59) chimeras with the chicken β -subunit in HeLa cells. Cells were infected with vaccinia virus then transfected with cDNAs encoding the β -subunit alone or together with CC[c/n], CC[c/n/c], CC[c/n], or CCC. Three hours post-infection, cells were metabolically labeled and extracts were prepared from these cells as described under "Experimental Procedures." Extracts were incubated with mAb- β 24 immunobeads, and the immune precipitations were analyzed by SDS-PAGE and fluorography. Positions of the molecular weight markers are indicated at the right. Positions of the CC[c/n] and CC[cnc] chimeras are indicated at the left in the order of molecular mobility by SDS-PAGE from slower (top) to faster (bottom). The protein bands representing the high-mannose forms of the β -subunit (β_{hm}) are also indicated. Endogenous α -subunit, which assembles with the chicken β -subunit at a much lower level, is barely detectable and thus not indicated.

include both the H7-H8 and H9-H10 domains (EC5911).

Sequence alignments of the amino acid acyl residues in the proposed H7-H8 domain of the α -subunit revealed a cluster of residues well conserved among all the known α -subunits of the Na,K-ATPase and H,K-ATPase families. This cluster is predicted to be located in the extracellular space just prior to the entry of the polypeptide into the proposed eighth membrane-spanning domain (Fig. 4). EC26 (Fig. 4), a chimera that contains 26 amino acids of the proposed H7-H8 domain of the α -subunit, including this conserved cluster of residues, does assemble with the β -subunit (Fig. 5, lane 5). The yields of EC26 are similar to the yields of CC[c/n] (Fig. 5, lane 2) and native α -subunit (1) in the assembly assay and significantly greater than the yields of EC59 and EC5911 (Fig. 5, lanes 3 and 4).

Assembly of Chimeric Proteins with the Extracellular Domain of Chicken β -Subunit—HeLa cells coexpressing chimeric catalytic subunits and a chimeric DPP β -subunit protein, retaining only the extracellular domain of the chicken β 1-subunit, were assayed for subunit assembly as described above. CC[c/n], EC59, EC5911, and EC26 (Fig. 5, lanes 8–11) assembled with the chimeric DPP β -subunit, although the yield of each of the chimeric proteins appeared to be less than the yield when the wild-type β 1-subunit was used. The CCC catalytic subunit did not assemble with the wild-type β -subunit and also failed to assemble with the chimeric DPP β -subunit (Fig. 5, lane 12).

To verify expression and to identify the characteristic apparent molecular weight of each of the chimeras, we recovered chimeras and CCC by immune precipitation from supernatants that had previously been incubated with mAb- β 24 immunobeads. As expected, mAb-5C3 (chicken-specific anti-Ca-ATPase

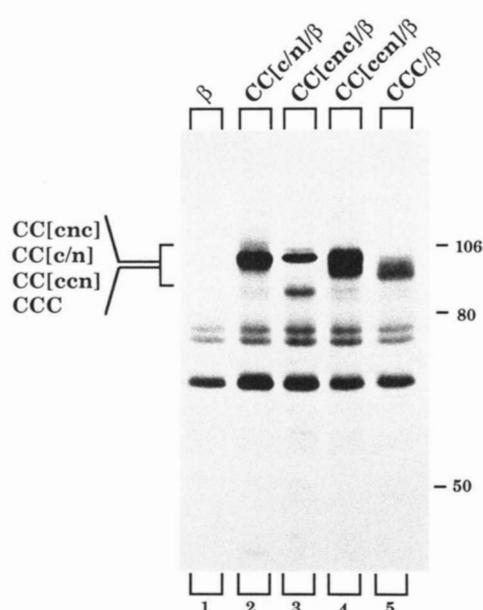


FIG. 3. Immune precipitations of CC[c/n], CC[c/n/c] (EC59), CC[c/c/n], and CCC proteins expressed in HeLa cells. Cells were infected with vaccinia virus then transfected with cDNAs encoding the β -subunit alone or together with CC[c/n], CC[c/n/c], CC[c/c/n], and CCC. Three hours post-infection, cells were metabolically labeled, and extracts were prepared from these cells as described under "Experimental Procedures." Extracts were first incubated with mAb- β 24 immunobeads followed by a second incubation with mAb-5C3 immunobeads, and the second immune precipitates were analyzed by SDS-PAGE and fluorography. Positions of the molecular weight markers are indicated at the right. Positions of each of the chimeras are indicated at the left in the order of molecular mobility by SDS-PAGE from slower (top) to faster (bottom). Note that the cell extracts used here are the same extracts used in Fig. 2.

antibody) immunobeads precipitated CC[c/n], EC59, EC5911, EC26, and CCC (Fig. 6, lanes 2–6). It was important to verify the presence of the EC26 chimera, since it has an apparent molecular weight (electrophoretic mobility) similar to that of the endogenous α -subunit, which coprecipitates with low yield (for endogenous α -subunit background see Fig. 5, DPP β set, lanes 7–12).

DISCUSSION

Transfection efficiencies and levels of expression were approximately equivalent throughout our experiments, and therefore the yields of different chimeras in the assembly assay should reflect relative efficiencies of assembly or relative stabilities of the assembled α - β complexes. In previous assembly experiments, chimeric catalytic subunits appeared to assemble with the β -subunit significantly more poorly than did the full-length α -subunit (1). In those experiments, the detergent used for solubilization and immune precipitation was Triton X-100. The assembly complexes, bound to mAb- β 24 immunobeads, were also washed once in buffer containing 0.1% SDS. When Triton X-100 was replaced with detergent C₁₂E₈ and SDS was removed from the wash buffer, there appeared to be little difference in the yield of the CC[c/n] and wild-type α -subunit in assembly assays. Therefore, differences observed in the yields from earlier work were due to differences in the stability of those chimera- β complexes rather than a decrease in the efficiency of subunit assembly *per se*.

Our results demonstrate that an extracellular domain consisting of 26 amino acyl residues (NDVEDSYGQQWT-FEQRKIVEFTCHTA) of the Na,K-ATPase α -subunit is sufficient for assembly with the Na,K-ATPase β -subunit when these 26 residues lie in the H7-H8 luminal loop of the SERCA1

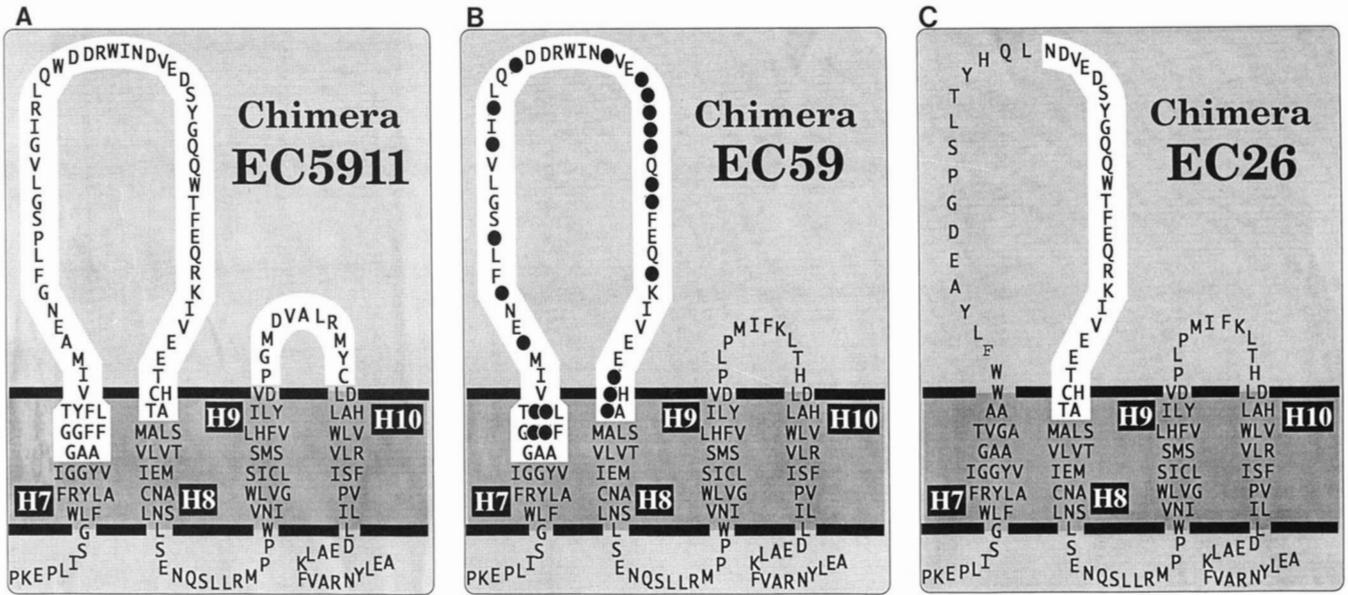


FIG. 4. Cartoon representation of carboxyl terminus of EC5911, EC59, and EC26 chimera H7-H8 domains depicted in the membrane. EC5911 chimera is predicted to have Na,K-ATPase α -subunit residues (highlighted) in both the extracellular H7-H8 domain and H9-H10 domain. EC59 and EC26 chimera have Na,K-ATPase α -subunit residues (highlighted) only in the predicted H7-H8 extracellular domain. EC59 shows black circles over amino acid residues found to be identical in both Na,K-ATPase and H,K-ATPase α -subunit sequences. Residues within transmembrane domains were fitted from hydrophobicity plots and are considered approximate. Ca²⁺-ATPase residues are shown outside highlighted areas. Not shown are the Ca²⁺-ATPase residues 1–823 that comprise that rest of each chimera.

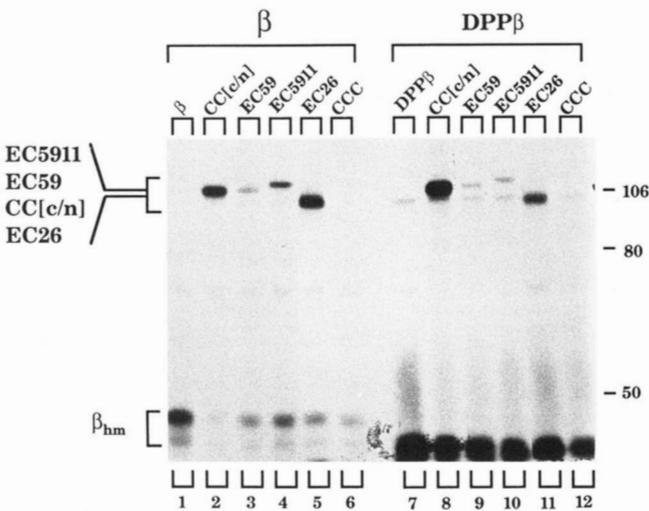


FIG. 5. Assembly of the CC[c/n], EC59, EC5911, and EC26 chimera with the chicken β -subunit or the DPP β -subunit chimera in HeLa cells. Cells were infected with vaccinia virus then transfected with cDNAs encoding the β -subunit or DPP β -subunit chimera alone or together with CC[c/n], EC59, EC5911, EC26, or CCC. Three hours post-infection, cells were metabolically labeled, and extracts were prepared from these cells as described under "Experimental Procedures." Extracts were incubated with mAb- β 24 immunobeads, and the immune precipitations were analyzed by SDS-PAGE and fluorography. Positions of the molecular weight markers are indicated at the right. Positions of the CC[c/n], EC59, EC5911, and EC26 chimera are indicated at the left in the order of molecular mobility by SDS-PAGE from slower (top) to faster (bottom). The protein bands representing the high-mannose forms of the β -subunit and DPP β -subunit chimera (β_{hm}) are also indicated. Endogenous α -subunit, which assembles with the chicken β -subunit at a much lower level and has approximately the same molecular mobility as the EC26 chimera, is detectable in the DPP β -subunit samples but not in the β -subunits samples.

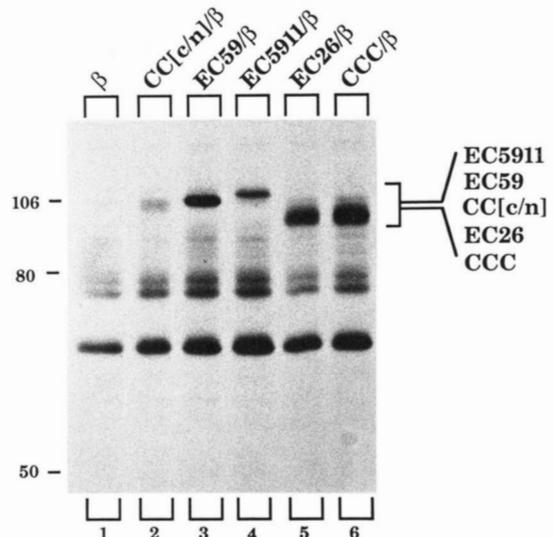


FIG. 6. Immune precipitations of CC[c/n], EC59, EC5911, EC26, and CCC proteins expressed in HeLa cells. Cells were infected with vaccinia virus, then transfected with cDNAs encoding the β -subunit alone or together with CC[c/n], EC59, EC5911, EC26, and CCC. Three hours post-infection, cells were metabolically labeled, and extracts were prepared from these cells as described under "Experimental Procedures." Extracts were first incubated with mAb- β 24 beads followed by a second incubation with mAb-5C3 immunobeads, and the second immune precipitates were analyzed by SDS-PAGE and fluorography. Positions of the molecular weight markers are indicated at the right. Positions of each of the chimera are indicated at the left in the order of molecular mobility by SDS-PAGE from slower (top) to faster (bottom). Note that the cell extracts used here were the same extracts that had previously been used for the experiments illustrated in Fig. 5.

Ca-ATPase. Furthermore, this chimera will assemble with a chimera that contains only the ectodomain of the Na,K-ATPase β -subunit (15). The assembled forms assayed in this study may represent intermediate states in the assembly-maturation process rather than mature functional forms. Since we know

that subunit assembly occurs in the endoplasmic reticulum, we can conclude that the interactions involved in assembly of the α - and β -subunits of the Na,K-ATPase must occur largely in the lumen of the endoplasmic reticulum. Subunit assembly involving extracellular domains has been observed with a few other oligomeric plasma membrane proteins (see review Ref. 22).

Two independent studies have defined epitopes between the

predicted seventh and eighth membrane-spanning domains of the Ca-ATPase that are exposed to the lumen of the sarcoplasmic reticulum (23, 24), and these results were interpreted to support a 10 membrane-spanning domain model of the calcium pump (25, 26). It is this epitope region in the chicken SERCA1 that we have replaced with 26 aminoacyl residues of the Na,K-ATPase. Since the resulting chimera, EC26, assembled with the β -subunit, one can infer that at least up to membrane span H8 the COOH-terminal end topologies of the Ca-ATPase and Na,K-ATPase α -subunit are the same.

Identification of an extracellular region between the seventh and eighth membrane spans of the gastric H,K-ATPase α -subunit has been presented (31). This region is homologous to the region of the Na,K-ATPase α -subunit our report identifies as part of the subunit assembly site. The Na,K-ATPase and H,K-ATPase α -subunits share ~62% amino acid sequence identity (32), and each associates with a β -like subunit. Sequence alignment of amino acids in the predicted H7-H8 domain of Na,K-ATPase and H,K-ATPase α -subunits reveals clusters of amino acids closest to the H8 domain that are identical throughout all species. Chimera EC26 contained these conserved aminoacyl residues.

The Na,K-ATPase α -subunit has been predicted to have from three to six membrane-spanning domains in its carboxyl-terminal ~300 amino acids (13, 27–29). These predictions were based upon hydrophobicity analysis and results of immunological and proteolysis studies. Recently Maguire and colleagues (30) showed more conclusively that a prokaryotic P-type Mg-ATPase has 10 membrane-spanning domains. In our experiments, EC5911, containing the predicted H7-H8 and H9-H10 extracellular domains, consistently appeared to assemble better with the β -subunit than did EC59. This suggests that subunit interactions stabilizing the assembled state may also involve the α -subunit's hypothetical H9-H10 domain. Complicating this interpretation is the fact that both EC59 and EC5911 appeared to assemble more poorly than EC26. Chimeras EC59 or EC5911 contain aminoacyl residues of the Na,K-ATPase α -subunit predicted to be part of the membrane-spanning H7 domain. Perhaps these residues partially disturb the optimal structure at the H7-H8 domain, distorting the presentation of the α -subunit assembly residues to the β -subunit.

It is difficult to speculate about what types of molecular interactions might be most important between the α - and β -subunit. Studies with the multisubunit ribulose-1,5-bisphosphate carboxylase/oxygenase have identified various types of interactions at the interfaces between assembled subunits (33). Flachmann *et al.* (33) found that replacement of a single critical aminoacyl residue completely abolished assembly. Amino acid substitutions in the Na,K-ATPase α -subunit assembly

domain may also allow us to identify a few of the critical amino acids.

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