Only the First and the Last Hydrophobic Segments in the COOH-terminal Third of Na,K-ATPase α Subunit Initiate and Halt, Respectively, Membrane Translocation of the Newly Synthesized Polypeptide

IMPLICATIONS FOR THE MEMBRANE TOPOLOGY*

(Received for publication, August 3, 1995, and in revised form, November 14, 1995)

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We studied the topogenic properties of five hydrophobic segments (H5–H9) in the COOH-terminal third of Na,K-ATPase α subunit using in vitro insertion of fusion proteins into endoplasmic reticulum membranes. These fusion proteins consisted of different lengths of truncated α subunit starting at Met729 and a reporter protein, chloramphenicol acetyltransferase, that was linked in frame after each hydrophobic segment. We found that membrane insertion of the newly synthesized COOH-terminal third was initiated by H5 and terminated by H9, indicating that here only H5 and H9 have topogenic function. The other three, H6–H8, did not have topogenic function in the native context and were translocated into the endoplasmic reticulum lumen. These results were in striking contrast to the previous models in which four or six hydrophobic segments were proposed to cross the membrane. Furthermore, the findings suggest a novel mechanism for achieving the final membrane topology of the COOH-terminal third of the α subunit.

Na,K-ATPase consists of two non-covalently linked subunits: a larger non-glycosylated α subunit (about 100 kDa) and a glycosylated β subunit (approximately 55 kDa) (Brotherus et al., 1983), both of which are transmembrane proteins. The complete amino acid sequence of the α subunit from different sources has been cloned (see review by Mercer (1993)). Hydrophathy analysis of the sequence has shown nine hydrophobic segments (H1–H9). Four segments (H1–H4) are in the NH2-terminal third and the rest (H5–H9) are in the COOH-terminal third of the subunit. However, H5 is often proposed to cross the membrane twice as H3 and H6, so that the remaining segments are often named H7–H10.

The four hydrophobic segments in the NH2-terminal third have been demonstrated to be transmembrane spans by immunochromical studies (Kano et al., 1990; Arystarkhova et al., 1992; Ning et al., 1993; Mohraz et al., 1994; Yoon and Guidotti, 1994). The sidedness of the NH2 terminus has been determined to be cytoplasmic by immunochromical methods (Felsenfeld and Sweadner, 1988; Antolovic et al., 1991; Ning et al., 1993; Canfield and Levenson, 1993; Yoon and Guidotti, 1994). The orientation of these four hydrophobic segments in the lipid bilayer has been determined by the in vitro ER1 membrane insertion experiments (Xie and Morimoto, 1995).

In contrast to the NH2-terminal third, the membrane topology of the COOH-terminal third of the α subunit has been quite controversial. Since this region is involved in several important functions of the Na,K-ATPase such as cation binding and occlusion (Karlish et al., 1990), ouabain binding, and conformational changes during the E1 to E2 transitions that occur during the catalytic cycle (see review by Vasillets and Schwartz (1993) and Lingrel and Kuntzweiler (1994)), structural information on this particular region is crucial for understanding the structure function relationship of the enzyme.

In this paper, we describe studies on the transmembrane disposition of the COOH-terminal third of the α subunit using an experimental strategy based on the topogenic property of hydrophobic segments. The fundamental principle of this approach is that the transmembrane disposition of a polypet protein is achieved cotranslationally by the action of a series of alternating insertion signals that initiate translocation of downstream portions of the polypeptide, and of halt transfer sequences that block the translocation of the downstream sequences (Blobel, 1980; Sabatini et al., 1982). Since the hydrophobic segments in the COOH-terminal third occur after a long cytoplasmic stretch, this principle implies that the first transmembrane segment in this region must be an insertion signal that has also anchoring function. Fusion proteins were made consisting of several different lengths of truncated α subunit, linked in frame at their COOH termini to a reporter protein, chloramphenicol acetyltransferase (CAT), that has a consensus N-linked glycosylation site (Gorman et al., 1982). Fusion proteins were designed such that the topogenic properties of all the hydrophobic segments could be examined individually and sequentially in their native context. Occurrence of N-linked glycosylation and sensitivity to protease digestion of the reporter protein were used as markers for luminal and cytoplasmic disposition, respectively. The results provide both structural

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*This work was supported by National Institutes of Health Grant GM20277. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: ER, endoplasmic reticulum; CAT, chloramphenicol acetyltransferase; kbp, kilobase pair(s); PCR, polymerase chain reaction; Endo H, endoglycosidase H.
(orientation with respect to the membrane) and functional (an insertion signal, a halt transfer signal, or an inactive element as signals) information on the hydrophobic segment in the context tested. Furthermore, these findings shed light on the membrane insertion mechanism of polytopic proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes, SP6 RNA polymerase, DNA polymerase I (Klenow enzyme) T4 DNA ligase, T4 polynucleotide kinase, human placenta RNase inhibitor, Taq DNA polymerase, m7G(5')ppp(5')G, and calf intestinal alkaline phosphatase were obtained from either Boehringer Mannheim or Life Technologies, Inc. (4-Butylamino)benzaldehyde 2-[(4-Chloro-2-methylphenyl)amino]ethyl hydroxy (tetracaine), phenylmethanesulfonyl fluoride, trypsin, chymotrypsin, and aprotinin (Trasyloid) were obtained from Sigma. A GeneClean kit was purchased from B101 (La Jolla, CA). Reticulocyte lysate was obtained from Promega Corp. (Madison, WI). [35S]Methionine was obtained from DuPont NEN. Rabbit antibody against chloramphenicol acetyltransferase (CAT) was obtained from 5Prime, Inc. (Boulder, CO). Microsomes were prepared from canine pancreas as described by Walter and Blobel (1983). Occasionally microsomes were obtained from Promega Corp.

**Plasmids and Constructs of Mutants**

Plasmids pGEM-3Z and pCAT basic were obtained from Promega Corp., and pT7/3 19R was obtained from Life Technologies, Inc. Okayama-Berg vector containing entire coding region of rat brain Na,K-ATPase α-1 subunit (pRNAK1A) was supplied by Dr. Hara (Tokyo Dental Medical School, Tokyo, Japan). cDNA Constructs—pN-5, -6, -7, -8, and -9, pC-5, -6, -7, -8, and -9, and pC-5B-6, which encode the fusion proteins of the truncated Na,K-ATPase α-1 subunit and CAT as a reporter protein, are derived from plasmids pF1 and pCAT (Xie and Morimoto, 1995).

pC-5—A 3.2-kbp fragment containing an entire coding region of the α-1 subunit including the modified 5'-end noncoding region was obtained by digesting pN-1 and Sall. An aliquot of 3.2-kbp fragment was digested with KpnI to generate a 2.1-kbp fragment with KpnI/Sall ends. Another aliquot was successively digested with Stul and HaeII, and the generated 0.64-kbp fragment was ligated to 8-mer Sall linker, followed by digestion with KpnI and Sall. The resultant 0.48-kbp fragment was inserted, together with 2.1-kbp Stul/KpnI fragment, into the plasmid pCAT that had been cut by Sall and Sall (this construct was defined as pN6). The generated 0.6-kbp fragment was then isolated and digested with KpnI, followed by digestion with Sall and Sall, to isolate the NoI-Cut plasmid pN5 that encodes the fusion protein C-5, which was self-ligated to construct plasmid pC5.

pC-6—A 3.2-kbp fragment (see "pC5") was successively digested with Stul and Xmn I to generate a 0.64-kbp fragment. After ligation of the fragment and 8-mer Sall linkers, the 0.6-kbp fragment was digested with Stul and Sall. The generated 0.6-kbp fragment and 2.1-kbp Stul/KpnI fragment from pC5 were inserted together into the pCAT vector that had been cut with Sall and Sall (defined as pN6). This construct contains an additional consensus N-linked glycosylation site at the ligation site between the truncated α subunit and CAT protein. Plasmid pC6 was constructed from pN6 in the same way as described for pC5 construction.

pC-7—After digestion of the 3.2-kbp Sall/Sadl fragment (see "pC5") with EcoR I, a generated 2.8-kbp fragment was subcloned into a PstI-EcoRI-cut plasmid pSP73, which was then digested with PstI and EcoR V. The PstI-EcoR V fragment was ligated to the pCAT vector that had been cut with PstI and HindI (defined as pN7). Plasmid pC7 was constructed from pN7 in the same way as described for pC5 construction. This construct contains an additional consensus N-linked glycosylation sequence like pC6.

pC-8—After digestion of pf1 with Bsu36I, a 2.95-kbp fragment was generated and its protruding ends were filled in with Klenow enzyme. The blunt ended fragment was then ligated to 8-mer Sall linker, digested with Sall and Sall, and subcloned into the plasmid pCAT that had been cut with PstI and Sall (defined as pN8). Plasmid pC8 was constructed from pN8 in the same way as described for pC5 construction.

pC-9—Plasmid pf1 was partially digested with HindIII and BgIII, and a generated 3.5-kbp fragment was blunt-ended with T4 DNA polymerase, followed by digestion with PstI. The resulting fragment was then subcloned into the pCAT that had been cut with PstI and HindI (defined as pN9). Plasmid pC9 was constructed from pN9 in the same way as described for pC5 construction.

pC5B-6—A 400-base pair DNA segment generated by PCR using a linearized plasmid pC6 as the template and two primers (upstream primer: 45-mer oligonucleotide described in the construction of plasmid pf1 (Xie and Morimoto, 1995) and downstream primer: 5'-TAGCTCCCT-GAAAATCTCCGCC-3') was digested with Dpn1. After ligation of the isolated 389-base pair fragment to 8-mer Sall linker, the ligated fragment was digested with Sall and PstI. The resulting 372-base pair fragment was inserted into the plasmid pCAT that had been cut with Sall and PstI to prepare plasmid pC5B-6.

pMT2-N5—A eukaryotic expression vector pMT2 was cut with EcoR I, and the resulting protruding ends were filled in with Klenow enzyme. The blunt-ended vector was further digested with PstI, and the isolated PstI/EcoRI-cut pMT2 was ligated with a fragment generated by digesting pN5 (see "pC5") with PstI and Sall to construct plasmid pMT2-N5.

pMT2-C5—This plasmid was constructed from plasmids pMT2 and pC5 in the same way as described for pMT2-N5 construction.

pX5, -6, -7, -8, and -9—These plasmids were prepared from plasmids p55p5GH and pC5, -6, -7, -8, and -9. About 800-base pair DNA segments containing one of the five hydrophobic segments and CAT were generated by PCR using two primers described below and a linearized plasmid that contains the hydrophobic segment interest as the template. The NH2-terminal side of the primers that contain BamHI site are given below.

| Primer | Name                      | Sequence                                    |
|--------|---------------------------|---------------------------------------------|
| Oligo 5 | pX5                       | TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT           |
| Oligo 6 | pX6                       | TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT           |
| Oligo 7 | pX7                       | TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT           |
| Oligo 8 | pX8                       | TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT           |
| Oligo 9 | pX9                       | TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT           |

pX5 for pX5: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT
pX6 for pX6: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT
pX7 for pX7: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT
pX8 for pX8: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT
pX9 for pX9: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT

The COOH-terminal side of the primer that contains BamHI site is given below.

Oligo C: GAATTCGAGCTCCGAGGGATTCAGGCGCTTCTGAT

These PCR products, which had been digested with PstI and BamHI, were ligated to p55p5GH, which had been digested with Eco471I and BamHI.

pXc and pX4—These two plasmids were prepared as a negative and a positive controls. Plasmid pXc was constructed by ligating an entire coding region of CAT cDNA to the p55p5GH that had been cut with Eco471I and BamHI in frame. For construction of plasmid pX4, DNA fragment that encodes a fusion peptide consisting of hydrophobic segment H4 and CAT protein was made by PCR using a linearized plasmid pN4 (Xie and Morimoto, 1995) as the template and two primers (Oligo C and Oligo 4: TGATGAGCTCCGAGGGATTCAGGCGCTTCTGAT).

The PCR product was inserted to plasmid p55p5GH as described for pX5 construction.

All plasmids encoding a fusion protein have been examined by DNA sequencing, the size of their primary translation products and immune reactivity to anti-CAT antibody in order to confirm the in frame ligation. These fusion proteins are shown schematically in Fig. 2.

**Site-directed Mutagenesis**

Replacement of Pro99 in α1-Leu was carried out basically according to the procedure developed by Deng and Nickoloff (1992) using the following two primers. TZ-12 contains the desired single point mutation, 5'-CAAGAGAGGGTGTACGGTCCGGA-3', which is complementary to the (-) strand of subunit coding region 2340–2358 except for a single nucleotide mismatch (C2351 and T2351). TZ-14, 5'-CAGGGATCTAGCTGAGACAGT-3', is complementary to the (-) strand of the multiple cloning sites of pGEM-3Z vector except for a single nucleotide mismatch (A29 and Tm of the pGEM-3Z sequence). This change converted a unique HindII site to a unique NheI site.

**Cell Culture, Transfection, Metabolite Labeling, and Sample Preparation for Immunoprecipitation Analyses**

COS-1 cells were grown in six-well culture plates containing 10% calf serum Dulbecco's modified Eagle's medium. At mid-log phase cells were transfected with plasmids containing cDNAs according to the procedure provided by Life Technologies, Inc. using Lipofectamine. At 44–48 h after the start of transfection, cells were metabolically labeled with 15N2methionine (50 μCi/ml) for 60 min and chased for 3 h. For immunoprecipitation analyses, at the end of chasing, the culture medium was saved and half was used for examining the amount of the secreted products, if any, and cells were washed three times with cold phosphate-buffered saline. After draining the residual buffer completely,
cells were solubilized in 0.5 ml/well of 10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin (Lysis buffer). Culture medium was added SDS to be a final concentration of 1%, and incubated in boiling water for 5 min. Upon being cooled down to room temperature, the medium was mixed with an equal volume of 1/3 Lysis buffer. Both cell lysate and medium were subjected to immunoprecipitation as described previously (Xie and Morimoto, 1995).

For preparation of the total membrane fraction, cells from four wells were used. After transfection, followed by pulse-chasing as described above, cells were washed three times with cold phosphate-buffered saline, and resuspended in 0.3 ml/well of 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 0.5 mM MgCl2, 1 mM phenylmethysulfonyl fluoride, 1 mM dithiothreitol, and 2 μg/ml aprotinin and immediately homogenized in a tight fitting Dounce homogenizer with 15 strokes. The homogenate was centrifuged at 40,000 rpm for 30 min at 4°C in a Beckman Ti-50 rotor. The pellet was suspended in 200 μl of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 3 mM Tetracaine, incubated at room temperature for 10 min, and then subjected to various treatments.

In vitro transcription, in vitro translation, isolation of mRNAs from the translation mixture, removal of globin from the translation reaction mixture, Endo H treatment, immunoprecipitation with anti-CAT antibody, protease digestion, and SDS-PAGE were carried out as described by Xie and Morimoto (1995).

RESULTS

Membrane Insertion of the COOH-terminal Third of the α Subunit Was Initiated by the Hydrophobic Segment H5 (Ile782–Ile805)—We used fusion proteins that contained different lengths of truncated α subunit starting at Met729, instead of Met5, which is the initiator methionine in the wild type α subunit (see Figs. 1 and 2). Because a molecular mass of the fusion proteins starting at Met5 becomes larger and unglycosylated and glycosylated forms, which differ by only 3 kDa, becomes indistinguishable in SDS-PAGE. In addition, we observed that translocation efficiency of hydrophobic segments became progressively lower as the peptide length increased. Met729 was chosen from the following reasons. (a) Since all hydrophobic segments in the COOH-terminal third occurred after a long cytoplasmic stretch, the first hydrophobic segment H5, which is located about 50 amino acid residues downstream of Met729, was considered as a signal/anchor type II (Sabatini and Adesnik, 1994), because the segment may function as an insertion signal that has an anchoring function and the NH2-terminal flanking region is predicted to be in the cytosol after insertion. (b) The H5 in this truncated form was considered to have the same topogenic properties as H5 in the wild type α subunit. (c) α subunit cDNA had two NcoI sites at Met525 and Met729 in frame. To examine whether the H5 in the truncated subunits initiates translocation of the downstream peptides in living cells, we constructed two eukaryotic expression vectors: (a) pMT2-N5, which encoded a fusion protein (N-5) of NH2-terminal 981 amino acid residues (containing five hydrophobic segments, H1, H2, H3, H4, and H5 in the native context) and the CAT protein (219 amino acid residues); and (b) pMT2-C5, which encoded a fusion protein (C-5) consisting of a truncated sequence (89 amino acid residues including H5) starting at Met729 and CAT. Membrane Insertion of the COOH-terminal Third of the α Subunit Was Initiated by the Hydrophobic Segment H5 (Ile782–Ile805)—We used fusion proteins that contained different lengths of truncated α subunit starting at Met729, instead of Met5, which is the initiator methionine in the wild type α subunit (see Figs. 1 and 2). Because a molecular mass of the fusion proteins starting at Met5 becomes larger and unglycosylated and glycosylated forms, which differ by only 3 kDa, becomes indistinguishable in SDS-PAGE. In addition, we observed that translocation efficiency of hydrophobic segments became progressively lower as the peptide length increased. Met729 was chosen from the following reasons. (a) Since all hydrophobic segments in the COOH-terminal third occurred after a long cytoplasmic stretch, the first hydrophobic segment H5, which is located about 50 amino acid residues downstream of Met729, was considered as a signal/anchor type II (Sabatini and Adesnik, 1994), because the segment may function as an insertion signal that has an anchoring function and the NH2-terminal flanking region is predicted to be in the cytosol after insertion. (b) The H5 in this truncated form was considered to have the same topogenic properties as H5 in the wild type α subunit. (c) α subunit cDNA had two NcoI sites at Met5 and Met729 in frame.

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glycosylated forms, NH2 terminus, and COOH terminus, respectively.

Fusion protein C-5 (37 kDa) from which the NH2-terminal protein corresponded to the expected size of the unglycosylated band by the same treatment. This membrane-protected 30-kDa transfected cells. The 33-kDa band shifted to a band at 30 kDa with antibody, two bands at 33 and 30 kDa were obtained from both precipitates. When the membrane fractions were digested with proteases and the other was used as a control. Both aliquots were subjected to immunoprecipitation. Each immunoprecipitate was dissolved in 30 μl of 1% SDS, 50 mM dithiothreitol, incubated in boiling water for 3 min, and then mixed with an equal volume of 0.2 M citrate buffer, pH 5.5. Each aliquot was further divided into two. One was treated with Endo H, and the other was the control. After incubation at 37°C overnight, samples were analyzed in SDS-PAGE as described under “Experimental Procedures.” Bottom, schematic drawing shows the orientation of the inserted chimera before (-P) and after (+P) protease digestion. Solid hearts, N, and C indicate glycosylated forms, NH2 terminus, and COOH terminus, respectively.

transiently in COS-1 cells.

As shown in Fig. 3, immunoprecipitation of the total membrane fractions by anti-CAT antibody yielded a single band with a molecular mass of about 110 kDa from pMT2-N5-transfected cells and two bands with molecular masses of 40 and 37 kDa from pMT2-C5-transfected cells. Upon treatment with Endo H, the 110-kDa protein band did not shift, but the 40-kDa protein band distinctly shifted to 37 kDa. The relative amount of the 40 kDa protein was about 50% of the total immunoprecipitates. When the membrane fractions were digested with proteases, followed by immunoprecipitation with anti-CAT antibody, two bands at 33 and 30 kDa were obtained from both transfected cells. The 33-kDa band shifted to a band at 30 kDa by Endo H treatment, but no shift was observed in the 30-kDa band by the same treatment. This membrane-protected 30-kDa protein corresponded to the expected size of the unglycosylated fusion protein C-5 (37 kDa) from which the NH2-terminal flanking region was cleaved. Since the consensus N-linked glycosylation site was only one in these fusion proteins and located within the CAT molecule downstream of H5, the results clearly demonstrated that H5 initiated translocation of both fusion proteins, and that the CAT protein was translocated across the membrane into the lumen where it was glycosylated. Since the membrane-protected 30-kDa protein was recovered by anti-CAT antibody as a major product from both pMT2-N5- and pMT2-C5-transfected cells, the 37-kDa protein from pMT2-N5-transfected cells was apparently translocated but failed N-linked glycosylation for unknown reason. Therefore, the extent of glycosylation was low, but the majority of the fusion proteins were considered to be translocated.

The translocation efficiency of H5 in the fusion protein C-5 was much lower than that of H1 in the fusion protein N-1 (Fig. 4, C-5 and N-1). The NH2-terminal flanking region of these two fusion proteins appeared to be similar, and the hydrophobicities of H1 and H5 were also very similar, except that H5 had 3 proline residues.
while H1 did not. Proline is known as an $\alpha$ helix breaker and could account for the low translocation efficiency of H5. If this were the case, change of 1 or more proline residues in H5 should increase its translocation efficiency. In fact, in parallel to this work, Homareda et al. (1992) observed improvement of the translocation efficiency of H5 by changing proline residues to hydrophobic amino acid residues. It was desirable to have an H5 with higher translocation efficiency for examination of the topogenic properties of the hydrophobic segments H6, H7, H8, and H9 that followed it in the native context. We mutated 1 proline residue at position 784 to leucine and examined the translocation efficiency. Among 3 proline residues, proline 784 was chosen because it was located very close to the beginning of the hydrophobic segment H5 and therefore this would cause the least effect on the structure of H5 in the lipid bilayer. (Constructs and fusion proteins that contain proline-mutated H5 are denoted by "$^\star"$ like pC*5 and C*-5, respectively.)

The immunoprecipitates from the translation of the pC*5 transcript in the presence of microsomes contained two major proteins with molecular masses of 36 and 39 kDa at a mass ratio of about 1:1 (Fig. 4, C*-5, lane 2). Endo H treatment resulted in the shift of 39 kDa band to 36 kDa (lane 3), indicating that the relative amount of the glycosylated form was about 50%. This value was much higher than the value obtained in the pC5 translation. Interestingly, the Endo H treatment also shifted the molecular mass of the 36-kDa protein to 33 kDa. This shift was presumably due to deglycosylation of a fusion protein that was initiated by an internal Met. These results clearly demonstrated that the mutation of Pro784 improved the translocation efficiency of H5. Thus, the plasmids that encoded fusion proteins containing "Pro$^\star$-mutated H5" (referred to as H$^\star$5) were used for testing the topogenic properties of the downstream hydrophobic segments H6–H9.

No Transmembrane Domains Existed between H5 and H6—The H5–H6 loop is a relatively long stretch, in which a residue (Arg$^{832}$) was reported to be present in the cytosolic side (Karlish et al., 1993), which suggests there may be a transmembrane span between the ligation site of the reporter protein and H6. To examine this possibility, the plasmid pCB$^\star$5–6 was constructed. This plasmid encoded a fusion protein that was similar to C*-5 but CAT was ligated at residue Ile$^{845}$ instead of Ala$^{818}$.

Two proteins (39 and 42 kDa) were immunoprecipitated by anti-CAT antibody from translation of the pCB$^\star$5–6 transcript in the presence of microsomes (Fig. 4, B$^\star$5–6, lane 2). The 42-kDa protein was converted by Endo H treatment to 39 kDa, which was the expected size of the primary translation product of the fusion protein (lane 3). From the comparison of the translocation efficiency between fusion proteins C*-5 and B$^\star$5–6, the H5–H6 loop was considered to be translocated into the ER lumen. Therefore, we concluded that there were no hydrophobic segments within the loop that functioned as a halt transfer signal. However, it cannot be ruled out completely the possibility that some regions in the loop may be inserted posttranslationally to microsomal membranes together with H6, H7, and/or H8.

Among Hydrophobic Segments H6 (Leu$^{844}$–Ala$^{869}$), H7 (Phe$^{911}$–Ile$^{931}$), H8 (Ile$^{948}$–Leu$^{973}$), and H9 (Thr$^{981}$–Tyr$^{996}$), Only H9 Functioned as a Halt Transfer Signal—The primary translation products of the pC$^\star$5–6 transcript had molecular masses of 43, 49, 53, and 57 kDa, respectively (Fig. 5, lane 1). In the presence of microsomes (lane 2) produced two additional proteins in C*-6 and C*-7 and one additional protein in C*-8, but no additional

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**FIG. 4.** Mutation of 1 proline residue in the hydrophobic segment H5 greatly increases its translocation ability in vitro. Left column, transcripts of pN-1, pC-5, pC*-5, and pCB$^\star$5–6 were translated in the absence (lane 1) and presence (lanes 2–6) of microsomes. The latter products were divided into two portions. One portion was used for Endo H treatment (lanes 2 and 3), and the other portion was further divided into three lanes 4, 5, and 6) and used for protease digestion. After treatment, the samples were subjected to immunoprecipitation, followed by SDS-PAGE as described under “Experimental Procedures.” Open circles indicate the position of glycosylated forms. Right column, schematic drawing presents the orientation of the inserted fusion proteins of lane 2. Solid hearts, N, and C indicate glycosylated form, NH$_2$ terminus, and COOH terminus, respectively.
protein in C*-9. These additional proteins were converted by Endo H treatment to the size of their respective primary translation products (lane 3). Majority of the glycosylated forms of C*-6, C*-7, and C*-8 were not digested when treated with proteases in the absence of Triton X-100, but C*-9 was digested into fragments that could not immunoprecipitate with anti-CAT antibodies under the same condition (lane 5). The glycosylated forms were, however, digested in the presence of Triton X-100 (lane 6). These results demonstrated that H6, H7, and H8 were not capable of halting the translocation and were translocated into the ER lumen where the downstream CAT peptide was glycosylated. Thus, the translocation initiated by H*-5 was halted only by H9.

Topogenic Properties of the Hydrophobic Segments Observed in the Native Context Seemed to Be Intrinsic—To examine whether the topogenic properties of H5, H6, H7, H8, and H9 observed in Figs. 4 and 5 were the same when examined in a different context, we constructed plasmids that encoded a fusion protein consisting of 66 amino acid residues (including the cleavable signal sequence) of the NH2-terminal rat growth hormone and one of the five hydrophobic segments, which was linked to the CAT protein. These plasmids were defined as pX5, pX6, pX7, pX8, and pX9 according to the location of the hydrophobic segment in the α subunit (Fig. 2). Two additional plasmids were constructed: pXc, which encoded a fusion protein of the NH2-terminal 66 amino acid residues of rat growth hormone and CAT protein; and pX4, which encoded a fusion protein of the NH2-terminal 66 amino acid residues of rat growth hormone and the hydrophobic segment H4, linked to CAT. Since the H4 in the NH2-terminal third of Na,K-ATPase α subunit has been confirmed to function as a halt transfer signal (Xie and Morimoto, 1995), pX4 served as a positive control and pXc was used as a negative control.

As shown in Fig. 6, a single protein with the same molecular weight as the primary translation product (33 kDa) was obtained from the translation of pXc transcript in the presence of microsomes (X-c, lane 2). This protein was converted to a 30-kDa protein by Endo H treatment (X-c, lane 3). Protease treatment in the absence of Triton X-100 did not cause any change in the molecular mass of the protein, but the same treatment in
the presence of detergent digested the protein (lanes 4 and 5).
Since the signal sequence of rat growth hormone has 26 amino
acid residues and the shift of molecular mass in SDS-PAGE
due to one N-linked glycosylation was about 3 kDa, the trans-
lation product in the presence of microsomes would be almost
the same size as the primary translation product if the trans-
location of a fusion protein initiated by the signal sequence
was not halted by the downstream segment, and this product
would be completely protected from digestion by exogenously added
proteases. However, if the translocation was halted by the
downstream segment, the translation product would be smaller
by about 3 kDa than the primary translation product and
digested by exogenously added proteases into fragments that
could not immunoprecipitate with anti-CAT antibody. Therefore,
the results showed that the translocation of the fusion
protein X-c was initiated by the cleavable signal sequence and
the following CAT protein was translocated into the ER lumen
where it was glycosylated.

The primary translation products of pX7, pX8, and pX9 tran-
scripts showed a single protein with a molecular mass of 36
kDa (Fig. 6, lane 1). The immunoprecipitates from the pX5 and
pX6 translation products showed two major proteins and one
minor protein (lane 1). The largest ones corresponded to the
expected size of the respective fusion proteins; the other two
could have been the products that were initiated by internal
methionines or degradation products. Translation of the pX5
and pX8 transcripts in the presence of microsomes yielded,
respectively, the same size of the protein as obtained in the
absence of microsomes (lane 2). Translation of the pX6 and pX7
transcripts yielded additional proteins (39 kDa in fusion pro-
teins X-6 and X-7, 33 kDa in fusion protein X-7) and the
translation of the pX9 transcript yielded a single protein with
smaller molecular mass (lane 2). Endo H treatment resulted in
the decrease by about 3 or 6 kDa of the molecular mass of each
fusion protein, except the pX9 translation products, whose
molecular mass was not changed (lane 3). Protease treatment
did not cause any change in the molecular mass of any of the
fusion protein except the smaller protein of the pX5 translation
products (X-5, lanes 2 and 4) and the pX9 translation products
(X-9, lane 4), both of which were digested into the fragments
that did not immunoprecipitate with anti-CAT antibody. These
data have confirmed the previous observation that among hy-
drophobic segments H6–H9, only H9 has a halt transfer signal
function. It should be pointed out here that H5 functions as an
insertion signal like H1, but, as opposed to H3, H5 has a weak
halt transfer function as seen in Fig. 6 (X-5, lanes 2 and 4).

Hydrophobic Segments H6, H7, and H8 That Were Translo-
cated into the ER Lumen Seemed to Remain in the ER without
Being Secreted—To examine whether H5, H6, H7, H8, and H9
have the same topogenic properties in living cells, plasmids
pXc, pX5, pX6, pX7, pX8, and pX9 were expressed transiently in COS cells. The immunoprecipitates from the pXc-expressing cells showed a single protein with a molecular mass of 33 kDa, which corresponded to the expected size of the unglycosylated form of the fusion protein X-c (Fig. 7, lane C). More than 50% of the 33-kDa protein was converted by Endo H treatment to a 30-kDa protein (lane M). The immunoprecipitates from the culture medium showed also a single protein (33 kDa), but Endo H treatment did not cause any shift in the molecular mass (lanes M − and M +). These results demonstrated that the fusion protein X-c was translocated into the ER lumen where the reporter protein was glycosylated and then secreted into culture medium like a regular secretory protein.

The immunoprecipitates from cells expressing plasmids pX5, pX8, and pX9 showed a single protein with molecular masses of 37, 36, and 36 kDa, respectively, and those from cells expressed with pX6 and pX7 showed three proteins with molecular masses of 33, 36, and 39 kDa (lane C). Endo H treatment did cause a decrease by 3 or 6 kDa in the molecular mass of each of the products except for the pX9 translation product, which remained unchanged (lane C ). No proteins were recovered from their culture media by the immunoprecipitation (lane M −), even after a 5-h chase (data not shown). These results have confirmed that all hydrophobic segments show the same topogenic properties both in the native context and in the different context. Interestingly, the fusion protein containing either H5, H6, H7, or H8 was totally translocated into the ER lumen, but no products were secreted into the culture medium, while the pXc products were secreted like regular secretory proteins. The only difference between the pXc translation product and pX5, -6, -7, and -8 translation products was that the latter group of the fusion proteins contained a hydrophobic segment (about 20 amino acid residues) at the ligation site between the truncated growth hormone peptide and the CAT protein. Since the glycosylated form was sensitive to Endo H treatment, the products may remain in the ER lumen by unknown mechanisms.

**DISCUSSION**

Unexpected Topogenic Properties of the Hydrophobic Segments in the COOH-terminal Third of α Subunit—Our data demonstrated the following. (a) Membrane insertion of the COOH-terminal third of the α subunit was initiated by H5 and terminated by H9 (Figs. 4 and 5). (b) H6, H7, and H8, located between H5 and H9, lacked halt transfer function and were translocated across the membrane into the ER lumen (Fig. 5). (c) H5 functioned as an insertion signal, but its halt transfer signal activity was weak (Fig. 6). This was quite a different property from H1 that initiated membrane insertion of the NH2-terminal third of the α subunit as shown by Xie and Morimoto (1995). (d) When H6, H7, and H8 were individually translocated into the ER lumen, they appeared to remain there without being secreted. These results clearly showed that the hydrophobic segments in the COOH-terminal third had very different membrane topology from those predicted by hydropathy analysis and other studies.

Why H6, H7, and H8 did not function as a halt transfer signal can not be explained by factors that are usually considered to be determinants of topogenic properties of hydrophobic segments. For example, the average hydrophobicity indices of the hydrophobic segments calculated using the residue hydrophobicity index taken from the data by Kyte and Doolittle (1982), were 2.19 (H1), 1.87 (H2), 2.42 (H3), 2.00 (H4), 2.07 (H5), 1.48 (H6), 1.69 (H7), 1.43 (H8), and 1.17 (H9). The values for H6, H7, and H8, which had no topogenic function, were significantly lower than those for H1, H2, H3, H4, and H5, which had topogenic function. However, the values for H6, H7, and H8 were higher than the value for H9, which had topogenic function. Thus, the topogenic properties of hydrophobic segments could not be defined by their average hydrophobic indices. Distribution of charged amino acids in the NH2- and COOH-terminal flanking regions of each hydrophobic segment is also considered to be an important factor that affects the topogenic properties of the segment (review by von Heijne (1994)). However, as seen in Fig. 1, there were no distinct differences in the distribution of charged amino acids of the flanking regions among the segments that had topogenic function and those who did not. These observations suggest that there must be other factors that determine the topogenic properties of the hydrophobic segments H6, H7, and H8. Similar examples have been reported with CHIP28 water channel protein (Skach et al., 1992, 1993) and H,K -ATPase (Bamberg and Sachs, 1994).

Another hydrophobic segment that displayed unexpected topogenic properties was H5. This segment could initiate translocation of COOH-terminal third of α subunit, but the efficiency of translocation was much lower than that of H1 (Fig. 4), and the halt transfer function was much weaker than that of H1 (Figs. 4 and 6). When these two segments were compared, their hydrophobicity indices were similar, but H5 contained 3 proline residues while H1 did not contain any. Since proline is known as an α helix breaker, the unusual topogenic properties of H5 may be to some extent due to the proline residues. This possibility was supported by the present observation (Fig. 4) that mutation of Pro784 to leucine resulted in a significant increase in the translocation efficiency (Fig. 4). Homareda et al. (1992, 1993) observed that increase in translocation efficiency...
proportional to the number of proline residues mutated. When the 10 residues flanking the NH2 termini of H1 and H5 were compared, H1 had 2 positively and no negatively charged amino acid residues, but H5 had no positively and 1 negatively charged residue (Fig. 1). Since H5 initiates translocation of the COOH-terminal third of the α subunit and not 1 membrane stretch, it is reasonably considered to have the same topogenic function as H1 in membrane insertion of its downstream peptide. Therefore, the positively charged amino acid residues in the NH2-terminal flanking region may play an important role in membrane insertion as indicated by several investigators (review by von Heijne (1994)). However, the NH2-terminal flanking region of H5 had only negatively charged residue. Such a distinct difference from H1 might be at least partially accounted for the unexpected topogenic properties of H5.

Sidedness of the Loop between Two Adjacent Hydrophobic Segments—Contradictory results have been reported on the membrane location of the sequence between H5 and H6. Our data (Fig. 4) demonstrated that the H5–H6 loop was located on the luminal side of the membrane, which supports the results of Mohraz et al. (1994). These authors demonstrated using immunochemistry with site specific antibody VG2 that the domain Val812–Ile826 was located on the extracellular side. However, several other investigators reported that the loop was located in the cytosolic side of the membrane. Those include the following. (a) Protease digestion of Na,K-ATPase in the right side out membrane vesicles in the presence or absence of detergent showed that Arg832 was located on the cytosolic side of the membrane (Karlish et al., 1993). (b) Immunochromatographic localization of the oligopeptide specific antibody on the two dimensional crystal of the oligopeptide showed that the domain Leu815–Arg830 was located in the cytosolic side of the membrane (Ning et al., 1993). (c) Yoon and Guidotti (1994) demonstrated that the domain Leu815–Arg830 was located in the cytoplasmic side of the membrane using “epitope insertion” also used by Canfield and Levenson (1993). In this method, the nucleotide sequence that encoded an immunoreactive peptide was inserted into various region within the α subunit cDNA, and the location of the peptide was determined by immunofluorescence method using cells that had been transiently expressed with the mutated cDNA.

In addition to the present data, five independent studies showed that the loop between H6 and H7 was located in the extracellular side. These studies are as follows. (a) Mohraz et al. (1994) showed that the domain Trp889–Phe911 was located on the extracellular side by immunogold method using site specific antibody IIIC9 and large membrane fragments rich in Na,K-ATPase. (b) Ning et al. (1993) localized the domain Asn889–Gln903 on the extracellular side by immunochemistry using an epitope-specific antibody. (c) Yoon and Guidotti (1994) demonstrated extracellular location of Gln903. (d) Schultheis et al. (1993) showed that Arg880 was a portion of ouabain binding site in the COOH-terminal third. (e) Lemas et al. (1994) demonstrated that a stretch of 26 amino acid residues (Asn831–Ala916), which included the beginning of H7, were involved in the assembly with β subunit.

Contradictory results have also been reported on the sidedness of the H7–H8 loop. Our data (Fig. 5) that showed the extracellular localization of the loop agreed with the results by Canfield and Levenson (1993). These authors found that using an epitope insertion a peptide inserted at Gly943 was positive to the peptide-specific antibody in the absence of detergent when the mutated cDNA was expressed transiently in cells. However, Yoon and Guidotti (1994) demonstrated that using the same “epitope insertion” the residues Val935 and Phe940 were located in the cytoplasmic side. Furthermore, Fisone et al. (1994) found that Ser936 was phosphorylated by protein kinase A, which indicates the cytoplasmic location of this amino acid residue.

Concerning the sidedness of the H8–H9 loop, our data indicated that it was in the extracellular side, but Canfield and Levenson (1993) showed by using “epitope insertion” method that Leu978 was located on the cytoplasmic side. The sidedness of the COOH-terminal flanking region of H9 was shown to be in the cytosolic side of the membrane by immunochromatographic method (Antolvic et al., 1991), epitope insertion method (Canfield and Levenson, 1993), and the present topogenic analysis of the hydrophobic segments.

Most of contradictory results stem from studies on the localization of antigenic sites by immunofluorescence microscopy of intact and permeabilized cells, and studies on the protease digestion of Na,K-ATPase-rich vesicles in the presence or absence of detergent. Positive results obtained by these methods in the absence of detergent can be safely interpreted as extracellular location. However, those obtained only in the presence of detergent can not always be interpreted as intracellular location. For example, if an antigenic site on the extracellular side is folded in such a way that it is covered by other domains of the peptide, it will not react with antibodies in the absence of detergent. However, when cells are permeabilized, conformation of membrane spanning helices may induce unfolding of the peptide which covers the antigenic site. Even the antigenic sites that are partially embedded in the lipid bilayer from the surface but not exposed to the cytosolic side will become exposed.

A Model for the Biogenesis of Na,K-ATPase α Subunit—In a previous study, we reported membrane topology of the NH2-terminal third of Na,K-ATPase α subunit (Xie and Morimoto, 1995). Based on the present and the previous studies, we propose a model for biogenesis of the α subunit in which the first hydrophobic segment H1 targets the nascent chain ribosome complex to the ER membrane and initiates translocation of the COOH-terminal flanking region. This translocation is terminated by the second hydrophobic segment H2. The second cycle of translocation is initiated by the third hydrophobic segment H3 and is terminated by the fourth hydrophobic segment H4. After a long cytoplasmic stretch of the middle third of the subunit, the third cycle of translocation is initiated by the fifth hydrophobic segment H5. This translocation continues until it is terminated by the ninth hydrophobic segment H9 that is located near the COOH terminus of the subunit. Hydrophobic segments H6, H7, and H8, which were located between H5 and H9, are translocated into the ER lumen. Thus, six membrane-spanning helices are established. H6, H7, and H8 may associate on the luminal surface of ER or may be embedded from the lumen into the lipid bilayer of the ER membrane during translocation or post-translationally shortly after the translocation is terminated.

The unique feature of this model is that the membrane insertion of the α subunit is achieved by combination of three cycles of alternate initiation and termination of translocation to establish transmembrane spans and or-post-translocational interaction of H6, H7, and H8 within the luminal surface of the ER to establish partially embedded membrane spans (Fig. 8).

Recently Bamberg and Sachs (1994) reported a study of the topological analysis of H+,K+-ATPase α subunit using a method similar to the one used in the present study to identify transmembrane segments of the subunit. 10 hydrophobic segments (M1–M10) were chosen from either biochemical analyses or hydrophathy plots, and their topogenic properties were examined. The results showed that four segments M1–M4 in the NH2-terminal portion were inserted into microsomal mem-
branes as in the case of four NH2-terminal hydrophobic segments (H1–H4) of Na,K-ATPase α subunit. Unexpectedly, M5, M6, and M7 did not have topogenic function like H6, H7, and H8 of Na,K-ATPase. M8 and M10 had a halt transfer function but not an insertion signal function, and M9 functioned as both an insertion and a halt transfer signals. Based on these results, the authors have proposed a model that H subunit seems to be inserted into microosomal membranes by similar mechanisms except that the insertion signal of the third cycle of translocation is M9 for H subunit and Na,K-ATPase α subunit shown in Fig. 1. Hydrophobic segment H6 has 2 consecutive glycine residues in the middle, and both flanking regions of the segment are definitely located on the extracellular side (Fig. 4). Since glycine is an α-helix breaker, the segment may be embedded in the lipid bilayer from the lumen forming a hairpin structure in which the two glycine residues make a turn. The hydrophobic segment H7 has 1 Asp near the COOH terminus and H8 has 2 Glu residues in the middle of the segment. The H7–H8 loop has 16 amino acid residues, of which 5 are positively charged and 2 are prolines, but no negatively charged amino acid residues are present. The H8–H9 loop has 7 amino acid residues, of which 2 are positively charged and 2 are proline residues, but no negatively charged amino acid residues are present. Since H9 was established as the last transmembrane segment whose COOH-terminal flanking region was on the cytoplasmic side, the H8–H9 loop must be on the luminal side. Therefore, if H7 and H8 are embedded in such a way that the NH2 terminus of H7 and COOH terminus of H8 face the lumen, the H7–H8 loop, which is hydrophilic, would be folded between H7 and H8, possibly by interaction between negatively charged amino acids in the segments and positively charged amino acids in the loop. The embedded H7 and H8 may form a complex with the other hydrophobic segments in the COOH-terminal third as well as hydrophobic segments in the NH2-terminal third.

Among hydrophobic segments that have topogenic function, only H5 has 3 proline residues. Because of this, the segment had weak halt signal function. As the COOH-terminal third of α subunit may be involved in occlusion and cation transport (Karlish et al., 1990), and this portion pops out when incubated at 50 °C (Arystarkhova et al., 1995), it is reasonable to consider that the complex formed by H6, H7, and H8 is movable within the lipid bilayer and the nature of the hydrophobic segment H5 may facilitate the movement of the complex. Thus, the unique nature of H5 found in this study may contribute not only to the folding process of the COOH-terminal third of the peptide during the enzyme biogenesis but also to the movement of the folded peptide complex within the membrane, coupling it to the gating activity of the enzyme. Such complexity in the structure-function relationship of the COOH-terminal third of the α subunit might have given rise, at least partially, to the contradictory results on the sidedness of the loops. Further studies on the role of the 3 proline residues in the topogenic function of H5, the role of hydrophobic segments H5, H6, H7, H8, and H9 in the folding process during enzyme biogenesis, and the involvement of β subunit in these processes, which are currently in progress, will provide crucial information on the conformational changes of the folded peptide complex that accompanies the ion transport function of the enzyme.

**Acknowledgment**—We thank Dr. David D. Sabatini for encouragement and support throughout this work. We also thank Dr. Y. Hala for the rat brain Na,K-ATPase α subunit cDNA, J. Culkin and F. Forcino
for their excellent photographic work, and H. Plesken for her excellent artwork. We are very grateful to Dr. Manijeh Mohraz for critical reading of the manuscript and helpful discussion and Dr. J. Shafland for advice during preparation of the manuscript.

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