A Transmembrane Segment Determines the Steady-state Localization of an Ion-transporting Adenosine Triphosphatase

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Abstract. The H,K-adenosine triphosphatase (ATPase) of gastric parietal cells is targeted to a regulated membrane compartment that fuses with the apical plasma membrane in response to secretagogue stimulation. Previous work has demonstrated that the α subunit of the H,K-ATPase encodes localization information responsible for this pump's apical distribution, whereas the β subunit carries the signal responsible for the cessation of acid secretion through the retrieval of the pump from the surface to the regulated intracellular compartment. By analyzing the sorting behaviors of a number of chimeric pumps composed of complementary portions of the H,K-ATPase α subunit and the highly homologous Na,K-ATPase α subunit, we have identified a portion of the gastric H,K-ATPase, which is sufficient to redirect the normally basolateral Na,K-ATPase to the apical surface in transfected epithelial cells. This motif resides within the fourth of the H,K-ATPase α subunit's ten predicted transmembrane domains. Although interactions with glycosphingolipid-rich membrane domains have been proposed to play an important role in the targeting of several apical membrane proteins, the apically located chimeras are not found in detergent-insoluble complexes, which are typically enriched in glycosphingolipids. Furthermore, a chimera incorporating the Na,K-ATPase α subunit fourth transmembrane domain is apically targeted when both of its flanking sequences derive from H,K-ATPase sequence. These results provide the identification of a defined apical localization signal in a polytopic membrane transport protein, and suggest that this signal functions through conformational interactions between the fourth transmembrane spanning segment and its surrounding sequence domains.

Key words: sorting • polarity • epithelia • Na,K-ATPase • H,K-ATPase

Introduction

Polarized epithelial cells localize distinct classes of transport proteins to different membrane surfaces in order to carry out their secretory and absorptive functions. The generation of apical and basolateral plasma membrane domains, which differ substantially in both their lipid and protein contents, requires polarized epithelial cells to target newly synthesized membrane components to their appropriate destinations and to retain them there preferentially after delivery. To become substrates for polarized sorting and retention, membrane proteins must be endowed with sorting signals that specify the proteins' destinations for the cellular sorting machinery. Currently, the nature of these sorting signals has been resolved for only a few membrane proteins. Tyrosine-based signals and dileucine motifs have been shown to direct proteins to the basolateral surface in many epithelial cell types (Ktistakis et al., 1990; Hunziker et al., 1991; Hunziker and Fumey, 1994; Thomas and Roth, 1994). Membrane association through a glycophosphatidyl inositol (GPI)[1] anchor can serve as an apical localization signal in most cell types (Brown et al., 1989; Lisanti et al., 1989). However, less is known about the methods through which non-GPI linked proteins are localized to the apical membrane.

The Na,K-ATPase and gastric H,K-ATPase are highly homologous proteins that are located at opposite membrane domains. These members of the P-type ATPase family are both composed of a 110-kD α subunit that spans the membrane 10 times, and a heavily glycosylated

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[1]*Abbreviations used in this paper:* GPI, glycophosphatidyl inositol; GSL, glycosphingolipid; TM4, fourth transmembrane domain.

55–60-kD β subunit that spans the membrane once. Catalytic activity has been attributed to the α subunit, although the β subunit may play a role in modulating ion affinity (Rabon and Reuben, 1990; Hersey and Sachs, 1995). At the amino acid level, the α subunits of these pumps are 63% identical, whereas the β subunits share 35% identity (Shull and Lingrel, 1986). Despite these similarities, the two proteins are targeted to different subcellular destinations. The gastric H,K-ATPase is restricted to subapical tubulovesicular elements in the resting stomach parietal cell. Stimulation results in the fusion of the tubulovesicular elements with the plasma membrane and relocalization of the H,K-ATPase to the apical membrane, where it actively pumps protons into the lumen of the stomach in exchange for potassium (Smolka et al., 1983; Urushidani and Forte, 1987). The H,K-ATPase β subunit appears to govern the protein's participation in this cycle of exo- and endocytosis through a tyrosine-based endocytosis motif that resides in its cytoplasmic tail (Courtois-Coutry et al., 1997). When expressed by transfection in the pig kidney epithelial cell line LLC-PK1, which does not contain a system of tubulovesicular elements, the H,K-ATPase is found exclusively at the apical membrane (Gottardi and Caplan, 1993a). The Na,K-ATPase, on the other hand, is localized to the basolateral membrane of most epithelial cell types, where it energizes the gradients that drive vectorial transepithelial fluid and electrolyte transport.

Through the generation of chimeric proteins incorporating complementary portions of these two ATPases, it has been possible to identify regions of the proteins that confer their distinctive plasma membrane distributions and catalytic properties (Blostein et al., 1999; Mense et al., 2000). In previous studies using α subunit chimeras expressed in LLC-PK1 cells, it was found that the first 519 amino acids (roughly the NH_2-terminal half) of the H,K-ATPase α subunit assures an apical distribution of a construct (H519N) in which this sequence is fused to the COOH-terminal half of the Na,K-ATPase polypeptide (Gottardi and Caplan, 1993a). The complementary chimera, in which the NH_2-terminal half of the Na,K-ATPase α subunit is paired with the COOH-terminal half of the H,K-ATPase protein (N519H), accumulates at the basolateral membrane in association with the β subunit of the H,K-ATPase (Muth et al., 1998a). These observations also demonstrated that the α subunits contain sorting information that is dominant over any that exists in the β subunits, since the apical chimera H519N assembled with and redistributed the normally basolateral Na,K-ATPase β subunit. Similarly, the N519H chimera redirected the normally apical H,K-ATPase β subunit to the basolateral cell surface. To narrowly define the sorting information that exists within the first 519 amino acids of the gastric H,K-ATPase, we have constructed several new chimeras from complementary portions of the Na,K- and H,K-ATPase α subunits. Here, we report the identification of a transmembrane domain in the gastric H,K-ATPase, which is sufficient for apical localization. Unlike previously identified localization signals in the transmembrane domains of single spanning membrane proteins, this transmembrane domain does not appear to function through glycolipid interactions. Rather, it acts through long range interactions with its flanking loop domains.

Materials and Methods

Construction of Chimeras

Chimeras were constructed between the rat Na,K-ATPase α1 subunit (cDNA provided by E. Benz, Johns Hopkins University, Baltimore, MD) and the rat gastric H,K-ATPase α subunit (cDNA provided by G. Shull, University of Cincinnati, Cincinnati, OH). All manipulations were performed on α subunit cDNAs subcloned into the Bluescript plasmid chimeras (Promega Corp.) at the ClaI and XbaI sites unless noted. The restriction sites ApaI, corresponding to H,K-ATPase amino acid number 85, AccI at amino acid 326, HpaI at amino acid 356, and NarI at amino acid 519, were used to construct the chimeras. Where necessary, sites were introduced at corresponding positions of both α subunit cDNAs through silent site-directed mutagenesis using the Kunkel method. Chimera I was made by subcloning the small ApaI fragment of the H,K-ATPase into the complementary portion of the Na,K-ATPase. To generate chimeras II–V, portions of the H,K-ATPase were excised at the appropriate restriction sites and ligated into the corresponding sites within chimera I. Chimeras VI and VII were made by ligating annealed oligos, encoding the appropriate amino acids into chimera I at the AccI and HpaI sites. Chimera VIII was made by ligating the ClaI/Hpa1 fragment of chimera VI into the corresponding sites within chimera IV. The constructs were sequenced through the ligation points. All chimeras were subcloned into the mammalian expression vector pCB6 (kindly provided by M. Roth, University of Texas Southwestern Medical Center, Dallas, TX) at the ClaI, XbaI sites before being transfected into LLC-PK1 cells.

Cell Culture and Transfection

LLC-PK1 cells were grown in α-MEM (GIBCO BRL) supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Subconfluent LLC-PK1 cells were transfected by the calcium phosphate method (Chen and Okayama, 1987) with chimera constructs that had been subcloned into the pCB6 vector that carries resistance to the antibiotic G418 (GIBCO BRL). After selection in 1.8 mg/ml G418, clones were screened for expression by immunofluorescence and Western blotting.

Immunofluorescence

Immunofluorescence was performed as described (Gottardi and Caplan, 1993a) on stably expressing cell lines. In brief, cells were grown to confluence on Transwell porous cell culture inserts (Corning Costar Corp.), or for screening clones, on 8-well lab-tek slides (Nalge Nunc International) and fixed in −20°C methanol for 7 min at room temperature. Cells were then permeabilized in a PBS-based wash buffer containing 0.3% Triton X-100 and 0.1% BSA for 15 min. Nonspecific binding of antibody was blocked by incubating the cells in goat serum dilution buffer (16% filtered goat serum, 0.3% Triton X-100, 20 mM NaPi, pH 7.4, 0.9% NaCl) (Cameron et al., 1991) at room temperature for 1 h. Chimeras were detected with the polyclonal antibody HK9 directed against the NH_2 terminus of the gastric H,K-ATPase α subunit (1:50). The endogenous Na,K-ATPase, a basolateral marker, was stained with mAb 6H (1:100), which is directed against the NH_2 terminus of the Na,K-ATPase α subunit. Secondary goat anti–mouse or anti–rabbit antibodies (1:200) were conjugated to either rhodamine or fluorescein (Sigma Chemical Co.). All antibody incubations took place in goat serum dilution buffer for 1 h at room temperature. Between primary and secondary antibody incubations, the cells were subjected to three 5-min washes in the PBS-based wash buffer. After incubation with the secondary antibody, cells were washed in PBS three times for 5 min, and finally in 10 mM NaPi for 10 min before being mounted on coverslips with Vectashield (Vector Laboratories). Confocal sections were taken using a Zeiss LSM 410 laser scanning confocal microscope. Images are the product of eightfold line averaging. xz cross sections were generated with a 0.2-μm motor step. Contrast and brightness were set so that all pixels were in the linear range.

Detergent Solubility Assay

The detergent solubility assay was performed as described previously (Arreaza et al., 1994), with minor modifications. LLC-PK1 cells expressing the chimera H519N were grown to confluence in 10-cm^2 tissue culture dishes and lysed in buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and 0.1 M sodium carbonate, pH 11,

on ice for 30 min. Lysates were passed through a 21-gauge needle to shear DNA, mixed with 80% sucrose to a final concentration of 40% sucrose, and transferred into an SW41 polyallomer tube and overlaid with 5 ml of 35% sucrose and 3 ml of 5% sucrose. The floatation gradient was spun at 120,000 *g* for 18 h at 4°C. The material at the 5–35% interface (corresponding to fraction 3) was harvested with a 23-gauge needle and 3-ml syringe. 1-ml fractions were then collected from the bottom. Alkaline phosphatase activity was detected in each fraction using a p-nitrophenylphosphate substrate (Kirkegaard and Perry Laboratories, Inc.). The reaction was stopped after 5 min with the addition of 5% EDTA, pH 8.0, in a 1:1 volume ratio. The product was quantitated on a Flow Labs plate reader at 410 nm and graphed as the percentage of the total absorbance of all fractions. All fractions were assayed by Western blot as described previously (Gottardi and Caplan, 1993b) for the presence of either the chimera or Na,K-ATPase. Aliquots of the fractions were run on 10% SDS-PAGE gels, transferred to nitrocellulose, and probed using the antibodies HK9 (1:250) and 6H (1:500) for the chimera and Na,K-ATPase α subunit, respectively, followed by either goat anti–mouse or goat anti–rabbit antibodies (1:1,000) conjugated to HRP (Sigma Chemical Co.). The resultant product was detected by ECL (Amersham Pharmacia Biotech) and quantified using an IS-1000 Digital Imaging System (Alpha Innotech Corp.) densitometer.

Ouabain Survival Assay

LLC-PK1 cells were plated in 6-well culture tissue dishes and allowed to attach overnight before media containing ouabain (Sigma Chemical Co.) at a concentration of 10 uM or 5 mM was added. The media were changed every 2 d during the assay. Cell survival was scored by light microscopy as the presence or absence of attached proliferating cells at the end of 5 d.

Acidification Assay

LLC-PK1 cells stably expressing chimera III and untransfected LLC-PK1 cells were grown to confluence on Transwell porous cell culture inserts (Corning Costar Corp.). The cells were rinsed in PBS++ and the media were replaced with weakly buffered DME containing 0.2 mM Hepes, pH 7.4. The cells were placed in a 37°C incubator with atmospheric CO_2 levels. At the time points indicated, 100-ul samples were removed from the apical and basolateral chambers, placed under oil, and the pH was measured on a Corning blood gas pH analyzer. All measurements were performed using three separate filters for each time point.

Results

A comparison of the amino acid sequences of the Na,K- and H,K-ATPase α subunits reveals a site of striking nonhomology at the extreme NH_2 terminus. Of the first 46 NH_2-terminal residues, only 9 are identical. Furthermore, the NH_2 terminus of the H,K-ATPase α subunit is 13 amino acids longer than that of the Na,K-ATPase α subunit. To examine the potential sorting function of this region, we constructed a chimera that consists of the first 85 amino acids of the H,K-ATPase fused to the complementary sequence of the Na,K-ATPase (Fig. 1, chimera I). When transfected into LLC-PK1 cells, this chimera was found exclusively at the basolateral membrane as shown by indirect immunofluorescence (Fig. 1, A and C). The endogenous Na,K-ATPase was also found at the basolateral membrane, as would be expected (Fig. 1, B and D). It is clear from this result that the first 85 amino acids of the H,K-ATPase are not responsible for the apical distribution seen with the first chimera, H519N.

Although the first 85 amino acids of the H,K-ATPase do not appear to mediate localization, this sequence does contain an epitope that enables us to discriminate between chimeric α subunits and the endogenous Na,K-ATPase with our panel of antibodies. The HK9 antibody, raised against a synthetic peptide whose sequence was derived

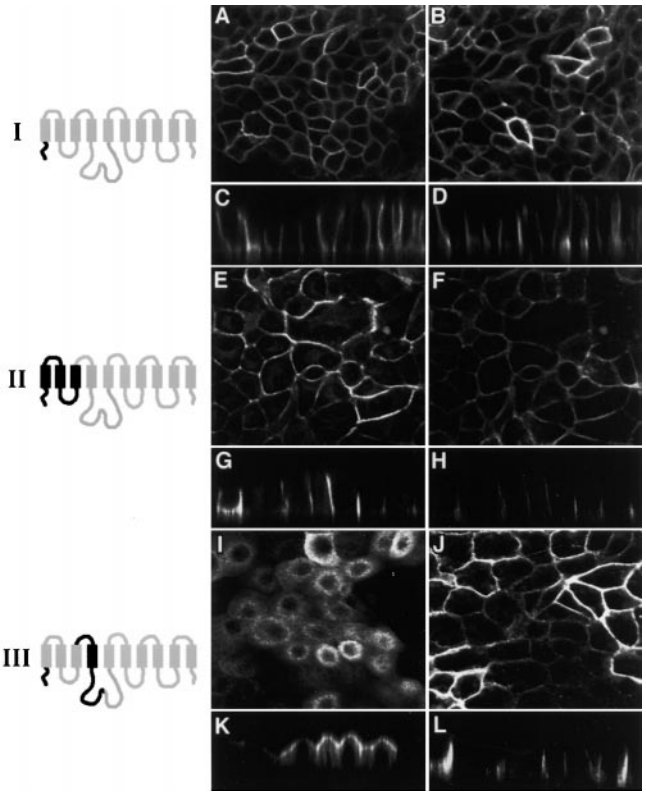

Figure 1. Localization of chimeras I–III in LLC-PK1 cells. Immunofluorescence was performed on LLC-PK1 cell lines stably expressing each chimera using antibodies that recognize the chimera (A, C, E, G, I, and K) or the endogenous Na,K-ATPase (B, D, F, H, J, and L). The structure of each chimera is shown on the left of the panel in which its localization is depicted. The Na,K-ATPase portions are shown in gray and the H,K-ATPase portions are shown in black. As can be seen above, the Na,K-ATPase is basolateral in all of the cell lines when viewed en face (B, F, and J) and in xz cross-section (D, H, and L). Chimeras I and II are located at the basolateral membrane as seen en face (A and E) and in xz cross section (C and G). However, chimera III demonstrates an apical staining pattern shown en face (I) and in xz cross section (K), suggesting that the H,K-ATPase sequence incorporated in this chimera contains apical localization information.

from rat gastric H,K-ATPase, recognizes an epitope at the extreme NH_2 terminus between amino acids 3 and 23. Our antibody directed against the Na,K-ATPase α subunit also recognizes an epitope within the NH_2 terminus between amino acids 1 and 21. Both of these antibodies have been well-characterized and do not show any cross-reactivity to other ATPases. By retaining the first 85 amino acids of the H,K-ATPase α subunit sequence as an epitope tag on the chimeras that were subsequently constructed, we are able to distinguish the chimeric α subunit proteins consisting largely of Na,K-ATPase α subunit sequence from the endogenous Na,K-ATPase. Western blots using the HK9 antibody show that chimeras prepared for this study migrated with the expected molecular weights in SDS-PAGE gels (data not shown).

To define the specific sequences within the remaining 434 amino acids that manifest sorting information, a set of overlapping chimeras was generated by taking advantage

of two engineered restriction sites, AccI and HpaI, at positions corresponding to H,K-ATPase α subunit amino acids 324 and 356, respectively. The chimera containing the NH_2-terminal 324 amino acids of the H,K-ATPase (Fig. 1, chimera II) does not appear to embody apical sorting information, since it is found at the basolateral membrane in transfected cells (Fig. 1, E and G). However, the chimera in which H,K-ATPase sequence constitutes the second ectodomain loop fourth transmembrane domain (TM4), and part of the large cytoplasmic loop (Fig. 1, chimera III) is localized predominantly to the apical membrane in LLC-PK1 cells (Fig. 1, I and K), indicating that the sorting information that leads to the apical distribution of the chimera lies between amino acids 324 and 519 of the H,K-ATPase α subunit.

We further dissected the region between amino acids 324 and 519 by examining two chimeras consisting of the 85–amino acid epitope tag and H,K-ATPase sequence between either amino acids 356 and 519 (Fig. 2, chimera IV) or amino acids 324 through 356 (Fig. 2, chimera V). The chimera containing H,K-ATPase sequence between amino acids 356 and 519, which corresponds to part of the large cytoplasmic loop, resides at the basolateral membrane in transfected LLC-PK1 cells (Fig. 2, A and C). The second chimera (Fig. 2, chimera V) on the other hand, is predominantly localized to the apical membrane when it is expressed in LLC-PK1 cells (Fig. 2, E and G) as seen from the microvillar staining. Therefore, the second ectodomain loop and TM4 of the gastric proton pump appear to contain information that is sufficient to allow the chimeric ATPase to reach the apical membrane.

It has been shown that both the Na,K-ATPase and H,K-ATPase α subunits must associate with their respective β subunits in order to leave the ER and be transported to the plasma membrane. The α subunit chimeras presented here contain the COOH-terminal half of the Na,K-ATPase, which has been shown to determine specificity in β subunit assembly (Gottardi and Caplan, 1993b). Therefore, we would expect these chimeras to dimerize with the Na,K-ATPase β polypeptide. Furthermore, no endogenous H,K-ATPase β subunit has been detected in LLC-PK1 cells by immunofluorescence. In the case of cells expressing apically localized chimeras, which incorporate the COOH-terminal half of Na,K-ATPase α subunit, we would expect that the Na,K-ATPase β subunit would assemble in the ER with both the Na,K-ATPase α subunit and chimeric α subunits, and thus be transported to both the apical and basolateral surfaces. Immunofluorescence localization performed on the cell line expressing chimera V shows that the chimeric protein is localized to only the apical membrane (Fig. 3, A and C), whereas the endogenous Na,K-ATPase β subunit protein is found at both the basolateral membrane and at the apical membrane in those cells expressing the chimera (Fig. 3, B and D). Identical results were found with all of the apical chimeras depicted in Figs. 1, 2, and 6 (data not shown). We conclude that these chimeras assemble with the endogenous Na,K-ATPase β subunit protein, and like the first chimera H519N, redirect this normally basolateral protein to the apical surface.

To discern whether it is the ectodomain or the transmembrane domain that mediates the apical localization

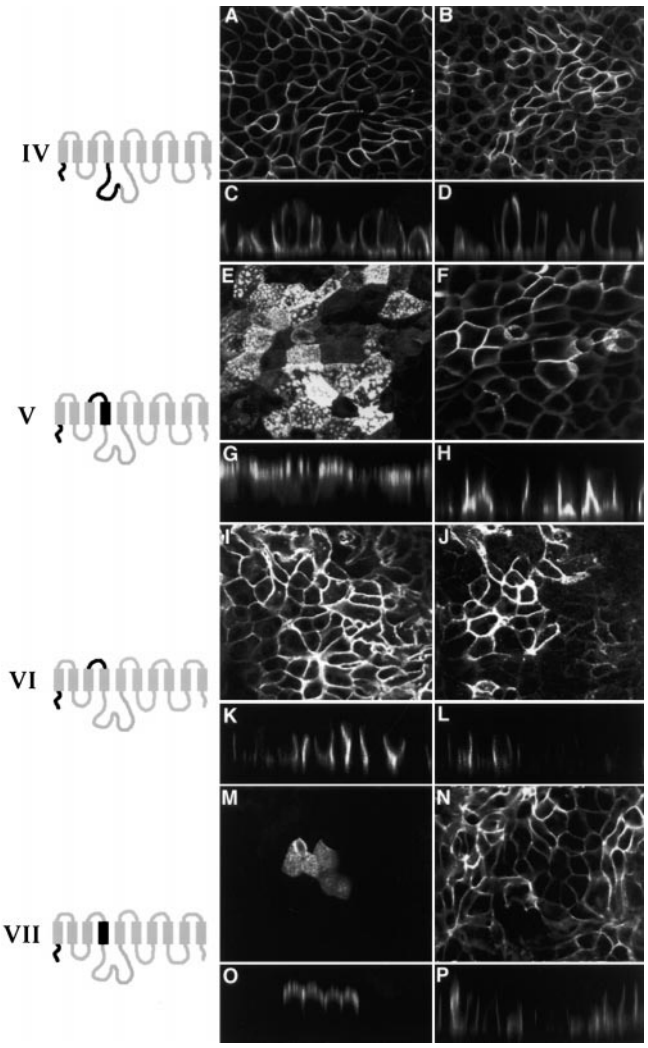

Figure 2. Localization of chimeras IV–VII. LLC-PK1 cells stably expressing chimeras IV–VII were stained as in Fig. 1. Confocal images were generated to show the localization of the chimeras (A, C, E, G, I, K, M, and O) or the endogenous Na,K-ATPase (B, D, F, H, J, L, N, and P). The Na,K-ATPase is localized to the basolateral membrane in all cell lines as shown in en face (B, F, J, and N) and xz cross sections (D, H, L, and P). Chimeras IV and VI are also localized to the basolateral membrane seen en face (A and I) and in xz cross section (C and K). In contrast, chimeras V and VII display a predominantly apical distribution when viewed en face (E and M) and in xz cross section (G and O), demonstrating that the TM4 of the H,K-ATPase is sufficient for apical localization in LLC-PK1 cells.

of the protein, another set of chimeras was designed to contain only the second ectodomain loop or TM4 of the H,K-ATPase. When transfected into LLC-PK1 cells, the chimeric protein, which includes the ectodomain of the H,K-ATPase α subunit (Fig. 2, chimera VI), is restricted to the basolateral membrane (Fig. 2, I and K). In contrast, the majority of the chimera incorporating the H,K-ATPase α subunit TM4 (designated as VII in Fig. 2) is localized to the apical membrane (Fig. 2, M and O). From these results, it is clear that the TM4 of the gastric H,K-ATPase α subunit is sufficient for the apical localization of these chimeric ion pumps.

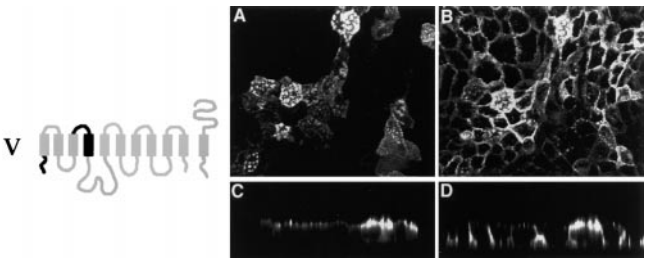

Figure 3. An apical chimera and the Na,K-ATPase β subunit colocalize at the apical membrane. Cells expressing chimera IV were fixed and stained for the presence of either the chimera or the Na,K-ATPase β subunit. The cells expressing the apical chimera (A, en face and C, xz section) express the Na,K-ATPase β subunit at the apical surface, where it is presumably assembled with the chimera, as well as at the basolateral surface, where it is normally found in association with the endogenous Na,K-ATPase α subunit (B, en face and D, xz section).

Comparison of the sequences of the TM4s of the two ion pump α subunits shows surprisingly little nonhomology, considering that this region can mediate the strikingly different membrane distributions exhibited by these proteins (Fig. 4 A). Of the 28 amino acids that comprise the putative transmembrane domain, only 8 are nonidentical. Seven of the nonconserved amino acids in this segment are found in the portion of the transmembrane domain that is predicted to pass through the outer leaflet of the lipid bilayer (Fig. 4 B). The outer leaflet of the apical plasma membrane of many epithelial cell types is enriched in glycosphingolipids (GSLs). Furthermore, there is evidence that GPI-linked proteins, as well as other apical polypeptides, are incorporated into GSL-rich domains during their biosynthetic passage through the Golgi complex (Brown and Rose, 1992). This interaction has been proposed to mediate their targeting to the apical plasma membrane (Simons and Wandinger-Ness, 1990). It has also been suggested that glycolipid sorting may be involved in the polarized delivery of the Na,K-ATPase to the plasma membrane (Mays et al., 1995). An MDCK line that lacks GSL polarity also mistargets the Na,K-ATPase. In this cell line, the Na,K-ATPase is delivered equally to both the apical and basolateral membrane. The population at the apical membrane is subsequently degraded, whereas the cohort delivered to the basolateral surface is stabilized, apparently through interactions with the cytoskeleton. The mistargeting of the Na,K-ATPase seen in this cell line may be directly linked to the lack of GSL polarity, since fumonisin, a drug that disrupts GSL synthesis, causes the same random pump delivery when applied to MDCK cell lines that ordinarily have a polarized distribution of GSLs. These experiments suggest that the Na,K-ATPase is normally exclusively sorted to the basolateral membrane by virtue of its exclusion from glycosphingolipid-rich membrane patches. We wondered whether TM4 of the H,K-ATPase exerts its effect on sorting by allowing the protein to partition into these GSL-rich domains.

GPI-linked proteins that have become associated with GSL-rich membrane domains are insoluble in 1% Triton X-100 at 4°C. When a cell lysate prepared in this fashion is fractionated on a sucrose floatation gradient, insoluble proteins are found near the top of the gradient, whereas soluble proteins remain in the heavier fractions (Arreaza et al., 1994). We lysed LLC-PK1 cells expressing the apically located chimera H519N on ice with 1% Triton X-100, and examined the distribution of the chimera, the endogenous Na,K-ATPase, and the endogenous GPI-linked alkaline phosphatase in fractions collected from a sucrose floatation gradient. Lysis took place in the presence of sodium carbonate to disrupt any cytoskeletal associations that could influence the solubility of the ion pumps. An alkaline phosphatase assay performed on each fraction shows that the enzymatic activity of this endogenous GPI-linked protein is restricted to fractions 2–4 (Fig. 5 A). These fractions correspond to the interface between the 5 and 35% sucrose layers, where GPI-linked proteins are expected to be found based on published reports (Arreaza et al., 1994). The endogenous Na,K-ATPase on the other hand, appears in the heavier fractions 6–10, a pattern typical for detergent-soluble membrane proteins (Fig. 5, A and B). If the chimera containing TM4 of the gastric H,K-ATPase partitions into insoluble glycolipid patches, we would have expected it to codistribute with the GPI-linked alkaline phosphatase. Instead, the chimera is found in the same fractions as the Na,K-ATPase, and is not present in any significant amounts in the fractions containing alkaline phosphatase activity (Fig. 5, A and B). Thus, under steady-state conditions, an apically located chimera containing the TM4 of the H,K-ATPase exhibits no differ-

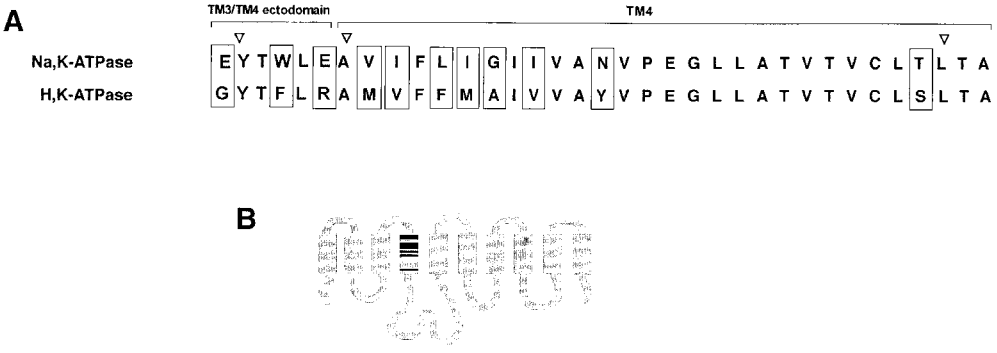

Figure 4. Amino acid sequence comparison. The linear sequences of the predicted second ectodomains (between TM3 and TM4) and the TM4 of the gastric H,K-ATPase and Na,K-ATPase α subunits are shown in A. Nonidentical amino acids within the ectodomain and TM4 are boxed. The amino acids corresponding to the junction points of the chimeras are shown by arrowheads. The locations of the eight nonidentical amino acids (black bars) within TM4 are shown in the context of the α subunit's predicted membrane topology in B.

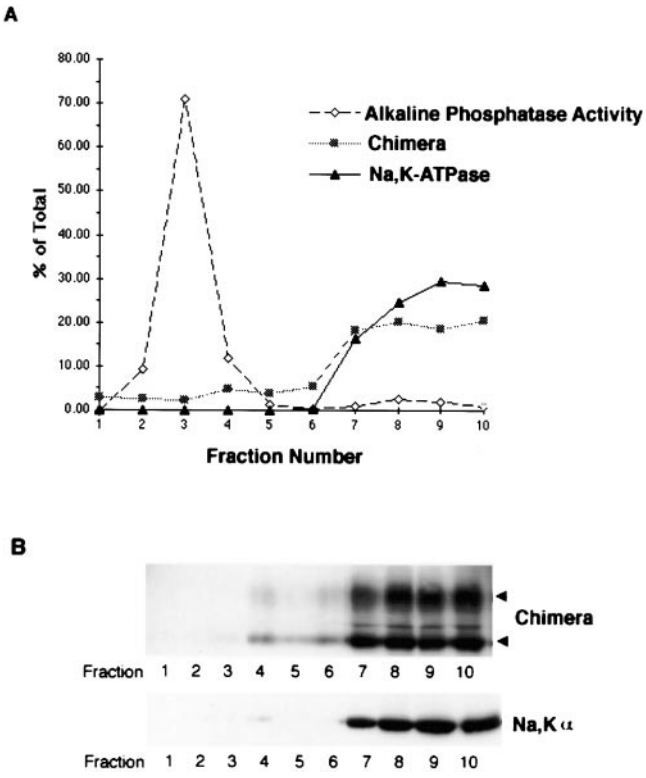

Figure 5. Detergent solubility of an apical pump chimera. A detergent solubility assay was performed to determine whether the apically located chimera H519N is associated with glycolipid-rich membrane domains. Cells were lysed on ice with Triton X-100 and loaded onto a sucrose floatation gradient. Fractions were collected and examined for the presence of alkaline phosphatase activity, the chimera, and Na,K-ATPase. As shown in A, the endogenous GPI-linked alkaline phosphatase is found in the lighter fractions of the gradient 2–4, as is typical for proteins associated with glycolipid-rich membranes (Arreaza et al., 1994). Western blotting reveals that both the chimera and the endogenous Na,K-ATPase appear in the heavier fractions 6–10 (B), which is characteristic of soluble proteins. The chimera α subunit runs as both a monomer (lower band) and a higher molecular weight α/β dimer. Densitometric quantification of the blots (A) clearly demonstrates that the chimera (squares) does not colocalize with the alkaline phosphatase (diamonds), and therefore is probably not associated with GSL-rich membrane domains; instead, the chimera is found in the same fractions as the Na,K-ATPase (triangles). The experiment presented in this figure is typical of three independent trials.

ence in its detergent solubility characteristics as compared with the basolaterally located Na,K-ATPase. Furthermore, no difference in solubility between the endogenous Na,K-ATPase and apically located chimeras is detected when lower concentrations of detergent are used (data not shown). Finally, the endogenous gastric H,K-ATPase of mouse stomach mucosa is also fully soluble under these detergent extraction conditions. These results suggest that mechanisms other than lipid association may be responsible for the sorting function of the transmembrane domain.

During the course of our dissection of the sorting information residing between residues 324 and 519 of the H,K-ATPase α subunit sequence, we constructed an eighth chimera to determine whether the TM4 of the H,K-ATPase was required for apical localization. This chimera contains the TM4 of the Na,K-ATPase flanked by the TM3-TM4 ectodomain and a portion of the large cytoplasmic loop of the H,K-ATPase. Since this chimera lacks the H,K-ATPase TM4, we anticipated that it would be expressed at the basolateral plasma membrane. Surprisingly, we found that it is predominantly located at the apical membrane when expressed in LLC-PK1 cells (Fig. 6, A and C). The apical polarity of this chimera is dependent upon the presence of both segments of H,K-ATPase sequences flanking the TM4, since the presence of either domain alone results in expression at the basolateral membrane (chimeras IV and VI). This result demonstrates that whereas TM4 of the gastric H,K-ATPase is sufficient to achieve apical localization of the chimeric ion pump, it is clearly not necessary. It also indicates that noncontiguous pump sequence domains predicted to lie on opposite sides of the bilayer can collaborate to create a signal for the pump's polarized distribution.

We wondered if the steady-state localization of the chimeras correlated with their enzymatic activities. To determine whether the chimeras can function as sodium pumps, we assayed the ability of the cells expressing chimeras to survive under conditions that block the endogenous Na,K-ATPase. Ouabain is a specific inhibitor of the Na,K-ATPase. The endogenously expressed pig Na,K-ATPase in LLC-PK1 cells has a K_i for ouabain of 10^{-7} μM, whereas the K_i for the rat Na,K-ATPase, which was used in the construction of the chimeras, is 10^{-4} μM. Taking advantage of this disparity in ouabain sensitivity, cells expressing the chimeras were tested for their ability to survive in the presence of 10 μM ouabain over the course of 5 d. This concentration is lethal to untransfected LLC-PK1 cells. As seen in Fig. 7 A, the cells expressing chimeras I, II, IV, VI, and VIII survived 10 μM ouabain. Presumably, the chimeras expressed in these cells are enzymatically active and can mediate K^+ influx and Na^+ efflux, since they were able to compensate for the ouabain-inhibited activity of the endogenous Na,K-ATPase. Extensive characterization of the activities catalyzed by chimeras I, IV, and VI is presented elsewhere (Blostein et al., 1999).

Chimeras III, V, and VII were not able to confer ouabain resistance, suggesting that they may be inactive. However, it is also possible that these chimeras possess

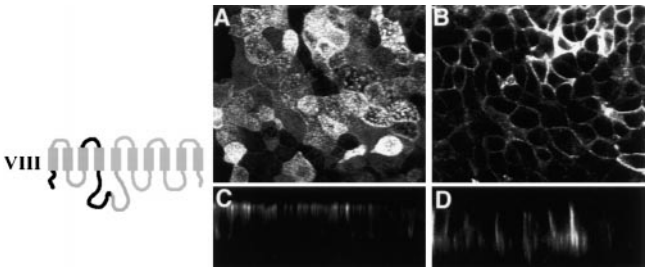

Figure 6. A chimera lacking the TM4 of the gastric H,K-ATPase is also found at the apical membrane. Cells expressing chimera VIII were fixed and stained as in Fig. 1. Chimera VIII is found predominantly at the apical membrane (A and C), demonstrating that the TM4 of the gastric H,K-ATPase is not necessary to ensure apical localization in LLC-PK1 cells.

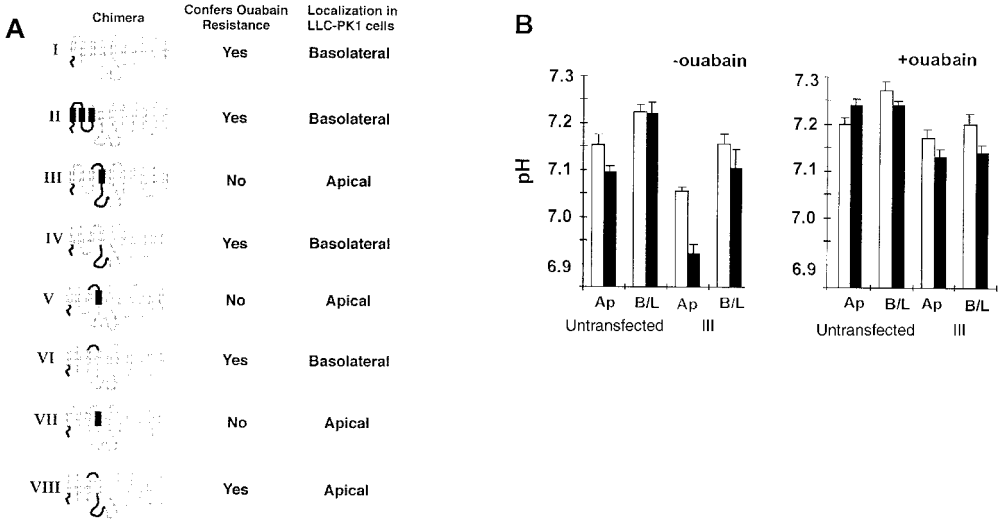

Figure 7. Several chimeras appear to be enzymatically active. The ability of chimeras to substitute for the sodium transporting activity of the endogenous Na,K-ATPase was assessed by determining whether expression of the chimera conferred ouabain resistance. All of the basolateral chimeras appears to be active as sodium pumps. However, the presence of Na,K-ATPase activity does not correlate with the steady-state distribution of the chimeras, since the apical chimera VIII confers ouabain resistance (A). The apically located chimera III also appears to be active, although it does not confer ouabain resistance. Expression of this apical chimera results in the acidification of the apical media when cells expressing this protein are grown on porous filters (B). Proton efflux was assessed by monitoring the change in pH of weakly buffered media bathing the apical or basolateral surfaces of transfected or untransfected LLC-PK1 cells after 2 (open bars) or 4 h (filled bars) of incubation. The acidification appears to be due to expression of the chimera, since the magnitude of the acidification of the apical media is not seen in untransfected LLC-PK1 cells and it is inhibited by high concentrations of ouabain added to the apical media as shown on the right.

activity that resembles that of the gastric H,K-ATPase. The H^+ efflux activity catalyzed by the gastric H,K-ATPase can not substitute for the essential Na,K-ATPase–driven sodium efflux. Hence, expression of the gastric H,K-ATPase does not confer ouabain resistance to transfected cells (Gottardi, C.J., and M.J. Caplan, unpublished observations). Qualitative evidence that at least one of these apical chimeras that did not confer ouabain resistance is indeed enzymatically active is provided by the observation that cells expressing chimera III acidify their apical media compartment when grown on permeable filter supports. Typical measurements of this acidification are presented in Fig. 7 B, which shows that over the course of 4 h there is a small but significant fall in the pH of the apical media bathing cells expressing chimera III. This phenomenon appears to be due to the expression of chimera III, since it is not seen in untransfected cells and the effect is abolished by high concentrations of ouabain added to the apical media.

The ability of chimeras I, II, IV, VI, and VIII to confer ouabain resistance strongly suggests that these chimeras are capable of mediating Na^+ efflux, since cells expressing gastric H,K-ATPase are not rendered ouabain-resistant. It is interesting to note that chimeras I, II, IV, and VI, which all appear to exhibit sodium pump function, are located at the basolateral plasma membrane, whereas the chimeras H519N and III, which exhibit H,K-ATPase–like function, are apical proteins (Blostein et al., 1999). However, chimera VIII is localized to the apical membrane and confers ouabain resistance. In addition, preliminary characterization of chimera VIII's enzymatic activity supports the conclusion that it functions as an Na,K-ATPase (data not shown). The fact that a chimera with Na,K-ATPase activity can accumulate at the apical plasmalemma demonstrates that a pump's localization to a particular cell surface domain need not correlate with its cation specificity.

Discussion

The evidence presented here demonstrates that the TM4 of the gastric H,K-ATPase contains information that is sufficient for this protein's apical localization in LLC-PK1 cells. Chimeras generated from complementary portions of the apical H,K-ATPase and the basolateral Na,K-ATPase are found at the apical membrane of LLC-PK1 cells if their TM4s are derived from the H,K-ATPase. Pulse-chase metabolic labeling experiments show that the apical chimera proteins appear to be at least as stable as the endogenous Na,K-ATPase, supporting the conclusion that the localization seen by immunofluorescence most likely reflects the true steady-state distributions of these proteins (data not shown).

Hydropathy plots predict that amino acids 329–356 of the gastric H,K-ATPase comprise the TM4. Although this type of analysis is not definitive proof that these amino acids pass through the lipid bilayer, in this case it is supported by biochemical evidence (Pedemonte and Kaplan, 1990; Canessa et al., 1993). All of the chimeras examined are able to assemble with the Na,K-ATPase β subunit, and several of the chimeras appear to be enzymatically active. Hence, the sorting behaviors observed in these studies are probably not due to misfolding of the chimeric proteins, but instead reflect the presence of an active apical localization signal in the H,K-ATPase α subunit TM4 region and/or its flanking sequences.

The steady-state localization of the chimeras does not appear to based on the ion selectivity of the pumps. Both apical and basolaterally located chimeras are capable of

pumping sodium, as measured by their ability to confer ouabain resistance. Several of these chimeras (I, IV, VI, and VIII) have also been found to possess Na^+-stimulated ATPase activity, further demonstrating that they are functional as sodium pumps (Blostein et al., 1999; Mense et al., 2000; Mense, M., and M.J. Caplan, personal communication). The apical distribution exhibited by chimera VIII clearly shows that a sodium-transporting ATPase need not, of necessity, be localized to the basolateral membrane. Finally, at least one apical chimera that did not confer ouabain resistance appears to be active as demonstrated by its ability to induce the acidification of the apical media. Thus, the localization of the chimeras does not appear to be correlated directly with their functional characteristics.

It must be noted, that although the apical sorting behavior of chimeras containing the H,K-ATPase TM4 (III, V, and VII) is consistent with our interpretation that the H,K-ATPase TM4 encodes apical localization information, it is also possible that the behavior of these chimeras is instead attributable primarily to the disruption of a basolateral localization signal in the TM4 of the Na,K-ATPase. According to this interpretation, these chimeras accumulate at the apical membrane by a default mechanism, as has been documented for other basolateral membrane proteins whose basolateral sorting signals have been perturbed (Mostov et al., 1987; Matter et al., 1992). We believe that this interpretation is unlikely to explain our observations. It has been demonstrated recently that in MDCK cells, proteins that completely lack targeting signals accumulate at both cell surface domains (Odorizzi et al., 1996; Muth et al., 1998b). Since at least one of the apical chimeras presented here also behaves as an apical protein when expressed in MDCK cells (data not shown), it would appear that their apical distributions are the product of an active localization process.

There is mounting evidence that transmembrane domains can play important roles in protein trafficking. The apical sorting of two influenza virus proteins, neuraminidase and hemagglutinin, appears to be encoded in their transmembrane domains (Kudru et al., 1996; Lin et al., 1998). The transmembrane domains of several resident Golgi proteins have been found to act as Golgi-retention signals. The transmembrane domain of the vesicular stomatitis virus G protein may be permissive for internalization, and the first transmembrane domain of the lamin B receptor was shown to be sufficient for the targeting of this protein to the inner nuclear membrane (Lazarovits et al., 1996). Thus, it would appear that despite their biochemical isolation from soluble components of the cellular protein sorting machinery, transmembrane domains are capable of performing dynamic signaling functions.

The fact that the amino acid residues of a transmembrane domain may be in direct contact with lipid molecules prompted us to examine whether the detergent solubility of the apical chimeras resembled that of GPI-linked proteins, which associate with GSLs in detergent-resistant domains (Brown and Rose, 1992). Interactions between GPI lipid anchors and glycolipid-rich domains have been proposed to mediate apical sorting of GPI-linked proteins (Simons and Wandinger-Ness, 1990). Furthermore, as noted above, the apical influenza proteins, neuraminidase and hemagglutinin, rely on their transmembrane domains for incorporation into insoluble, GSL-rich membrane domains (Kudru et al., 1996; Scheiffele et al., 1997). The data from our detergent extraction assay do not support the proposal that the apically located chimeras associate with detergent-insoluble patches of glycosphingolipid-rich membranes. Both the endogenous Na,K-ATPase and an apical chimera, H519N, appear to be fully soluble in Triton X-100. This observation suggests that the apical pump chimeras do not achieve their polarized distribution through any long-term, stable interactions with GSL-rich membrane domains. However, it must be noted that the reliability of the detergent insolubility assay, when applied to polytopic membrane proteins, is unclear. More specific assays for GSL association may be required to determine if the TM4 signal exerts its influence by determining the parent protein's affinity for various lipid milieus. Finally, it is also possible that an interaction between the H,K-ATPase TM4 and GSLs is transitory, occurring only during the protein's biosynthetic processing and not persisting after its surface delivery. However, this is probably not the case, since data from experiments in which the extraction assay was paired with a pulse-chase labeling protocol failed to demonstrate an interaction at any point in the chimera's postsynthetic course (data not shown).

In the absence of evidence for specific lipid associations, it is tempting to propose that protein–protein interactions are involved in the recognition and interpretation of this novel sorting signal. In one line of MDCK cells, cytoskeletal interactions appear to play an important role in establishing the basolateral distribution of the Na,K-ATPase. These MDCK cells, which missort glycolipids, target newly synthesized Na,K-ATPase to both the apical and basolateral membrane domains (Mays et al., 1995). It has been suggested that the Na,K-ATPase at the apical membrane is degraded, since it is unable to interact with stabilizing cytoskeletal elements such as ankyrin, which are present only at the basolateral plasma membrane in those cells (Hammerton et al., 1991). Both the Na,K-ATPase and H,K-ATPase α subunits have been shown to bind ankyrin and share two conserved putative ankyrin-binding sites located in the cytoplasmic loops between transmembrane domains 2 and 3 and between transmembrane domains 4 and 5 (Smith et al., 1993; Jordan et al., 1995). All of the chimeras generated in this study incorporate ankyrin-binding sites derived either from their Na,K-ATPase or H,K-ATPase sequence domains. Metabolic labeling studies demonstrate that both the apical and the basolateral chimeras are not subject to rapid degradation, suggesting that the entire cohort of each newly synthesized pump chimera becomes stably associated with the plasma membrane (data not shown). Therefore, it is unlikely that the steady-state polarity of the pump chimeras is achieved by random delivery followed by selective degradation of one surface population.

The TM4 of the gastric H,K-ATPase could function as a localization signal by interacting with other proteins within the plane of the membrane. Evidence for such intra-membranous protein associations has been obtained in studies of the assembly of major histocompatibility complex class II molecules. The transmembrane helices of the major histocompatibility complex class II α and β subunits

appear to be necessary and sufficient to ensure the interaction of these two polypeptides (Cosson et al., 1991). Similarly, the interaction between charged residues embedded within transmembrane helices appears to drive assembly of the α chain of the T cell receptor with the CD3 δ chain (Cosson and Bonifacino, 1992). There is also evidence that synaptobrevin and syntaxin 1a interact through their transmembrane domains (Margittai et al., 1999). Future experiments will be required to determine whether a comparable mechanism is involved in the interpretation of the pump localization signal by a putative transmembrane receptor.

The gastric H,K-ATPase TM4 sequence shows no significant homology to any other apically located proteins. The gastric H,K-ATPase's closest molecular relatives are the nongastric H,K-ATPases. These pumps are expressed in colonic and renal epithelial cells and appear to reside at the apical plasma membrane (Del Castillo et al., 1991; Grishin et al., 1999). The gastric H,K-ATPase, nongastric H,K-ATPases, and the Na,K-ATPase α subunits are each 63% identical to one another at the amino acid level (Crowson and Shull, 1992; Modyanov et al., 1995). It is interesting to note that the TM4s of the nongastric H,K-ATPase α subunits are almost identical to that of the Na,K-ATPase. There is mounting evidence that the similarity of the TM4 of the nongastric H,K-ATPases to that of the Na,K-ATPase may confer a shared ability to transport Na^+ ions (Grishin et al., 1996; Cougnon et al., 1998; Grishin and Caplan, 1998). The fact that the nongastric and gastric H,K pumps share an apical localization despite the disparity in their TM4 sequences suggests that these ATPases either contain different apical localization signals or rely on different regions of the α subunit sequence to generate similar apical localization signals through modification of the conformations of their TM4s.

In light of the apical localization of the nongastric H,K-ATPases, the behavior of chimera VIII, which contains the TM4 of the Na,K-ATPase, is especially interesting. This chimera resides at the apical plasma membrane of LLC-PK1 cells, demonstrating that TM4 of the H,K-ATPase is sufficient but not necessary for apical localization. Although it is possible that this chimera contains an apical localization signal that is completely distinct from that present in TM4 of the H,K-ATPase, it must be noted that the apical polarity of this chimera is dependent upon the simultaneous presence of two stretches of H,K-ATPase amino acids flanking TM4. Alone, each of these sequences is unable to direct the chimera to the apical membrane. The predicted ectodomain loop between TM3 and TM4 is only six amino acids in length and differs at only three positions between the Na,K and gastric H,K-ATPases. As only two of the nonidentical amino acids were exchanged in chimeras incorporating the H,K-ATPase TM3-TM4 ectodomain, one or both of these amino acids must account for the change in localization seen between chimera IV and chimera VIII. This ectodomain region could play a role in creating an apical localization signal by cooperating with the large cytoplasmic loop to cause TM4 to adopt a particular conformation or orientation. The H,K-ATPase TM4 segment alone may independently achieve this same conformation. Consistent with this interpretation, recent structural analysis of the P-type Ca-ATPase suggests that cytosolic segments of the enzyme may anchor the transmembrane helices in specific positions (Shi et al., 1998). Similarly, it is possible that TM4 of the gastric H,K-ATPase α subunit does not itself carry a specific localization signal. Instead, it may exert its effects on localization by imposing certain conformations on other parts of the α subunit, such as the ectodomain or cytoplasmic domain adjacent to TM4. These conformational motifs could then be recognized by components of the cellular sorting machinery. Future studies will determine the specific residues of both the TM4 and its flanking regions, which are responsible for apical localization.

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