Transmembrane Organization of the Na,K-ATPase Determined by Epitope Addition†

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ABSTRACT: The Na,K-ATPase is a membrane-associated enzyme that establishes the internal Na^+/K^+ environment of most animal cells. The catalytic (α) subunit of the Na,K-ATPase contains multiple transmembrane segments, but the number and location of these domains has not been clearly established. We have used epitope addition to determine the transmembrane topology of the α subunit. An immunoreactive peptide was inserted into various regions of the cDNA encoding the rat α1 subunit, and the constructs were expressed in transfected mammalian cells. The intra- or extracellular location of the epitiope tags was determined by immunofluorescence analysis. Our results indicate that the amino and carboxyl termini of the α subunit are situated intracellularly, and the polypeptide is likely to possess eight membrane-spanning segments. The systematic application of epitope tagging may be useful for analyzing the topology of membrane proteins of unknown structure.

Information on the overall structure of a protein is crucial for understanding the relationship between protein structure and function. Crystallographic analysis of bacteriorhodopsin (Henderson et al., 1990), the bacterial photosynthetic reaction center (Deisenhofer et al., 1985), and porin (Weiss et al., 1991; Cowan et al., 1992) has provided important information on the structure of these membrane proteins. However, the high-resolution structure of other integral membrane proteins has not yet been determined. Secondary structure models of membrane topology are commonly derived from algorithms designed to predict the location of putative hydrophobic α-helical membrane spanning segments (Kyte & Doolittle, 1982; Engelman et al., 1986). A number of approaches have been developed to test these predictions. Reagents that react within the lipid bilayer have been used to identify membrane-spanning segments (Brunner, 1981). Membrane impermeant reagents (Lemke et al., 1982), proteases (Dumont et al., 1985), and sequence-specific antibodies (Carrasco et al., 1986) have also been used to identify intra- and extracellular regions within a protein.

Here we have used a genetic approach to determine membrane protein topology. This approach is based on identification of the intra- versus extracellular location of an epitope tag inserted into an expressed transmembrane protein. To test this strategy, we have used the Na,K-ATPase, a well-characterized membrane-associated protein that catalyzes the coupled translocation of Na^+ and K^+ across the plasma membrane. The enzyme has been shown to consist of two subunits. The α or catalytic subunit contains the site for ATP hydrolysis (Cantley, 1981). The β subunit is a glycosylated polypeptide of unknown function.

The cDNA for the rat α1 subunit, encoding one of three highly conserved rodent α subunit isoforms (Shull et al., 1986; Herrera et al., 1987), has been shown to be capable of conferring ouabain resistance upon ouabain-sensitive primate cells in DNA transfection experiments (Kent et al., 1987; Emanuel et al., 1988). Based on hydropathy analysis of the predicted amino acid sequence, several secondary structure models have been proposed in which the α subunit has from 6 to 10 membrane-spanning domains (Kawakami et al., 1985; Shull et al., 1985; Herrera et al., 1987; Takeyasu et al., 1990). The orientation of the carboxyl terminus of the α subunit is also a matter of uncertainty. This region has been placed at the extracellular surface (Ovchinnikov et al., 1988; Bayer, 1990), intracellularly (Antolovic et al., 1991; Thibault, 1993), or embedded in the membrane (Ball & Loftice, 1987). To help resolve these issues, we generated a panel of epitope-tagged α1 subunits and determined directly whether the epitope is located on the inside or the outside of the cell.

EXPERIMENTAL PROCEDURES

DNA Constructs. Epitope-tagged Na,K-ATPase α1 subunits were generated by introduction of a nine amino acid peptide from the hemagglutinin (HA) of influenza virus into rat α1 subunit cDNA. Two strategies were used for epitope addition. In the first, sense and antisense oligonucleotides corresponding to the nine amino acid (aa) HA epitope (YPYDVPDYA) were synthesized, annealed, and inserted directly into blunt-ended restriction sites within α1 subunit cDNA. The second approach involved the generation of a set of HA oligonucleotides that could be introduced in-frame into any natural or engineered blunt-ended restriction site in the cDNA. To do this, a set of three sense and three antisense HA oligonucleotides were generated containing 0, 1, or 2 additional nucleotides at the 5′ end. The oligonucleotides were annealed pairwise and made blunt-ended using the Klenow fragment of DNA polymerase I. Oligonucleotide tags were introduced at the following restriction sites (numbered from the initiating methionine): *Pvu*II (aa 14); *Bam*H1 (aa 514); *Eco*47III (aa 608); *Stu*I (aa 662); *Nco*I (aa 734); *Bsp*EI (aa 785); *Bsp*HI (aa 832); *Bcl*I (aa 851); *Pfl*MI (aa 862); *Eco*RI (aa 941); *Bsu*36I (aa 978); *Sma*I (aa 1013). Each mutant α1 cDNA was verified by dideoxynucleotide sequencing and subcloned into the eukaryotic expression vector pCB6.

Cell Culture and DNA Transfection. African green monkey kidney CV-1 cells and human embryonic kidney 293 cells were used as recipients for transfection. CV-1 and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM)

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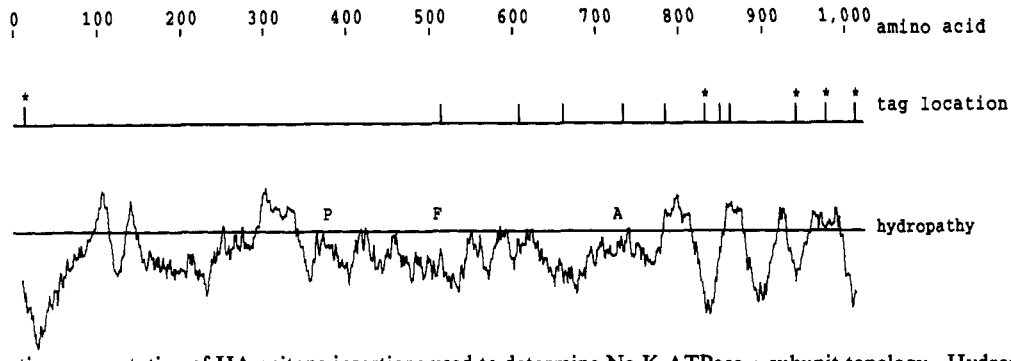

FIGURE 1: Schematic representation of HA epitope insertions used to determine Na,K-ATPase α subunit topology. Hydropathy analysis of the rat α1 subunit was obtained using the Goldman–Engleman–Steitz scale using a 15-residue window. The phosphorylation (P), FITC-reactive (F), and FSBA-reactive (A) sites are depicted above the hydropathy plot. The scale (every 100 aa) is shown at the top. Insertion sites of the HA epitope tag are represented by vertical lines on the linear map. Asterisks denote functional epitope-tagged constructs capable of conferring ouabain resistance to CV-1 cells.

supplemented with 10% fetal calf serum (FCS). Transfections were performed essentially as described previously (Kent et al., 1987). For stable transfectants, CV-1 or 293 cells were exposed to a calcium phosphate precipitate of plasmid DNA (10 μg of plasmid DNA per approximately 10^6 cells). Forty-eight hours later, cultures were split 1:4. Medium containing ouabain (0.5 μM) was added 4 h later. Colonies of transfected cells were picked in 2–3 weeks and expanded into cell lines that were maintained in medium containing 0.5 μM ouabain. For transient transfection, 293 cells were plated on glass coverslips and transfected under conditions described above. Following transfection, cells were maintained on coverslips in DMEM supplemented with 10% FCS.

Membrane Preparation and Immunoblotting. Crude membrane fractions from transfected CV-1 cells were prepared essentially as described previously (Shyjan & Levenson, 1989), and protein concentrations were determined as described by Bradford (1976). Solubilized membrane proteins were fractionated on an SDS-containing 7.5% polyacrylamide gel (80 μg of protein/lane) and transferred to a nitrocellulose filter as described (Towbin et al., 1979). The filter was quenched overnight in Tris-buffered saline (TBS) containing 5% dry milk and 0.1% Tween 20 and then incubated with the HA-specific monoclonal antibody (MAb) 12CA5 (Babco Labs, Richmond, CA). The blot was rinsed with wash buffer (TBS, 0.1% Tween 20) and then incubated with horseradish peroxidase conjugated goat anti-mouse (second) antibody for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL) using an ECL kit (Amersham).

Immunofluorescence and Confocal Microscopy. Transfected human 293 cells grown on glass coverslips were examined 72 h after transfection. For experiments using permeabilized cells, 293 cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized and blocked with 0.05% Nonidet P-40 in PBS, 5% goat serum, and 1% bovine serum albumin at room temperature for 15 min and then incubated in the same medium containing the 12CA5 MAb (diluted 1:500) for 1 h. Secondary antibody (Texas Red-conjugated rabbit anti-mouse IgG) was diluted 1:200 and applied in the same buffer. Nonpermeabilized 293 cells were incubated with MAb 12CA5 in DMEM, 10% FCS, and 20 mM Hepes, pH 7.4 at 4 °C for 1 h. Cells were fixed, permeabilized, and incubated with secondary antibody as described above. Immunofluorescence microscopy was performed using standard epifluorescence optics (Zeiss Axiophot) or by confocal laser scanning microscopy using a Bio-Rad MRC 600 confocal microscope (GHS filter block, 514-nm excitation wavelength).

RESULTS

To analyze the membrane topology of the Na,K-ATPase, we determined the intra-versus extracellular location of an HA epitope tag inserted into the expressed α1 subunit polypeptide. A panel of gene fusions was produced in which the HA epitope was inserted at the amino (N-) terminal end of the α1 subunit, at various sites within the carboxyl (C-) terminal third of the protein, or at sites within the large cytoplasmic domain located between putative transmembrane segments 4 and 5 (Figure 1). To test biological activity, each of the full-length epitope-tagged α1 cDNAs was introduced into the eukayrotic expression vector pCB6 downstream of the cytomegalovirus promoter and transfected into ouabain-sensitive CV-1 or 293 cells. Transfected cells were selected for their ability to proliferate in 0.5 μM ouabain, a concentration of drug that is normally cytotoxic to these cells (Canfield et al., 1990). As a positive control, wild-type rat α1 subunit cDNA was introduced into CV-1 or 293 cells. A construct, HA14, carrying the tag at the N-terminus of the α1 subunit (residue 14) gave ouabain-resistant colonies with an efficiency similar to that of wild-type α1 cDNA. Constructs HA832, HA941, HA978, and HA1013, containing the tag within the C-terminal third of the α1 subunit at residues 832, 941, 978 and 1013, respectively (Figure 1), also produced ouabain-resistant colonies with high efficiency. The ability of the tagged constructs to transfer ouabain resistance indicates that the α1 fusion protein is capable of forming an active Na,K-ATPase.

Constructs carrying the HA epitope within the cytoplasmic domain situated between transmembrane segments 4 and 5 failed to confer ouabain resistance in our assay. One construct was tagged at residue 514, a site that maps close to the FITC (fluorescein 5′-isothiocyanate)-reactive lysine at position 508 (Farley et al., 1984), while another was tagged at residue 734 in close proximity to the FSBA [5′-(*p*-fluorosulfonyl)-benzoyladenosine]-reactive lysine at position 726 (Ohta et al., 1985). These sites are highly conserved among Na,K-ATPase α subunit isoforms and have been implicated in ATP binding (Farley et al., 1984; Ohta et al., 1985). Two constructs carrying the HA tag in the C-terminal region (residues 851 and 862) also failed to transfer ouabain resistance. The presence of the epitope at these sites may interfere with proper enzymatic activity or folding.

Expression of the epitope-tagged α subunits was determined by immunoblot analysis. Microsomal membrane fractions prepared from ouabain-resistant CV-1 transfectants were probed with 12CA5, a MAb specific for the HA epitope (Wilson et al., 1984). The presence of the epitope-tagged

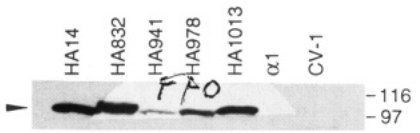

FIGURE 2: Detection of epitope-tagged α1 subunits in cell membranes by immunoblot analysis. Crude membranes were prepared from CV-1 cells stably transfected with HA epitope-tagged or wild-type rat α1 subunit cDNAs, or from untransfected CV-1 cells. Solubilized microsomal proteins (80 μg/lane) were resolved on an SDS-containing 7.5% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the HA-specific MAb 12CA5. Immunoreactivity was detected by ECL. The positions of molecular mass markers (in kDa) are shown at the right.

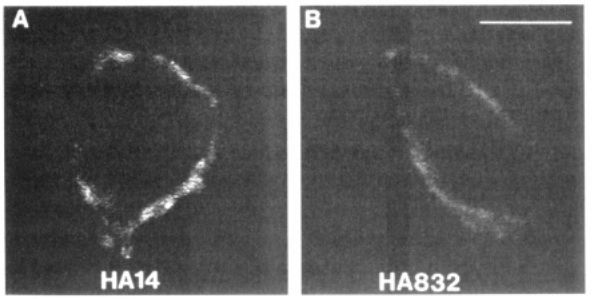

FIGURE 3: Subcellular localization of epitope-tagged α1 subunits visualized by confocal laser microscopy. Human 293 cells transiently expressing the HA14 (A) and HA832 (B) constructs were fixed and permeabilized, and the HA epitope was detected using MAb 12CA5 and Texas Red-conjugated anti-mouse antibodies. Optical sections were scanned through cells ~2 μm above the surface of the coverslip. In permeabilized cells, bright staining is visualized at cell margins. No staining was observed in nonpermeabilized cells. Scale bar = 10 μm.

protein in all ouabain-resistant transfectants was indicated by expression of a MAb-reactive polypeptide of ~100 kDa (Figure 2), a size consistent with that of the Na,K-ATPase α1 subunit. The epitope-tagged polypeptide was not detected in untransfected CV-1 cells or in CV-1 cells transfected with wild-type rat α1 subunit cDNA (Figure 2).

Examination of epitope-tagged α1 subunit distribution in stable and transiently expressing cells by immunofluorescence microscopy revealed a uniform pattern of staining, consistent with the localization of the polypeptide in the plasma membrane. To more precisely determine α1 subunit distribution, epitope-tagged α1 polypeptides were visualized by confocal laser microscopy. Examination of 293 cells transiently expressing the HA14 (Figure 3A) or HA832 (Figure 3B) constructs revealed bright staining at cell margins. These results support the view that tagged α1 subunits are present at the cell surface.

To determine the membrane orientation of the epitope-tagged domains, the staining specificity of the 12CA5 MAb was characterized in 293 cells transiently expressing epitope-tagged α1 subunits. Stable and transient expression of the epitope-tagged constructs gave identical patterns of immunoreactivity. However, transient expression facilitated detection of the epitope-tagged constructs because quantitatively higher levels of the tagged protein were produced compared to stably transfected 293 cells. In 293 cells expressing the HA14 construct, MAb 12CA5 was immunoreactive with permeabilized cells (Figure 4A) but did not detect tagged α1 subunits in nonpermeabilized cells (Figure 4B). These results are consistent with the proposed intracellular disposition of

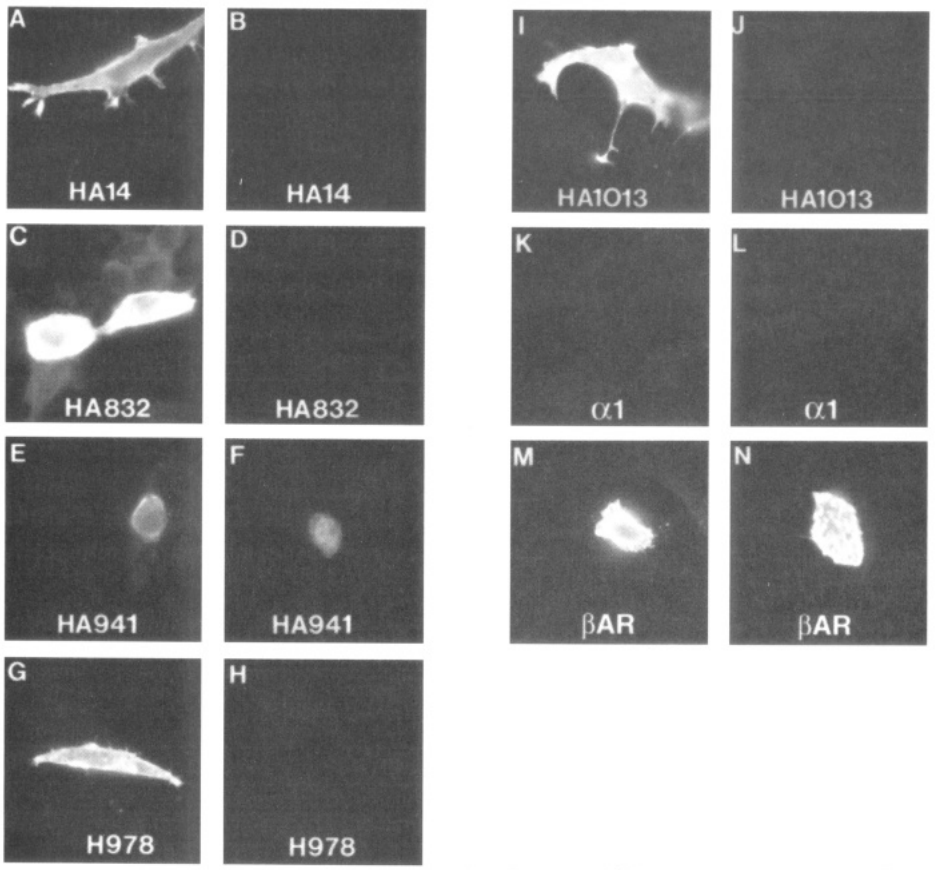

FIGURE 4: *In situ* detection of epitope-tagged α1 subunits by immunofluorescence. Human 293 cells transiently expressing epitope-tagged rat α1 subunit cDNAs were examined for MAb 12CA5 immunoreactivity 3 days after transfection. For each construct, fields of permeabilized (left column) and nonpermeabilized (right column) cells are shown. The epitope tags in constructs HA14 (A, B), HA832 (C, D), HA978 (G, H), and HA1013 (I, J) were only detected in permeabilized cells, while the tag in HA941 (E, F) was visualized in both permeabilized and nonpermeabilized cells. Cells expressing wild-type rat α1 subunits (K, L) were not reactive with the 12CA5 MAb, whereas the N-terminal-tagged β2 adrenergic receptor (M, N) was detected extracellularly.

the N-terminus of the α1 subunit (Felsenfeld & Sweadner, 1988). Human 293 cells transfected with wild-type α1 subunit cDNA were not immunoreactive with MAb 12CA5 (Figure 4K,L). For comparison, we monitored immunoreactivity of an expressed β2 adrenergic receptor carrying an N-terminal HA epitope-tag. The N-terminus of this receptor has previously been shown to be extracellular (von Zastrow & Kobilka, 1992). The 12CA5 MAb specifically detected the epitope-tagged receptor in both permeabilized and nonpermeabilized 293 cells (Figure 4M,N). This method is therefore capable of distinguishing between regions located on the outside versus the inside of the cell. Examination of 293 cells expressing the HA832, HA978, and HA1013 constructs revealed that MAb 12CA5 specifically detected each of the tagged proteins in permeabilized but not nonpermeabilized transfectants (Figure 4C–D,G–J), suggesting the intracellular disposition of these epitope-tagged segments. In contrast, the HA941 epitope tag was detectable in both nonpermeabilized and permeabilized cells (Figure 3E,F), suggesting that this region is exposed extracellularly.

DISCUSSION

The results presented here demonstrate the utility of epitope tagging for testing computational predictions of membrane topology. The rat Na,K-ATPase α1 subunit serves as a useful model for testing these predictions because it is possible to assay the biological activity of the tagged polypeptide by transfection. The ability of a tagged α1 subunit to transfer ouabain resistance demonstrates that the polypeptide is capable of forming functional Na,K-ATPase in both CV-1 and 293 cells, thus providing confidence that the structural integrity of the fusion protein has not been compromised.

Previous attempts to determine the orientation of the C-terminus of the α-subunit have relied primarily on indirect assays of antibody binding to vesicles and led to conflicting conclusions (Ovchinnikov et al., 1988; Bayer, 1990; Antolovic et al., 1991; Thibault, 1993; Ball & Loftice, 1987). Direct examination of cells expressing the C-terminal-tagged HA1013 construct shows that MAb 12CA5 is only reactive with the epitope when cells are permeabilized, suggesting that the C-terminus is located on the cytoplasmic side of the plasma membrane. The intracellular distribution of the region defined by the HA832 tag was originally mapped as an ectodomain (Ovchinnikov et al., 1988). Recently, this region has been localized intracellularly using biochemical techniques (Karlish et al., 1993). By immunofluorescence microscopy, we detected the HA832 epitope only in permeabilized cells. The localization of the HA832 fusion protein to the plasma membrane by confocal microscopy provides further evidence for the intracellular disposition of the HA832-tagged region.

A model of α1 subunit topology suggested by our epitope-tagging experiments is shown in Figure 5. Hydropathy and biochemical analysis are consistent with the presence of four transmembrane segments in the N-terminal portion (residues 1–775) of the α1 subunit (Shull et al., 1985; Kawakami et al., 1985; Herrera et al., 1987; Ovchinnikov et al., 1988). However, the number of membrane-spanning segments in the C-terminal region (residues 776–1023) has not been clearly established. The intracellular localization of residues 832, 978, and 1013, coupled with the extracellular localization of residue 941, suggests the presence of one membrane-spanning segment between residue 941 and the C-terminus and a single transmembrane segment between residues 832 and 941. Hydropathy analysis identifies a region between residues 776 and 832 which may contain one or two transmembrane

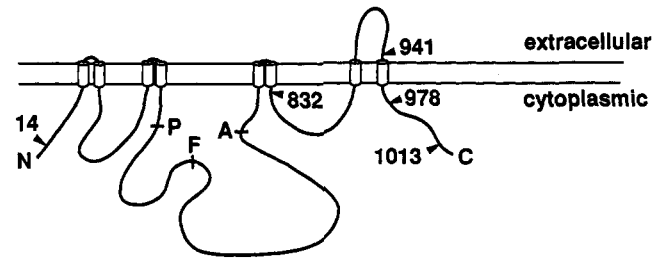

FIGURE 5: Proposed membrane topology of the Na,K-ATPase α1 subunit based on immunolocalization of HA epitope tags. The eight suggested membrane-spanning segments are shown in relation to the lipid bilayer. Insertion sites of the HA epitope are numbered (aa residue) and depicted by arrowheads. N and C refer to the amino- and carboxyl-terminal ends of the protein, respectively. The positions of the phosphorylation (P), FITC- (F), and FSBA-reactive (A) sites are marked by lines.

segments. The biochemically defined intracellular location of residue 726 (Ohta et al., 1988), coupled with the cytoplasmic location of the HA tag at residue 832, suggests the presence of two transmembrane segments in this region. Taken together, our results predict the existence of four transmembrane segments situated between residues 776 and the C-terminus and are most consistent with an eight transmembrane model of α1 subunit topology.

The further application of epitope addition to the Na,K-ATPase α subunit should permit the development of a more detailed structural map for this polypeptide, including the precise location of all transmembrane segments. The experimental system developed in this study should be applicable to other transmembrane proteins, such as ion channels and receptors, particularly in situations where functional assays are available.

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