An Ion-transporting ATPase Encodes Multiple Apical Localization Signals

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Abstract. Epithelial cells accumulate distinct populations of membrane proteins at their two plasmalemmal domains. We have examined the molecular signals which specify the differential subcellular distributions of two closely related ion pumps. The Na,K-ATPase is normally restricted to the basolateral membranes of numerous epithelial cell types, whereas the H,K-ATPase is a component of the apical surfaces of the parietal cells of the gastric epithelium. We have expressed full length and chimeric H,K-ATPase/Na,K-ATPase cDNAs in polarized renal proximal tubular epithelial cells (LLC-PK1). We find that both the α and β subunits of the H,K-ATPase encode independent signals that specify apical localization. Furthermore, the H,K-ATPase β-subunit possesses a sequence which mediates its participation in the endocytic pathway. The interrelationship between epithelial sorting and endocytosis signals suggested by these studies supports the redefinition of apical and basolateral as functional, rather than simply topographic domains.

The plasma membranes of polarized epithelial cells are divided into apical and basolateral domains whose markedly different biochemical compositions reflect their distinct functional roles (Caplan and Matlin, 1989; Rodriguez-Boulan and Nelson, 1989). To generate and maintain these domains, cells must be capable of distinguishing among newly synthesized membrane proteins and of ensuring that they are concentrated at their sites of ultimate functional residence. In many cases, this sorting process appears to occur during the course of a protein’s initial transit through the organelles of the biosynthetic processing pathway, before its first appearance at the plasma membrane (Fuller et al., 1985; Matlin and Simons, 1984; Misek et al., 1984; Pfeiffer et al., 1985). Evidence from other systems indicates that sorting can involve selective stabilization at a plasmalemmal domain, subsequent to a protein’s random delivery to both cell surfaces (Hamerton et al., 1991). Whether sorting takes place at the level of the Golgi or at the plasmalemma, this process must make use of sorting information, or sorting signals embedded within the structure of the sorted protein itself. These signals are interpreted by the cellular sorting machinery and thus serve to dictate each polypeptide’s ultimate subcellular distribution.

Efforts to characterize sorting signals have generally involved the generation of chimeric or truncated constructs prepared from portions of apical and basolateral membrane proteins. Through analysis of the subcellular distributions of the resulting proteins, portions of the parent molecules necessary for the sorting process have been identified. Over the past several years, a large number of such chimeric/truncated proteins have been prepared, but only a few general rules have emerged from this work. It has been shown that the cytosolic tails of several basolateral proteins possess basolateral sorting signals (Casanova et al., 1991; Hunziker et al., 1991), while the ectodomains of a number of apical polypeptides seem to carry targeting information (McQueen et al., 1986; Mostov et al., 1987; Roth et al., 1987). Recent evidence also indicates that sequences which allow proteins to be endocytosed can double as basolateral sorting signals (Hunziker et al., 1991; LeBivic et al., 1991; Brewer and Roth, 1991). The resolving capacity of these methods has been limited in part by the necessity to generate chimeras from portions of unrelated polypeptides. The tertiary structures of the resultant chimeras are thus likely to differ substantially from those of either parent molecule, which may exert unpredictable effects on sorting behavior. To avoid this potential problem, we have chosen to apply the chimera approach to a novel example of two proteins which share an extremely high degree of structural and functional similarity and are sorted to distinct compartments of polarized epithelial cells.

We have examined the sorting signals which mediate the localization of two members of the closely related E1-E2 family of ion-transporting ATPases. The Na,K-ATPase, or sodium pump, is a component of the basolateral plasma membranes of many epithelia (Matlin and Caplan, 1992). In contrast, the highly homologous H,K-ATPase is restricted to the apical membrane and related structures in its native gastric parietal cells (Smolka and Weinstein, 1986). Both...
ATPases are composed of 100 kDa α subunits (which are 62% identical to one another, see Fig. 1) and richly glycosylated β subunits (which manifest ~31% identity) (Jorgensen, 1982; Okamoto et al., 1990; Reuben et al., 1990; Shull et al., 1986). While the pumps' catalytic functions appear to be accomplished by their alpha subunits (Jorgensen, 1982), assembly of the α/β heterodimer appears to be a prerequisite for cell surface delivery as well as functional activity (Ackermann and Geering, 1990). Our studies take advantage of the structural homology relating these ATPases in order to examine the molecular signals underlying their differential localizations. Because these proteins share a highly conformation-dependent catalytic cycle, the potential exists to assay the enzymatic activity and hence the structural integrity of each chimeric protein. Our results demonstrate that in contrast to the Na,K-ATPase, both subunits of the H,K-ATPase encode dominant information specifying accumulation at the apical membrane.

**Materials and Methods**

**Plasmid Construction**

The rat gastric H,K-ATPase α cDNA was kindly provided by G. Shull (Shull and Lingrell, 1986) as two overlapping fragments in separate pBR322 plasmids labeled by pBR322-3.3. The NH2-terminal coding region encoded by the Clal-EcoRI fragment of pBR322-1.8 was ligated to the COOH-terminal region encoded by the EcoRI-ScaI fragment of pBR322-3.3, and then subcloned into the Clal/Smal polylinker sites of the Bluescript vector (Promega Corp., Madison, WI). The full-length H,K-ATPase cDNA was cut with Clal/XbaI and subcloned into the mammalian expression vector pCB6 (Fertis et al., 1990) provided by M. Roth, D. Russell and C. Brewer, University of Texas, Dallas, TX; Brewer and Roth, 1991) which carries resistance to the antibiotic (3418. The full-length Na,K-ATPase cDNA was kindly provided by Ed Benz (Yale University, New Haven, CT) in a pGEM vector. This eDNA was subcloned into the HindIII site of the Bluescript vector (Stratagene, Inc., San Diego, CA).

The H519N cDNA was generated by cutting the 5' half of the H,K-Bluescript coding region with Clal and NarI endonucleases and subcloning the 1.7-kb fragment into the 3' half of the Na,K-Bluescript coding region generated with NarI and Clal (4.8 kb). The conserved NarI site lies within the putative ATP-binding domain (amino acids 513-523) shared between these two proteins. The full-length H519N chimeric cDNA was cut from the Bluescript vector with Clal and XbaI and then directly subcloned into the same sites of the mammalian expression vector pCB6. Thus, the H519N chimera comprises an H,K-ATPase/Na,K-ATPase chimera that encodes the amino half of the H,K-ATPase α subunit up to residue 519. Residues 520-1034 are encoded by the Na,K-ATPase (see Fig. 1).

The H,K-ATPase β cDNA was kindly provided by M. Reuben and G. Sachs (University of California at Los Angeles, Los Angeles, CA) (Reuben et al., 1990) in pGEM7(+) vector. The coding fragment was excised with Clal and XbaI and subcloned into the pCB6 vector for expression studies.

**Cell Culture and Transfection**

LLC-PK1 cells (clone 4; kindly provided by K. Amsler [UMDNJ, Robert W. Johnson Medical School]) were maintained in a humidified incubator at 37°C under 5% CO2 atmosphere in minimal essential media alpha (α-MEM) that was supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Cells were plated at 50% confluence in a 10-cm tissue culture dish 12-24 h before transfection. DNA transfection was performed by coprecipitating ∼40 μg DNA with calcium phosphate as described by Puddington et al. (1987). The folding factor must be included in order to correct for the relative membrane densities which characterize the basolateral and apical surfaces. This number is determined from electron microscopic stereological measurements by calculating the ratio of the apical:basolateral surface volume densities. This value reflects the degree to which the two membrane surfaces are amplified through apical microvilli and basolateral infoldings. The folding factor has been determined for LLC-PK1 cells using the conditions employed in this study and corresponds to the background pixel intensity measured over several intracellular regions and varied within each experiment by less than 5% from point to point; and F is a folding factor. The basolateral pixel intensity was divided by 2 in this equation because our measurements were made over lateral domains and corresponds to 0.7 (Pfaller et al., 1990). Where R is the apical:basolateral polarity ratio: a and b correspond to the apical and basolateral pixel intensities, respectively; k represents the background pixel intensity measured over several intracellular regions and varies within each experiment by less than 5% from point to point; and F is a folding factor. The basolateral pixel intensity was divided by 2 in this equation because our measurements were made over lateral domains and correspond to the summed contributions of two adjacent cells. The folding factor must be included in order to correct for the relative membrane densities which characterize the basolateral and apical surfaces. This number is determined from electron microscopic stereological measurements by calculating the ratio of the apical:basolateral surface volume densities. This value reflects the degree to which the two membrane surfaces are amplified through apical microvilli and basolateral infoldings. The folding factor has been determined for LLC-PK1 cells using the conditions employed in this study and corresponds to 0.7 (Pfaller et al., 1990).

**Microsomal Membrane Preparation**

Transfected and untransfected LLC-PK1 cells were grown to confluence on 25-mm Cyclopore transparent cell culture inserts (FALCON catalog 3090; Becton Dickinson Labware, Lincoln Park, NJ). Monolayers were fixed in 3% paraformaldehyde in 125 mM NaPi pH 7.5, where indicated. Cells were then permeabilized in a PBS buffer (wash buffer) containing 0.3% Triton X-100 and 0.1% BSA for 15 min at room temperature. Nonspecific antibody binding was blocked by preincubating in goat serum dilution buffer solution (16% filtered goat serum, 0.3% Triton X-100, 20 mM NaPi pH 7.4, 0.9% NaCl) for 30 min. All antibody incubations were carried out in the GSDB buffer; all washes between antibody incubations were carried out using the wash buffer. Monolayers were incubated for 1 h at room temperature with one or a combination of the following nonspecific antibodies: anti-H,K-ATPase α (HK9 diluted 1:100), anti-Na,K-ATPase α (mAb 6H diluted 1:100), anti-Na,K-ATPase β (mAb 8A 1:100), and anti-H,K-ATPase β (kindly provided by J. Forte (University of California at Berkeley, Berkeley, CA), diluted at 1:1,000). FITC conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) and rhodamine conjugated anti-rabbit IgG (Boehringer-Mannheim Biochemicals, Indianapolis, IN) secondary antibodies were used for 1 h at room temperature at a 1:100 dilution. Monolayers were washed in low ionic strength detergent-free buffer (10 mM NaPi pH 7.5) for 10 min before mounting the filters in a 15% glycerol/PBS solution containing 0.1% p-phenylenediamine to retard FITC bleaching. En face immunofluorescence images were visualized using the Zeiss Axioshot microscope and photographed using Kodak T-MAX 100 ASA film (Eastman Kodak Co., Rochester, NY). Confocal images were generated with a Zeiss laser scanning confocal microscope.

**HRP Uptake.** Colocalization of H,K-ATPase β and endocytozed HRP was carried out according to the following protocol: mAb specific for the H,K-ATPase α subunit was diluted 1:1000 in PBS with Ca/Mg (0.1 mM/1 mM) and added to the apical chamber of the cell culture insert (Cyclopore) for 1 h at 4°C with gentle horizontal rotation. The monolayer was washed in ice-cold PBS/Ca/Mg and then warmed to 37°C in the presence of HRP (P-8250, Sigma Chemical Co.) at 10 mg/ml in α-MEM for the specified length of time, before being fixed in 3.5% paraformaldehyde for immunofluorescence analysis (Fig. 9).

**Electron Microscopy.** H,K-ATPase β transfected LLC-PK1 cells were grown to confluence on 25 mm Cyclopore filters. Monolayers were fixed in 3.0% paraformaldehyde/0.05% glutaraldehyde for 1 h at room temperature, and then processed for immunocytochemistry (peroxidase method) as described by Machamer et al. (1990) and Caplan et al. (1986).

**Quantitative Confocal Microscopy.** Domain-specific fluorescence intensity was quantified on xz images by applying the measurement software associated with the Zeiss laser scanning confocal microscope. A 4 μm2 box was placed over the domain to be measured and the mean pixel intensity (on a scale of 0-254) within that box was determined. The histograms of pixel intensity were examined in order to ensure that all points considered had values within the linear range. Three apical and three basolateral measurements were recorded and averaged for each cell. At least eight cells were examined for each determination. Apical to basolateral polarity ratios were calculated according to a slight modification of the formula presented in Vega-Salas et al. (1987):

\[ R = (a - k)(b - k)/2, \]

where R is the apical:basolateral polarity ratio: a and b correspond to the apical and basolateral pixel intensities, respectively; k represents the background pixel intensity measured over several intracellular regions and varied within each experiment by less than 5% from point to point; and F is a folding factor. The basolateral pixel intensity was divided by 2 in this equation because our measurements were made over lateral domains and correspond to the summed contributions of two adjacent cells. The folding factor must be included in order to correct for the relative membrane densities which characterize the basolateral and apical surfaces. This number is determined from electron microscopic stereological measurements by calculating the ratio of the apical:basolateral surface volume densities. This value reflects the degree to which the two membrane surfaces are amplified through apical microvilli and basolateral infoldings. The folding factor method has been determined for LLC-PK1 cells using the conditions employed in this study and corresponds to 0.7 (Pfaller et al., 1990).
aprotinin. Cells were recovered by gentle centrifugation (500 g, 3 min) and then resuspended in 2 mls of hypotonic buffer (10 mM Tris-HCl pH 7.5, 0.02 M sucrose, 0.24 M Tris-HCl pH 7.5, with PMSF/aprotinin) and allowed to sit on ice for 10 min. The cells were broken by 25 strokes in a glass dounce homogenizer, then 2 mls of homogenization buffer (0.5 M sucrose, 0.24 M Tris-HCl pH 7.5 with PMSF/aprotinin) were added and the suspension was subjected to another 25 strokes. Post nuclear supernatants were prepared by centrifugation at 100,000 g for 90 min (SW50.1 rotor; Beckman Instruments, Inc., Palo Alto, CA). The membrane pellet was reinserted in the homogenization buffer and total protein concentrations were determined by the Bio-Rad protein assay (BioRad Labs, Richmond, CA). EndoH and EndoF studies were performed according to product instructions (Boehringer Mannheim Biochemicals, Indianopolis, IN).

**SDS-PAGE and Western Blot Analysis**

For SDS-PAGE, samples were brought to a final concentration of 80 mM dI-dithiothreitol, 56% SDS, 0.008% bromophenol blue, 0.2 M M Tris-HCl pH 8.9, 16% glycerol, and then warmed to 80°C for 5 min before loading onto 8.5% SDS-PAGE (Laemmli, 1970). The gels were transferred to nitrocellulose as described by Towbin et al. (1979) with the addition of 0.02% SDS to the transfer buffer. Blots were blocked in blocking buffer, which contains 5% powdered milk in TBS (20 mM Tris HCl pH 7.5, 150 mM NaCl), and 0.1% Tween 20. Primary incubations were performed in the same buffer with the following specific antibodies: anti-H,K-ATPase α (HK9 1:250), anti-Na,K-ATPase α (mAb 6H 1:500), anti-Na,K-ATPase β (mAb 8A 1:500), and anti-H,K-ATPase β (mAb 1:0000). Blots were then incubated with either goat anti-mouse or anti-rabbit secondary antibodies conjugated to HRP (working dilution 1:2,000; Sigma Chemical Co.). Labeled proteins were visualized by the enhanced chemiluminescence detection method (ECL; Anerham Corp., Arlington Heights, IL).

**Cell Surface Immunoprecipitation.** A 25-mm (Cyclopore) filter of confluent H,K-ATPase β-transfected LLC-PK1 cells was labeled with 200 μCi [35S]methionine in methionine-free media for 6 h. The monolayer was washed and then anti-H,K-ATPase β was diluted in PBS-Ca/Mg and added to the apical chamber of the cell culture insert for a 1 h incubation at 4°C. After extensive washing, the monolayer was lysed in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris HCl pH 7.4, and 0.5 mM EDTA). The supernatant was cleared by centrifugation for 10 min at 14,000 g and then incubated with immobilized anti-mouse IgG (Calbiochem Corp., La Jolla, CA) for 2 h at 4°C with end over end rotation. Pellets were washed five times with lysis buffer, followed by two washes in 0.1% NP-40 lysis buffer. The sample was subjected to SDS-PAGE, and after fixation and incubation with Autofluor (National Diagnostics Inc., Manville, NJ), gels were dried and exposed to Kodak XOMAT-AR film for 12 h at -70°C.

**Results**

To generate a probe which would detect the H,K-ATPase and not cross react with the Na,K-ATPase, we prepared a polyclonal antipeptide antibody (HK9) directed against the amino-terminal region of the H,K-ATPase (Fig. 1, amino acids 3-22). A detailed description of the production and full characterization of this antibody is provided elsewhere (Okusca, M., C. J. Gottardi, V. M. Rajendron, H. Binder, and M. J. Caplan, manuscript in preparation). As can be seen in Fig. 2, immunostaining performed on 0.5 μ sections of rat stomach reveals that this antibody reacts exclusively with the H,K-ATPase-rich parietal cells which line the gastric glands (Fig. 2 A). This staining pattern is consistent with previous H,K-ATPase localizations (Smolka and Weinstein, 1986). Electron microscopic immunocytochemistry employing this antibody demonstrates specific H,K-ATPase labeling of the cytoplasmic surfaces of tubulovesicular and apical canalicula membranes (Fig. 2 B). This antibody does not label the parietal cell basolateral membrane (arrowheads), which is instead endowed with a high concentration of Na,K-ATPase (Soroka et al., 1992). Thus, despite extensive sequence homology, the H,K-ATPase and Na,K-ATPase enzymes occupy distinct membranous domains within the gastric parietal cell.

**Expression and Distribution of H,K-ATPase Subunits in LLC-PK1 Cells**

We have generated a stably transfected LLC-PK1 cell line expressing both the α and β subunits of the H,K-ATPase. LLC-PK1 cells form a highly polarized epithelium and are capable of supporting high levels of expression of exogenous proteins (Roth, 1989). Preliminary experiments with MDCK...
Figure 2. Synthetic peptide antibody directed against the H,K-ATPase specifically labels gastric parietal cells. (A) The HK9 (α subunit specific) antibody was used to label 0.5 μm frozen sections of rat stomach fixed in 4% formaldehyde. Specific labeling was detected using a rhodamine conjugated secondary antibody. As can be seen above, the antibody reacts exclusively with parietal cells which line the gastric glands. (B) The HK9 antibody was used to localize H,K-ATPase at the EM level. Immunostaining was performed on 20 μm cryostat sections of rat stomach fixed by perfusion with PLP. Specific labeling was detected using a secondary antibody conjugated to HRP. DAB reaction product can be seen decorating the cytoplasmic surfaces of the parietal cell's tubulovesicular and canalicular membranes (white arrows). No labeling is associated with the basolateral membrane (arrowheads), or the adjacent chief cell (*).

cells suggested that the level of expression achievable with the pump constructs was not adequate for the purpose of our studies. It may be possible that the LLC-PK1 cell type is better able to tolerate the physiological activities of the pump molecules employed in this work. The western blot in Fig. 3 demonstrates that both the α and β subunits of the H,K-ATPase are present in our transfected cells and can be detected by our antibody probes (lanes 2 and 9). The H,K-ATPase α-specific antibody (HK9) described in Fig. 2 reacts exclusively with a 100-kD protein in the transfected cells (lane 2). No immunoreactivity is detected in untransfected cells (lane J), confirming that this antibody does not cross-react with the endogenous Na,K-ATPase (compare with lanes 3 and 4). With a mAb specific for the H,K-ATPase β subunit (kindly provided by J. Forte, University of California at Berkeley, Berkeley, CA), both ER (45 kD) and mature (≥66 kD) forms of the β subunit can be detected (lanes 9, and 12–15). The ER and mature forms are revealed by their sensitivity to endoH and endoF, respectively (Kobata, 1979; lanes 12–15). As expected, this antibody does not cross-react with endogenous LLC-PK1 proteins, as there is nothing detected in membranes from untransfected cells (compare lane 11 with lanes 6–8).

We next wished to determine the subcellular localization of the H,K-ATPase in our transfected cells. Indirect immunofluorescence was performed on the cell line expressing both subunits of the H,K-ATPase using the HK9 antibody characterized in Fig. 2. The results of this experiment are shown in Fig. 4. En face (Fig. 4, A and B) and confocal optical sectioning (C and D) confirm that both subunits of the H,K-ATPase immunolocalize predominantly to the apical brush border. The normal basolateral distributions of both the α and β subunits of the endogenous Na,K-ATPase remain unperturbed in these cells (E–J). Thus, this cell line is able to distinguish the H,K-ATPase α/β complex from the Na,K-ATPase α/β complex and to maintain each on its appropriate and distinct membrane domain. Efforts to employ surface labeling techniques to quantify the surface distributions of these pump subunits have been frustrated by our observation that these methods cannot be applied quantitatively to LLC-PK1 cells, due to marked differences in labeling efficiency at the two surface domains (Gottardi and Caplan, 1992). Consequently, we have employed quantitative confocal fluorescence microscopy in order to measure the polarized distributions of pump molecules. Because cells are fixed and
Table I. Quantitative Confocal Microscopy

| Cell line      | Antibody | Average A/B ratio (± SD) |
|----------------|----------|-------------------------|
| Untransfected  | Na,K-α   | 0.071 ± 0.007           |
|                | Na,K-β   | 0.175 ± 0.012           |
| H,K-ATPase α/β | H,K-α    | 42.1 ± 4.65             |
|                | H,K-β    | 31.2 ± 3.92             |
| H519N          | H,K-α    | 30.0 ± 3.19             |
|                | Na,K-α   | 0.254 ± 0.013           |
|                | Na,K-β   | 2.64 ± 0.228            |

The apical to basolateral (A/B) fluorescence intensity ratios were calculated according to the formula described in Methods.

Salas et al., 1987). As can be seen in Table I, the ratio of apical/basolateral signal is 42.1 and 31.2 for H,K-ATPase α and β subunits, respectively.

Co-expression of the H,K-ATPase α and β subunits appears to be necessary in order to ensure the α-subunit's surface delivery. While we have been unable to generate a cell line that expresses only the H,K-ATPase α subunit, transient transfection studies in COS-1 cells demonstrate that the H,K-ATPase α can only be expressed on the cell surface when it is co-transfected with its H,K-ATPase β subunit (Gottardi, C., and M. J. Caplan, manuscript submitted for publication). This observation strongly suggests that the pump subunits exhibit strict fidelity with respect to assembly, since Na,K-ATPase β does not appear to chaperone H,K-ATPase α to the cell surface. This conclusion, which argues against the formation of hybrid pump dimers, is further supported by the observed spatial segregation of H,K-ATPase from Na,K-ATPase subunit polypeptides in the LLC-PK₁ cell line.

**H,K-ATPase β Expressed Alone Is Localized at the Apical Surface and in a Subapical Vesicular Compartment**

A stable cell line expressing only the H,K-ATPase β subunit was generated in order to explore the possibility that this subunit encodes apical sorting information. A western blot performed on this cell line (Fig. 3, lanes 5, 8, and 10) shows that both mature and immature forms of the protein are present. Immunofluorescence analysis (Fig. 5) reveals that the H,K-ATPase β subunit is localized to the apical brush border (Fig. 5, A and B) as well as to a population of large subapical vesicles (C is same cell as B, with different plane of focus). The endogenous Na,K-ATPase α subunit is found in its normal basolateral distribution, and therefore does not appear to escort the H,K-ATPase β subunit to the cell surface (data not shown).

We wondered whether the H,K-ATPase β subunit is, in fact, able to depart the ER by itself or if another, as yet unidentified protein permits its transit to the cell surface. We addressed this problem by carrying out a cell surface immunoprecipitation of H,K-ATPase β from [35S]methionine-labeled cells under conditions that normally allow for the coprecipitation of Na,K-ATPase α/β complexes (Fig. 3, lane J6). We were unable to resolve any proteins which specifically interact with the H,K-ATPase β. This negative result does not rule out the possibility that H,K-ATPase β forms a
Figure 5. H,K-ATPase β localizes to the apical brush border and to a population of subapical vesicles. LLC-PK cells stably transfected with the H,K-ATPase β subunit were grown on transparent microporous filter supports, fixed and permeabilized for indirect immunofluorescence as described in Materials and Methods. The en face image in A shows a field of H,K-ATPase β-expressing cells detected with the β specific mAb; both apical cell surface and intracellular staining is evident (40x). At higher magnification (100x), the H,K-ATPase β subunit is more clearly visualized at both the apical brush border (B) and within a population of intracellular vesicles (C), same cell different plane of focus. D and E show a cluster of H,K-ATPase α/β cotransfected LLC-PK1 cells double labeled with H,K-ATPase α (D) and H,K-ATPase β (E) specific antibodies, respectively. Note that the H,K-ATPase β vesicles (E) are devoid of H,K-ATPase α staining (D). The * in E shows a cell that weakly stains with the H,K-ATPase α antibody, but stains strongly with the H,K-ATPase α antibody, giving the impression that the H,K-ATPase α subunit can get to the cell surface by itself. This is not the case, rather, the variable staining intensities reflect these two antibodies' preferences for different methods of fixation.

Expression and Distribution of H519N in LLC-PK1 Cells

The apical localization of the H,K-ATPase β subunit could be interpreted to suggest that the β subunit encodes the information responsible for the polarized cell surface distribution of the H,K-ATPase α/β dimer. To test this hypothesis, we have prepared a stably transfected LLC-PK1 cell line which expresses a high level of the H519N chimera (Figs. 1 and 6). Western blots probed with the HK9 antibody (Fig. 2) reveal the presence of a strong immunoreactive band at ~100 kD in transfected cells that is not present in control cells (Fig. 6, lanes 1 and 2). This chimeric protein has the same mobility as the full length H,K-ATPase α subunit and is not detected with an antibody directed against the carboxy half of the H,K-ATPase α (mAb kindly provided by A. Smolka, University of South Carolina, Columbia, SC, data not shown). The tertiary structure of the H519N chimera appears to be largely intact, because it is functionally active as an ion-transporting ATPase, sensitive to both SCH28080 and ouabain, which are inhibitors of the H,K-ATPase and Na,K-ATPase, respectively (Blostein et al., 1993).

Indirect immunofluorescence and quantitative confocal microscopy were employed in order to determine the cell surface distribution of this chimera. Fig. 7 shows that the H519N chimera is almost exclusively localized to the apical membrane (Fig. 7, A and C) with an average apical/basolateral ratio of ~30:1 (Table I). The endogenous Na,K-ATPase α subunit maintains its steady-state basolateral distribution, demonstrating that expression of this chimera does not alter the steady-state distribution of this similar protein (Fig. 7, A and B, E; Table I). Interestingly, the Na,K-ATPase β subunit colocalizes both with the chimera at the apical brush border as well as with the Na,K-α subunit at the basolateral surface (Fig. 7, C and D, G; Table I). Both the chimera and the Na,K-ATPase β subunit are actually intercalated in the apical membrane and are exposed at the apical surface. This fact is demonstrated by incubating the mono-
Figure 7. H519N chimera immunolocalizes to the apical brush border of LLC-PK1 cells in association with the endogenous Na,K-ATPase β subunit. H519N transfected (A-E, G, and I) and untransfected (F, H, and J) LLC-PK1 cells were grown to confluence on transparent microporous membrane supports (FALCON cell culture inserts). Monolayers were fixed in methanol and permeabilized in a Triton X-100 buffer and processed for immunofluorescence as described in Materials and Methods. The HK9 polyclonal antibody was visualized with a rabbit rhodamine conjugated secondary; Na,K-ATPase α, Na,K-ATPase β, and H,K-ATPase β monoclonal antibodies were detected with an anti-mouse FITC conjugated secondary. A and B and C and D represent paired, enface views of the following double-labeling experiment performed on transfected cells: (A) H,K-ATPase α (HK9); (B) Na,K-ATPase α; (C) H,K-ATPase α; (D) Na,K-ATPase β. Confocal optical sections generated in E and G correspond to the A and B and C and D pairs, respectively. The left panels of the split screen confocal images in E and G represent H,K-ATPase α rhodamine staining, the right panels detect Na,K-ATPase α or Na,K-ATPase β/FITC staining, respectively. F and H represent confocal sections of untransfected cells that have been double labeled with H,K-ATPase α/Na,K-ATPase α and H,K-ATPase α/Na,K-ATPase β antibodies, respectively. Note that in transfected cells, Na,K-ATPase β appears to be present at both the apical and basolateral surfaces. I and J represent confocal split screen images (H,K-ATPase α [left]; Na,K-ATPase β [right]) demonstrating that the Na,K-ATPase β subunit can be accessed from the apical side of H519N transfected cells (I) but not untransfected (J) cells. Cells were incubated from the apical surface with the mAb Na,K-ATPase β for 30 min at 4°C. The monolayers were washed to remove unbound antibody, then fixed and permeabilized for double immunolabeling with the H,K-ATPase α antibody.

Figure 8. EM immunolocalization of the H,K-ATPase β subunit in H,K-ATPase β transfected LLC-PK1 cells. Immunostaining was performed with the mAb H,K-ATPase β on a confluent monolayer of paraformaldehyde/glutaraldehyde fixed cells. Specific labeling was detected using a secondary antibody conjugated to horseradish peroxidase. The DAB reaction product can be seen at the apical membrane, along the microvilli (open arrows) and within coated pits (inset), in addition to structures that resemble the recycling portions of tubular endosomes (black arrows). Note that there is little reaction product along the basolateral membrane (arrowheads).
the H,K-ATPase β was detected in a population of subapical vesicles, in addition to its cell surface localization (Fig. 5C). This distribution has never been observed for the Na,K-ATPase α or β subunits (data not shown) or for the H,K-ATPase α subunit (Fig. 5, D and E). To better understand the unique behavior of the H,K-ATPase β, it is necessary to establish the identity of this vesicular compartment. Fig. 8 shows an electron micrograph in which the localization of the H,K-ATPase β is revealed by the immunoperoxidase technique. Dense reaction product can be detected along the microvilli, in coated pits and within compartments that resemble tubular endosomes.

To further characterize these structures, we wished to determine whether this compartment could be loaded with a fluid phase endocytosis marker such as HRP (Fig. 9). Fluid phase markers can be found in early endosomes within 5 min and late endosomes/lysosomes within 20 min of their internalization (Stoorvogel et al., 1991). Stably transfected cells expressing solely the H,K-ATPase β subunit (Fig. 3) were incubated with the H,K-ATPase β mAb at 4°C to prebind reactive epitopes at the apical cell surface. After the cells were rewarmed to 37°C in the presence of HRP for various lengths of time they were fixed, permeabilized and processed for indirect immunofluorescence. As can be seen in Fig. 9 (A, C, E, and G), prebinding with the H,K-ATPase β antibody produces a clear cell surface pattern that gives way to a punctate, vesicular localization after the cells have been incubated for various lengths of time. As early as 10 min both HRP and H,K-ATPase β colocalize within the same intracellular compartment (Fig. 9, C and D). They continue to colocalize for as long as 45 min after warming the cells (Fig. 9, G and H). The structure they occupy is not labeled by antibodies to LGP 120, a lysosomal marker (data not shown; Kornfeld and Mellman, 1989). It seems likely, therefore, that the H,K-ATPase β positive vesicles can be derived from the cell surface and thus represent an endosomal compartment. While the pattern observed in these uptake experiments is virtually indistinguishable from the steady-state vesicular pattern seen in fixed permeabilized cells (Fig. 5 C), we cannot rule out that a subpopulation of these vesicles may represent a post-golgi/preapical compartment. The H,K-ATPase β in the intracellular vesicles observed in these experiments is unlikely to have been internalized as a result of crosslinking effected by our bivalent mAb, because a large literature suggests that bivalent mAbs (e.g., Hopkins, 1983) and non-cross-linking antibodies (e.g., Lazarovits and Roth, 1988) fail to induce endocytosis of surface bound molecules which lack endocytosis signals.

Discussion

We have transfected LLC-PK1 cells with cDNAs encoding wild-type and chimeric α and β subunits of the H,K-ATPase. We find that efficient surface expression of the H,K-ATPase α subunit requires co-expression with its H,K-ATPase β subunit. No such assembly requirement appears to exist for the H,K-ATPase β, which seems to be able to depart the ER unaccompanied by an α subunit. We find that the assembled H,K-ATPase α/β complex accumulates exclusively in the apical plasmalemma. Both the α and β subunits of the endoge-
nos Na,K-ATPase maintain their steady-state basolateral distribution in this cell line. Thus, LLC-PK, cells are capable of distinguishing between these two highly homologous enzymes and of maintaining them at their appropriate domains.

The experiments performed in this study examine the steady-state distribution of pump subunits and subunit chimeras in transfected LLC-PK, cells. Recent work suggests that the steady-state basolateral distribution of the Na,K-ATPase is not the result of sorting at the level of the TGN, but rather is due to selective retention afforded by a preexisting basolateral cytoskeletal network (Hammerton et al., 1991). Definitive exploration of the mechanism through which our proteins are differentially distributed must await the results of targeting assays. Our efforts to carry out biosynthetic targeting assays in this system have been complicated by several factors. We have recently demonstrated that dramatic differences in biotinylation efficiency prevail at the apical and basolateral surfaces of LLC-PK, cells (Gottardi and Caplan, 1992), rendering this technique inapplicable. Furthermore, surface immunoprecipitation protocols cannot be applied, because antibodies recognizing ectodomain epitopes of the H,K-ATPase α-subunit have yet to be successfully produced.

It should be noted, however, that recent work from two laboratories (Gottardi and Caplan, 1993; Zurzolo and Rodriguez-Boulan, 1993) suggests that the Na,K-ATPase in at least one clone MDCK cells, as well as in thyroid epithelial cells (FRT) is sorted intracellularly and vectorially targeted in keeping with earlier observations (Caplan et al., 1986). The recently reported detection of random sodium pump delivery in MDCK cells (Hammerton et al., 1991) may be referenceable to differences in the particular subclone of MDCK cells used (Siemens, C. [Krzeminski] et al., 1993). In the context of these studies, which reconfirm the earlier conclusion that the Na,K-ATPase is competent to serve as a substrate for vectorial targeting in MDCK cells, we believe that the H,K-ATPase and the chimeric subunits are likely to be sorted intracellularly and vectorially targeted in LLC-PK, cells. While it is true that our analysis of the steady-state distributions of these ion pump and pump chimera molecules does not allow us to draw conclusions with respect to the pathways these molecules follow, our experiments do, however, establish domains of these pumps which are necessary for ensuring their steady state distributions. Whether the signal domains we have described are interpreted at the level of the Golgi, or serve to anchor these proteins at one or the other surface domain after random delivery, they are clearly necessary and sufficient to ensure the ultimate polarized distributions of their parent proteins.

**Signals Contributed by α Subunits**

In our efforts to characterize the signal responsible for the apical localization of the H,K-ATPase complex, we have examined the contributions of each subunit to the steady-state apical distribution of the holoenzyme. To our surprise, we found that both the α and β proteins encode information which specifies apical accumulation. The contribution of the H,K-ATPase α subunit is revealed in the cell surface localization of the H,K-ATPase/Na,K-ATPase α chimera, H519N. This protein comprises the amino-terminal half of the H,K-ATPase and the carboxy-half of the Na,K-ATPase and assembles exclusively with the endogenous Na,K-ATPase β subunit. The resulting complex localizes predominantly to the apical brush border of transfected LLC-PK, cells, suggesting that a dominant apical localization signal may reside within the amino-half of the H,K-ATPase. As can be seen in Fig. 1, <20% of the H519N chimera is encoded by amino acids unique to the H,K-ATPase. Furthermore, analysis of the Na,K-ATPase and H,K-ATPase sequences reveals a single region of extensive nonhomology (Shull and Lingrel, 1986). The amino terminus of the H,K-ATPase α extends 13 residues beyond that of the Na,K-ATPase α. Significant identities are not found until residue 23 of the H,K-ATPase sequence. It is therefore possible that this unique amino acid sequence encodes the information that constitutes the signal which ultimately accounts for the apical steady-state distribution of this protein. It is interesting to note that a colonic isoform of the H,K-ATPase, which is 63% identical to the gastric H,K-ATPase and is also localized to the apical membrane, shares an 8 amino acid stretch of identity in this amino terminal region (Crowson and Shull, 1992). Chimeric constructs which will test this hypothesis are currently being generated.

**Signals Contributed by β Subunits**

It has been speculated that these pumps' β subunits may somehow be necessary for escorting α subunits to specific cell surface domains. Our results show that this is not the case for the Na,K-ATPase β subunit. The data presented in Fig. 7 demonstrate that the H519N chimera colocalizes with an endogenous Na,K-ATPase β subunit at the apical cell surface. Because the Na,K-ATPase β subunit colocalizes with both the H519N chimera at the apical membrane, as well as with the endogenous Na,K-ATPase α at the basolateral membrane, we conclude that this β subunit is unlikely to encode a dominant distribution-determining signal. The behavior of the H,K-ATPase β subunit (~31% identical to rat Na,K-ATPase β; Reuben et al., 1990), however, is markedly different and supports the argument that at least this β subunit possesses a localization signal. When expressed by itself or in concert with H,K-ATPase α, the H,K-ATPase β localizes to the apical brush border as well as to elements of the endocytic pathway (Figs. 5, 8, and 9). The presence of H,K-ATPase β in components of the endocytic apparatus suggests the existence of an endocytosis signal within the 36 amino acid cytoplasmic domain of this polypeptide (Goldstein et al., 1985).

As can be seen in Fig. 10, the H,K-ATPase β shares with the transferrin receptor a sequence which has been implicated in forming a tight turn structural recognition motif for endocytosis (Collawn et al., 1990). This region is highly conserved between the varied species of cloned H,K-ATPase β subunits (Fig. 10). While the H,K-ATPase motif is opposite in orientation to the transferrin receptor sequence (i.e., -FRXY- vs. -YXRF-), crystallographic data predicts that tetrapeptide sequences such as -FEKY- and -FSKY- are capable of forming the requisite tight turn structures (Collawn et al., 1990; Alvarez et al., 1990). Furthermore, studies performed on the transferrin receptor demonstrate that this sequence mediates internalization when expressed in either orientation (Girones et al., 1991). The -FRXY- sequence is not found in β, or β subunits of the Na,K-ATPase (Martin-Vasallo et al., 1989; Shull et al., 1986), neither of which appear to localize to similar vesicular structures (data not shown). It remains to
be determined whether this sequence, and/or residues which flank it are responsible for the H,K-ATPase β's participation in the endocytic pathway.

It is interesting to consider why the H,K-ATPase might be well served by some manner of endocytosis signal. It its native gastric parietal cell, the H,K-ATPase α/β complex resides within a preapical compartment, which in response to physiologic stimuli for gastric acid secretion, rapidly fuses with the apical membrane (Hirst and Forte, 1985). When the stimulatory signal abates, the H,K-ATPase, along with most of the apical membrane, is rapidly endocytosed and retrieved into this preapical tubulovesicular compartment. Clearly, a mechanism must exist to ensure that these pumps can be rapidly and efficiently removed from the apical membrane. The -FRXY- signal is likely to serve this function. We are in the process of generating site-directed mutants that delete this sequence or alter the critical tyrosine residue in order to test this hypothesis.

In light of this model, it is surprising that in cells transfected with both H,K-ATPase α and β subunits, the H,K-ATPase β appears to accumulate in endocytic vesicles in the absence of any accompanying α subunit (Fig. 5). In stimulated parietal cells the α subunit of the H,K-ATPase has been shown to form a complex with ezrin/ankyrin/fodrin cytoskeletal elements at the apical membrane (Hanzel et al., 1991; Mercier et al., 1989). It is possible, therefore, that the subpopulation of H,K-ATPase β subunit that is participating in an H,K-ATPase α/β dimer cannot be endocytosed because it is tethered to analogous apical cytoskeletal elements. H,K-ATPase β subunits that are expressed at the cell surface without an H,K-ATPase α would not be linked to the cytoskeleton, and would, therefore, be free to be endocytosed.

Endocytosed Signals and Protein Targeting

It has recently been shown that the presence of an endocytosis signal is sufficient to ensure a protein's basolateral targeting in MDCK cells (Brewer and Roth, 1991; Hunziker et al., 1991; LeBivic et al., 1991). The first demonstration of this property involved the influenza hemagglutinin protein, which is normally targeted to the apical membrane of MDCK cells (Rodriguez-Boulan and Sabatini, 1978). Brewer and Roth found that this protein could be rerouted to the basolateral surface if a single amino acid in its cytoplasmic domain is altered to create a functional endocytosis signal. These observations have now been extended to a number of proteins. In light of this behavior, it is surprising that the endocytosis competent H,K-ATPase β accumulates at the apical surface of LLC-PK1 cells. We hypothesize that this behavior reflects the fact that LLC-PK1 cells, derived from the renal proximal tubule, are specially adapted to perform apical endocytosis.

Proximal tubule cells carry out bulk resorption of the glomerular filtrate (Mandel and Balaban, 1981). Their apical domains are richly endowed with coated pits and endocytic profiles, which participate in the retrieval of small proteins and peptides which have been filtered through the glomerular basement membrane (Maunsbach, 1976). The preponderance of apical endocytic machinery may also be associated with the apical accumulation of proteins whose targeting is mediated by virtue of their coated pit localization sequences. According to this model, a coated pit localization sequence specifies neither apical nor basolateral sorting, but instead ensures that proteins so endowed accumulate at the most endocytically active domain. This interpretation receives support from the surprising observations of Pathak et al. (1990), who generated a transgenic mouse expressing the low density lipid (LDL) receptor. This protein has been shown to possess a coated pit localization sequence as well as an autonomous basolateral targeting signal, both of which can independently mediate basolateral sorting of receptor constructs expressed in MDCK cells (Hunziker et al., 1991). The distribution of the wild-type LDL receptor in transgenic mice was found to be basolateral in the epithelia of intestine and liver. In the proximal tubule of the kidney, however, this protein was restricted to the apical brush border. It is possible that in this tissue the LDL receptor's coated pit localization sequence exerts a dominant effect which results in the protein being sorted to the endocytically active apical surface. To test this theory, we are currently comparing the sorting behavior of a number of endocytically competent proteins exogenously expressed in both MDCK and LLC-PK1 cells. Preliminary results suggest that, consistent with this model, H,K-ATPase β accumulates at the basolateral surface of MDCK cells. It would appear, therefore, that sorting signals may specify delivery to functionally rather than topologically defined plasma membrane domains.

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1. Abbreviation used in this paper: LDL, low density lipoprotein.
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