H,K-ATPase α subunit C-terminal membrane topology: epitope tags in the insect cell expression system

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The H,K-ATPase responsible for gastric acidification is a heterodimeric (α and β subunit) P-type ATPase, an integral protein of parietal cell apical membranes, which promotes the electroneutral exchange of K^+ for protons, is stimulated by K^+ and is inhibited by 2-methyl-8-(phenylmethoxy)imidazo[1,2- α]pyridine-3-acetonitrile (SCH 28080). Hydropathy analysis of the catalytic α subunit has been interpreted in terms of four N-terminal transmembrane domains, a cytoplasmically oriented segment containing ATP binding and phosphorylation sites, and a C-terminal region with four or six putative transmembrane domains. Several lines of evidence implicate the C-terminal region of P-type ATPases in cation-binding and occlusion, conformational changes, and interactions with the β subunit (HK β), making the definition of topology a prerequisite for understanding the structural basis of these functions. Influenza haemagglutinin epitopes (YPYDVPDYA; flu tag) were inserted in predicted hydrophilic segments of the α subunit (HK α) to establish the membrane orientation of two amino acids with different predicted topologies in the C-terminal four- and six-transmembrane

models. Wild-type and mutated HK α and HK β cDNA species were expressed in insect cells (Sf9) via recombinant baculovirus infection, and expression of H,K-ATPase was verified by immunoblotting with HK α - and HK β -specific and flu-tag-specific antibodies. Functional assays showed K^+ -stimulated, SCH 28080-sensitive ATPase activity, confirming neo-native topology in H,K-ATPase heterodimers expressed in Sf9 cells. The topology of flu tags was determined by microsomal protease protection assays in Sf9 cells and immunolabelling of HK α and HK β in intact and permeabilized Sf9 cells. In addition, MS of native H,K-ATPase tryptic peptides identified cytoplasmically oriented HK α residues. The results indicated cytoplasmic exposure of Leu⁸⁴⁴ and Phe⁹⁹⁶, and luminal exposure of Pro⁸⁹⁸, leading to a revised secondary structure model of the C-terminal third of HK α .

Key words: acid secretion, antibody probes, proton transport, secondary structure.

INTRODUCTION

Gastric acid secretion is mediated by a Mg^{2+} -dependent, K^+ -stimulated, H^+ -transporting adenosine triphosphatase (H,K-ATPase, EC 3.6.1.36) [1,2]. The enzyme is a polytopic integral protein of tubulovesicular and secretory canalicular membranes in acid-secreting gastric epithelial parietal cells, and catalyses the electroneutral exchange of luminal K^+ for cytoplasmic H^+ . The resulting 10^6 -fold H^+ gradient across the secretory membranes makes the H,K-ATPase an attractive model for structure–function studies of epithelial proton pumping. The enzyme is a vanadate-sensitive P-type ATPase with closely interacting α and β subunits (HK α and HK β). Enzyme activity is specifically and reversibly inhibited by the substituted imidazo [1,2- α]pyridine 2-methyl-8-(phenylmethoxy)imidazo[1,2- α]pyridine-3-acetonitrile (SCH 28080), a high-affinity K^+ -competitive agonist [3] acting at the extracellular surface of the first two transmembrane (Tm) domains of HK α [4].

The primary structures of HK α from several species (reviewed in [5]) are more than 95% similar, comprising approx. 1033 residues, with deduced molecular masses of approx. 114 kDa. The molecular mass of HK α estimated by SDS/PAGE is 94 kDa; sequence similarity to the Na,K-ATPase α subunit is approx. 65%. HK α is closely associated with HK β [6,7], whose primary structures consist of 290–294 residues with a molecular mass of approx. 33 kDa. Glycosylation at six or seven tripeptide consensus sites gives a diffuse molecular mass of 60–80 kDa. Cysteine

residues in the C-terminal half of HK β form three disulphide bonds in the native enzyme, reduction of which is dose-dependently accompanied by loss of activity [8].

Hydropathy plots of HK α primary structure predict an integral membrane protein, with four Tm α -helices (H1–H4) in the N-terminal third, a hydrophilic cytoplasmic domain containing the phosphorylation and nucleotide-binding sites, and four [9–11] or six [5,12] Tm α -helices in the C-terminal third. A large body of evidence, mostly proteolytic and immunochemical, from the closely related Na,K-ATPase is also interpreted in terms of four or six C-terminal Tm domains [13–16]. Cytoplasmic N- and C-termini have been confirmed [17,18], as have the assignments of H1–H4 as Tm domains. However, the topology of the C-terminal third of both HK α and Na,K-ATPase α subunits remains unclear. Figure 1 shows two conflicting models of the secondary structure of gastric H,K-ATPase, in which quite different topologies have been proposed for residues encompassing the putative H5, H6 and H7 Tm domains. The eight-Tm model predicts a luminal Leu⁸⁴⁴ and a cytoplasmic Pro⁸⁹⁸, whereas the ten-Tm model predicts a cytoplasmic Leu⁸⁴⁴ and a luminal Pro⁸⁹⁸.

In the present study, mutant HK α carrying epitope inserts adjacent to these amino acids was expressed in insect cells (Sf9 cells) with the use of recombinant baculovirus (rBV) vectors. Native conformation of the heterologously expressed HK α and HK β heterodimers was ensured by measurement of K^+ -stimulated, SCH 28080-sensitive ATPase activity in membranes de-

Abbreviations used: HK α , gastric H,K-ATPase α subunit; HK β , gastric H,K-ATPase β subunit; mAb, monoclonal antibody; MALDI-MS, matrix-assisted laser desorption ionization MS; MOI, multiplicity of infection; NGS, normal goat serum; rBV, recombinant baculovirus; SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2- α]pyridine-3-acetonitrile; Tm, transmembrane domain.

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Figure 1 Models of the secondary structure of H,K-ATPase

(A) An eight-Tm-domain model based on Kyte–Doolittle hydropathy analysis of rat gastric H,K-ATPase, with the use of a nine-residue span and averaging of each set of five data points [9]. (B) A ten-Tm-domain model of rabbit gastric H,K-ATPase based on Kyte–Doolittle hydropathy analysis with the use of an 11-residue span and other data [30]. Amino acids at N- and C-termini of putative Tm helices (H1–H10) are numbered, as are the C-terminal amino acids of α and β subunits. Locations of flu tag epitope inserts are labelled ft1, ft2, ft3, and β ft.

rived from rBV-infected SF9 cells. The results of protease protection assays and immunolabelling of cells expressing epitope-tagged HK $\alpha\beta$, and identification by MS of cytoplasmic HK α C-terminal peptides, indicated cytoplasmic Leu⁸⁴⁴ and Phe⁸⁹⁶ and luminal Pro⁸⁹⁸, and led to a revised model of the C-terminal secondary structure of HK α .

EXPERIMENTAL

Materials

Pig H,K-ATPase α and β subunit cDNA species (pHK α and pHK β) were a gift from Dr. Masatomo Maeda (Osaka University, Osaka, Japan). Restriction enzymes and T4 ligase were purchased from Promega (Madison, WI, U.S.A.). Cell culture supplies and baculovirus donor vectors were from Gibco BRL (Gaithersburg, MD, U.S.A.) and ovarian cells of *Spodoptera frugiperda* (Sf9 cells) were from American Type Culture Collection (CRL 1711; Rockville, MD, U.S.A.). Influenza haemagglutinin epitope (flu tag) monoclonal antibody (mAb) HA.11 was from BAbCO (Richmond, CA, U.S.A.), immunoblotting reagents were from Bio-Rad Laboratories (Richmond, CA,

U.S.A.) and immunochemistry supplies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). [³²P]-ATP was from New England Nuclear (Boston, MA, U.S.A.). Proteinase K was from Sigma (St. Louis, MO, U.S.A.) and SCH 28080 was donated by Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.). All other reagents were of molecular biology grade or the highest grade of purity available.

cDNA constructs

Nucleotide sequences encoding the flu tag (YPYDVPDYA) were inserted into wild-type HK α and HK β cDNA species by PCR mutagenesis (XL-PCR kit; Perkin-Elmer) or ligation of synthetic oligonucleotides. Flu tag cDNA species encoded an *Nde*I restriction site to facilitate screening for recombinant clones, and all flu tag insertions were confirmed by dideoxy sequencing. rBVs were generated with pFastBac Dual (pFBD) donor vectors carrying both HK α and HK β cDNA species under polyhedrin (very late) and p10 (late) promoter control respectively. HK β cDNA (wild-type or mutated) constructs were placed first into pFBD, followed by HK α cDNA constructs (wild-type or mutated). Three separate insertions of the flu tag into HK α were made: between Leu⁸⁴⁴ and Arg⁸⁴⁵ (HK α_{ft1}), between Pro⁸⁹⁸ and Gln⁸⁹⁹ (HK α_{ft2}) and between Gly⁶⁷⁷ and Met⁶⁷⁸ (HK α_{ft3}). One flu tag insertion was made into HK β , between Lys²⁸⁵ and Leu²⁸⁶ (β_{ft}).

HK $\alpha_{wt}\beta$ construct

Plasmid pHK β containing HK β cDNA in pBlueScript II KS⁺ was digested with *Xho*I and cloned into pFBD vector downstream of the p10 promoter, producing pFBD.HK β donor vector. HK α cDNA was excised from pUC18 with *Xba*I, then cloned into pFBD.HK β vector downstream of the polyhedrin promoter.

HK β_{ft} cDNA construct

HK β cDNA was ligated into the *Xho*I cloning site of pGem7zf (Promega) from which the *Hind*III restriction site had been ablated. A 150 bp segment containing the HK β 3' terminus was excised with *Hind*III and *Bam*HI and replaced with a pair of annealed complementary oligonucleotides encoding the 27 bp flu tag, an *Nde*I restriction site, and *Hind*III and *Bam*HI cohesive termini (5'-AGCTTTACCCATATGACGTTCCGGACTACGCAAA-GATCCAGAAGCTCGAG-3' and 5'-ATGGGTATACTGCA-AGGCTGATGCGTTTCTAGGTTCTTCGAGCTCCTAG-3'). HK β_{ft1} cDNA was excised from pGem7zf with *Xho*I and cloned into pFBD vector downstream of the p10 promoter.

HK α -flu tag cDNA constructs

For HK α_{ft1} , HK α cDNA was ligated into the *Xba*I cloning site of pGem7zf. A 44 bp segment (nt 2498–2542 in HK α cDNA) was excised with *Nde*I and *Sac*II and replaced with a pair of annealed complementary oligonucleotides encoding the 27 bp flu tag and *Nde*I and *Sac*II cohesive termini (5'-TATGAGAAGGCCGA-GAGTGACATCATGCACCTGTACCCATATGACGTTCCGGACTACGCACGCCCGC-3' and 5'-GGGCGTGCGTAG-TCCGGAACGTCATATGGGTACAGGTGCATGATGTC-ACTCTCGCCTTCTCA-3'). For HK α_{ft2} , HK α cDNA was ligated into the *Xba*I cloning site of *Bam*HI-ablated pGem7zf to form pGem7zfba.HK α . A 448 bp *Sac*II–*Bam*HI HK α cassette (nt 2539–2987) was ligated into pBlueScript II KS⁺ and served as a template for forward (5'-TACCTGACTACGCACAAT-GGGAGAACCACCACCT-3') and reverse (5'-CATCATATG-GATATGGACGCAGCCCCACACACAG-3') phosphorylated PCR primers (eight cycles of 94 °C for 60 s, 60 °C for 90 s and

68 °C for 5 min). After the digestion of template DNA with *DpnI*, and the removal of 3' single-base overhangs with *Pfu* polymerase, the linear blunt-ended PCR product was self-ligated and cloned into XL1 Blue *Escherichia coli*. The mutated cassette was excised with *SacII* and *BamHI*, then ligated into *SacI*-/*BamHI*-linearized pGem7zfb α .HK α . For HK α_{118} , the plasmid pBSch was formed by adding an *MluI* restriction site to the multiple cloning site of pBlueScript II KS⁺. A 1160 bp *MluI*-/*SacII* HK α cassette (nt 1379–2539) was cloned into pBSch and served as the template for forward (5'-GTCCCAGACTACGCAATGCAACTGAAGGACATGG-3') and reverse (5'-GT-CGTATGGATAACCGTTGATCAGCAGG-3') phosphorylated PCR primers (eight cycles of 94 °C for 1 min, 55 °C for 90 s and 68 °C for 5 min). After the digestion of template DNA with *DpnI*, and the removal of 3' single-base overhangs with *Pfu* polymerase, the linear blunt-ended PCR product was self-ligated and cloned in XL1 Blue *E. coli*. The mutated cassette was excised with *MluI* and *SacII*, then ligated into *MluI*/*SacII*-linearized pBS.HK α . All three HK α -flu tag constructs were excised from host plasmids with *XbaI* for cloning into pFBD.HK β vector.

rBVs and Sf9 cell infection

Site-specific transposition of cDNA species to a baculovirus shuttle vector (bacmid) was used to form rBVs [19]. HK α and HK β cDNA cloned in pFBD donor vectors were transformed into DH10B_{AC}-competent cells containing the bacmid shuttle vector in accordance with protocols provided by Gibco BRL. Bacmids were extracted from overnight bacterial cultures and used to transfect Sf9 cells. After 4 days, cell culture supernatants containing rBVs were harvested. Viral stocks were amplified to approx. 10⁸ plaque-forming units/ml as measured by plaque assay [20] by infecting 150–200 ml cultures of Sf9 cells (approx. 10⁶ cells/ml) in spinner flasks. Insect cells (Sf9) were routinely grown to 2 × 10⁶ cells/ml as suspension cultures in spinner flasks, in Grace's insect cell medium supplemented with yeastolate and lactalbumin (TNM-FH medium) with 10% (v/v) fetal bovine serum, as described previously [21]. For functional expression of HK α and HK β , (6–8) × 10⁷ Sf9 cells in spinner flasks were infected at a multiplicity of infection (MOI) of between 5 and 10; ethanol (1%, v/v) was added to infected Sf9 cell cultures to augment H,K-ATPase synthesis [22].

Preparation of microsomal membranes

Sf9 cells were washed in K⁺-free PBS and centrifuged (1000 *g*, 10 min, 4 °C), then resuspended in homogenization buffer (HB) [10 mM Pipes/Tris (pH 6.8)/0.25 M sucrose/2 mM EDTA/1 mM benzamidine/10 μ g/ml leupeptin/10 μ g/ml pepstatin/1 μ g/ml aprotinin]. Cells were sonicated on ice (three times for 15 s each) (Branson) with 1 min incubations on ice between sonications. Homogenates were centrifuged at 13000 *g* for 20 min; the resulting supernatants were centrifuged at 100000 *g* for 1 h (Beckman 50Ti rotor). Membrane pellets were resuspended in HB and homogenized (glass/Teflon, 2000 rev./min, ten strokes), then aliquots were snap-frozen in liquid N₂ and stored at –70 °C. The HK α content of Sf9 cells was measured by quantifying densitometric scans of mAb HK 12.18 immunoblot reactivity at 94 kDa relative to native H,K-ATPase immunoblots with the same antibody. Native H,K-ATPase (more than 90% of total protein with a molecular mass of 94 kDa) was prepared from pig gastric mucosal homogenates by differential and sucrose/Ficoll step gradient centrifugation [23]. Protein concentrations were measured by the Bradford assay [24], with BSA as standard.

Antibodies

HK α expression in Sf9 cells was detected immunocytochemically with a synthetic peptide antibody (HK α C2) against pig HK α C-terminal sequence GVACCPGSWWDQELYY [17]. SDS/PAGE immunoblots of Sf9 microsomes were probed for HK α by using mAb HK 12.18 against pig gastric H,K-ATPase α subunit [25]. Conflicting reports of HK 12.18 epitope location [10,26] precluded the use of this antibody for HK α topology assignments. HK β expression in Sf9 cells was detected both immunocytochemically and in immunoblots with a synthetic peptide antibody (HK β C3) against pig HK β C-terminal sequence CPYEGKVEFKLTIQK [27]. Flu tags expressed in HK α and HK β were detected with mAb HA.11.

ATPase assay

Reaction mixtures for assay of K⁺-stimulated ATPase activity in Sf9 membranes contained 5 μ g of membrane protein, 100 mM Tris/acetate, pH 7.0, 1 mM MgCl₂, 1 mM NaN₃, 0.1 mM EGTA, 5 μ M ATP ([γ -³²P]ATP, 10 Ci/mmol; NEN, Boston, MA, U.S.A.), 0–10 mM KCl and 0–100 μ M SCH 28080. After incubation for 20 min at 37 °C, reactions were stopped by the addition of 10% (w/v) activated charcoal (Sigma)/5.5% (w/v) trichloroacetic acid, vortex-mixed vigorously and centrifuged at 14000 *g* for 10 min at 4 °C. The [γ -³²P]P_i content of the supernatant was measured by scintillation counting. H,K-ATPase-specific ATP hydrolysis was calculated as the difference between K⁺-dependent ATPase in the presence and in the absence of SCH 28080, and was expressed as nmol of P_i/h per mg of HK α .

Protease treatments

For limited trypsin hydrolysis, native H,K-ATPase (1 mg/ml) was incubated for 30 min at 37 °C with tosylphenylalanylchloromethane ('TPCK')-treated trypsin (12700 units/mg of protein, 15 μ g/ml) in 10 mM KCl/250 mM sucrose/20 mM Tris/HCl (pH 7.2); hydrolysis was stopped by the addition of a 10-fold excess (over trypsin) of soybean trypsin inhibitor. Reaction mixtures were centrifuged at 100000 *g* (70000 rev./min for 30 min in a Beckman TL 100 rotor); HK α peptides in the supernatant were identified by MS. For digestion with proteinase K, Sf9 microsomes (0.4–0.8 mg/ml) suspended in 10 mM Tris/HCl (pH 7.5)/250 mM sucrose/10 mM CaCl₂ were incubated for 1 h on ice with 50 or 250 μ g/ml proteinase K, in the presence or absence of 1% (v/v) Triton X-100 as a control to distinguish between intravesicular protease protection and inherent protease resistance. Digestion was stopped and microsomal proteins were precipitated by the addition of trichloroacetic acid to 12.5% (w/v). After 1 h on ice, microsomes were centrifuged at 100000 *g* for 20 min (Beckman TL 100.4 rotor), and resuspended in SDS/PAGE sample buffer for immunoblot analysis.

Matrix-assisted laser desorption ionization (MALDI) MS

MALDI-MS was performed on a custom-built linear time-of-flight mass spectrometer equipped with a nitrogen laser source (Mass Spectrometry Facility, Medical University of South Carolina, Charleston, SC, U.S.A.). In a typical experiment, 1 μ l of a 1 mg/ml protein sample was mixed with 3 μ l of matrix [50 mM α -cyano-4-hydroxycinnamic acid in 50% (v/v) aqueous propan-1-ol]. Aliquots (1 μ l) of this solution were air-dried on the probe tip, and data from 10–200 laser shots were summed to produce mass spectra. Data were acquired and processed with a LeCroy 9400A digital oscilloscope interfaced with a Zenith 386-SX computer running Tofware software. Mass assignments were made with bovine insulin (*m/z* 5733.6) and ubiquitin (*m/z*

8565.9) as internal standards to bracket the singly charged HK α peptide masses. This two-point calibration was also used to assign m/z values to other peaks in the mass spectrum.

Immunoblots

Microsomal membranes from Sf9 cells infected with HK α and/or HK β rBVs were diluted with equal volumes of SDS/PAGE sample buffer [28] and heated to 37 °C for 10 min, then applied to 4–20 % (w/v) polyacrylamide gradient or Tricine/SDS/PAGE minigels [16 % (w/v) gel], and electrophoresis was performed for 90 min at 125 V. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.) for 1 h at 150 mA per membrane in 0.25 M Tris/1.92 M glycine/0.1 % SDS transfer buffer. Replicas were blocked for 1 h with 5 % (w/v) non-fat dried milk in 20 mM Tris/150 mM NaCl (pH 7.5) (TBS), then incubated for 2 h with 1:1000 dilutions of HK α -, HK β - or flu tag-specific antibody in 1 % (w/v) non-fat dried milk in 1 % (v/v) Tween 20/TBS (TTBS). Replicas were washed three times in TTBS and then incubated for 1 h with a 1:2000 dilution in TTBS of goat anti-mouse Ig or goat anti-rabbit Ig, conjugated to horseradish peroxidase as appropriate. After a wash, signal was developed by incubating replicas in chemiluminescent substrate (ECL[®]; Amersham, Arlington Heights, IN, U.S.A.) followed by exposure for 1–10 s to X-ray film.

Immunocytochemistry

Adherent Sf9 cells in six-well culture plates (750 000 cells per well) were infected with rBVs at an MOI of 10. After 48 h cells were washed (this and subsequent cell washes were three times with PBS); then cycloheximide (100 mg/ml, 1 h) was added to halt protein synthesis. For the detection of extracellularly oriented HK α or HK β epitopes, cells were incubated with HK α -, HK β - or flu tag-specific antibody diluted in 5 % (v/v) normal goat serum (NGS)/1 % (w/v) BSA for 1 h at 4 °C. Cells were washed, fixed with 2 % (w/v) paraformaldehyde for 15 min, washed, then treated with 100 mM glycine to quench excess paraformaldehyde. After being washed, cells were permeabilized and blocked for 15 min with 0.1 % saponin in 5 % NGS/1 % BSA, then incubated for 1 h with FITC-conjugated secondary antibody (1:200 in 5 % NGS/1 % BSA/0.1 % saponin). Cells were washed and coverslips were applied over mounting medium [90 % (v/v) glycerol in PBS/0.1 % *p*-phenylenediamine]. For the detection of cytoplasmically oriented epitopes, Sf9 cells were fixed, permeabilized and blocked as described above, then incubated overnight at 4 °C with HK α -, HK β - or flu tag-specific antibody. Cells were washed, incubated with FITC-labelled secondary antibody (1:200 in 1 % NGS/0.1 % saponin) for 45 min at room temperature, then washed and mounted as above. Immunolabelling of cells was recorded by video imaging on a Zeiss Axioplan Microscope with 496 nm FITC filters. Digital images were imported to Adobe Photoshop, and printed from TIFFs.

RESULTS

Expression of HK α and HK β in Sf9 cells

Pig H,K-ATPase subunit cDNA species were integrated into rBVs by using pFastBac Dual donor vectors. These vectors allowed the concurrent expression of two heterologous cDNA species after the infection of Sf9 cells with a single rBV. Sf9 cells were infected at an MOI of 10 with rBVs carrying only HK α cDNA species (α rBVs) or both HK α and HK β cDNA species ($\alpha\beta$ rBVs). All HK α constructs were expressed under very late

Table 1 Biological activity of wild-type and epitope-tagged H,K-ATPase heterodimers expressed in Sf9 cells

Sf9 cells were infected for 72 h with rBVs as indicated, then the H,K-ATPase expression level and SCH 28080-sensitive K⁺-ATPase activity in isolated microsomal membranes were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for measurements on membranes from at least three independent infections with each rBV. *Significantly different from $\alpha_{wt}\beta$ (Student's *t* test, *P* > 0.05).

Mutant	Sequence	Expression level (pmol/mg of protein)	Specific activity (nmol of P _i /h per mg of HK α)
Wild-type virus	No HK α or HK β	0	0
$\alpha_{wt}\beta$	Wild-type	322 $\pm$ 185	213 $\pm$ 122
$\alpha_{fl}\beta$	α Leu ⁸⁴³ -tag-Arg ⁸⁴⁴	338 $\pm$ 163	691 $\pm$ 108*
$\alpha_{fl}\beta$	α Pro ⁸⁹⁸ -tag-Gln ⁸⁹⁹	263 $\pm$ 175	687 $\pm$ 327
$\alpha_{fl}\beta$	α Gly ⁶⁷⁷ -tag-Met ⁶⁷⁸	363 $\pm$ 132	517 $\pm$ 145
$\alpha_{wt}\beta_{fl}$	β Lys ²⁸⁵ -tag-Leu ²⁸⁶	690 $\pm$ 200	443 $\pm$ 70

Figure 2 Immunoblot detection of the expression of wild-type and mutated HK α with mAb HK 12.18

Pig gastric microsomal membranes enriched in H,K-ATPase (HK α , lane 1, 50 ng), uninfected Sf9 microsomal membranes (lane 2, 1 μ g) and Sf9 microsomal membranes expressing wild-type and flu-tagged HK α and HK β 72 h after infection with rBVs (lanes 3–9, 1.0 μ g per lane) were resolved by SDS/PAGE, transferred to PVDF membranes and probed with HK α -specific mAb HK 12.18. The positions of molecular mass markers are indicated at the left.

promoter (polyhedrin) control; HK β constructs were expressed under late promoter (P10) control. After 72 h cells were processed to yield membranes, which were then resolved by SDS/PAGE and transferred to PVDF membranes for SDS/PAGE immunoblot analysis.

Wild-type and epitope-tagged HK α were detected by using the mAb HK 12.18 against the wild-type HK α sequence. Expression levels of HK α , measured from immunoblots with native H,K-ATPase as a standard (Table 1), varied considerably between preparations, ranging from 1 % to 10 % of total Sf9 membrane protein. Immunoblots of rBV-infected Sf9 membrane proteins resolved by SDS/PAGE exhibited immunoreactivity with mAb HK 12.18, as shown in Figure 2. The antibody labelled a 94 kDa band in native H,K-ATPase (lane 1), and showed no immunoreactivity with membrane proteins from uninfected Sf9 cells (lane 2), confirming the absence of endogenous HK α in Sf9 cells. The antibody labelled 94 kDa bands in membranes from Sf9 cells infected only with wild-type or epitope-tagged HK α rBVs (Figure 2, lanes 3 and 5) or with HK $\alpha\beta$ rBVs (lanes 4 and 6–9). The presence of flu tags adjacent to Leu⁸⁴⁴ (Figure 2, lane 6), Pro⁸⁹⁸

Figure 3 Immunoblot detection of the expression of wild-type and mutated HK α and HK β with HK β C3 and mAb HA.11

Pig gastric microsomal membranes enriched in H,K-ATPase (HK $_n$, lanes 1 and 2, 50 ng per lane), Sf9 microsomal membranes expressing wild type and flu-tagged HK α and HK β 72 h after infection with rBVs (lanes 3 and 4, 1 μ g per lane) and uninfected Sf9 microsomal membranes (lane 5, 1 μ g) were resolved by SDS/PAGE, transferred to PVDF membranes and probed with HK β -specific antibody HK β C3. In lanes 6–10, Sf9 microsomal membranes expressing flu-tagged HK α and HK β 72 h after infection with rBVs were resolved by SDS/PAGE (1 μ g per lane), transferred to PVDF membranes and probed with flu-tag mAb HA.11. The positions of molecular mass markers are indicated at the left.

(lane 7) or Gly⁶⁷⁷ (lane 8) had no apparent effect on immunoblot reactivities of HK 12.18 with expressed HK α , suggesting that flu tags in these positions did not physically interrupt the HK 12.18 epitope. Co-expression of HK β with HK α had no effect on HK 12.18 immunoblot reactivity, with or without HK α flu tag inserts, suggesting that sites of functional interaction of HK α with HK β do not involve the HK 12.18 epitope.

HK β expression in Sf9 membranes was detected with antiserum HK β C3 against an HK β C-terminal synthetic peptide. Immunoblots of pig gastric and rBV-infected Sf9 membranes probed with HK β C3 are shown in Figure 3 (lanes 1–5). The antibody labelled a diffuse 60–80 kDa band in native H,K-ATPase microsomes (lane 1). Treatment of gastric microsomes with N-glycosidase F, which cleaves the N-glycan linkage of glycoproteins between asparagine and the oligosaccharide substituent, resulted in HK β C3's labelling a band of molecular mass 34 kDa (Figure 3, lane 2), corresponding to the molecular mass of the HK β core peptide [8]. With membranes from HK $\alpha\beta$ rBV-infected Sf9 cells (Figure 3, lanes 3 and 4), the antibody labelled a band of 34 kDa (the HK β core peptide) and bands of 39, 45 and 49 kDa, representing partly glycosylated forms of HK β . The presence of a flu tag on HK α seemed to promote the glycosylation of HK β core peptide, although the overall expression level of HK β was somewhat decreased (Figure 3, lanes 3 and 4). Membranes from uninfected Sf9 cells showed no immunoreactivity with HK β C3 (Figure 3, lane 5). The absence from Sf9 cells of full HK β glycosylation, expressed as a spectrum of 60–80 kDa native HK β species (Figure 3, lane 1), did not preclude K⁺-stimulated, SCH 28080-sensitive ATPase activity by Sf9 cell-expressed wild-type or flu-tagged HK $\alpha\beta$ (see below and Table 1).

Flu-tagged HK α and HK β were also detected with mAb HA.11 against YPYDVPDYA epitope inserts at Leu⁸⁴⁴ (α_{ft1}), Pro⁸⁹⁸ (α_{ft2}) and Gly⁶⁷⁷ (α_{ft3}) in HK α , and Lys²⁸⁵ (β_{ft}) in HK β . Immunoblots of Sf9 membranes probed with HA.11 are shown in Figure 3 (lanes 6–10). Membranes derived from Sf9 cells infected with flu-tagged α or $\alpha\beta$ rBVs showed immunoreactivity

at 94 kDa when α subunits were tagged (lanes 6–9), and immunoreactivity at 34, 45 and 49 kDa when β subunits were tagged (lane 10). In contrast with the effect of an HK α flu tag, the presence of an HK β flu tag seemed to retard HK β core peptide glycosylation (Figure 3, lanes 3 and 10). Taken together, these results demonstrate that flu tag insertion did not compromise either the expression of mutated HK α and HK β in Sf9 cells or the immunoreactivity of the flu tag in the Sf9 cell setting.

Functional activity of H,K-ATPase expressed in Sf9 cells

Microsomal membranes from Sf9 cells expressing wild-type or flu-tagged HK α and HK β after infection with rBVs were assayed for K⁺-stimulated, SCH 28080-sensitive ATPase activity. Membrane preparations showed a wide range of H,K-ATPase-like activity; because measured activities were normalized for HK α content, the variability might reflect differences in H,K-ATPase activation in different Sf9 cell compartments (e.g. endoplasmic reticulum compared with plasma membrane), or the effect of the flu tag on the conformation of the H,K-ATPase active site. These results are shown in Table 1, in which specific activity (measured as nmol of inorganic P_i released/h per mg of α subunit) is expressed as the difference in K⁺-stimulated (0.5 mM) ATPase activity in the presence and in the absence of 100 μ M SCH 28080. The K⁺-dependence and SCH 28080-sensitivity of ATPase activity of membranes from Sf9 cells infected with $\alpha_{wt}\beta$ rBVs is shown in Figures 4(A) and 4(B). The apparent K_m for K⁺ (with 5 μ M ATP as substrate) was 0.4 mM, maximal stimulation was at approx. 0.6 mM KCl, and 1 mM KCl was inhibitory. The corresponding results for native pig H,K-ATPase (with 100 μ M ATP as substrate) are a K_m for K⁺ of 0.3 mM, with maximal stimulation at 1 mM KCl [3]. The sensitivity of Sf9 cell-expressed H,K-ATPase to 1 mM KCl is a function of the low (5 μ M) ATP concentration in the Sf9 cell ATPase assay, which is needed to minimize the basal ATPase activity of Sf9 microsomes [29]. SCH 28080 inhibited K⁺-dependent ATPase activity with moderate affinity (Figure 4B), with an apparent K_i of 7 μ M in the presence of 0.5 mM KCl. In contrast, the apparent K_i for SCH 28080 for actively-transporting native pig H,K-ATPase under comparable conditions was 0.4 μ M [3]. The lower potency of SCH 28080 in our assay of Sf9 microsomal ATPase activity reflects buffering of the intramicrosomal potential acid space, which prevents the accumulation of SCH 28080 (a weak base with a pK_a of 5.5). Flu tag insertion into the selected HK α and HK β sites had little effect on sensitivity to SCH 28080; the K⁺-dependent ATPase activity of four Sf9 cell-expressed mutated heterodimers ($\alpha_{ft1}\beta$, $\alpha_{ft2}\beta$, $\alpha_{ft3}\beta$ and $\alpha_{wt}\beta_{ft}$) was inhibited dose-dependently by SCH 28080 (Figures 4C–4F). Taken together, the activity results confirm that Sf9 cell-expressed wild-type and flu-tagged HK $\alpha\beta$ heterodimers display ATPase activity that is stimulated by sub-millimolar K⁺ concentration and is inhibited by low micromolar SCH 28080 concentration. We conclude that HK α and HK β assume heterodimeric topology in Sf9 cell membranes similar to that assumed by native H,K-ATPase in parietal cell apical membranes.

Protease protection assays of Sf9 microsomal HK α and HK β

The membrane topology of inserted flu tags was studied by exposing Sf9 microsomes expressing flu-tagged HK α and HK β to proteinase K digestion, and probing membrane-bound residual peptides resolved by SDS/PAGE with flu tag antibody. The interpretation of protease protection assays of microsomal proteins depends on the orientation of the microsomes. Treat-

Figure 4 Wild-type and flu-tagged H,K-ATPase subunits expressed in Sf9 cells are functionally active

Sf9 cells were infected for 72 h with $\alpha_{wt}\beta$ (A, B), $\alpha_{ft1}\beta$ (C), $\alpha_{ft2}\beta$ (D), $\alpha_{ft3}\beta$ (E) or $\alpha\beta_{ft}$ (F) rBVs, and the specific activity of H,K-ATPase in isolated microsomal membranes was measured as a function of K⁺ (A) and SCH 28080 (B–F) concentration. In (A), specific activity was defined as the difference between K⁺-stimulated ATPase activities in the presence and the absence of 10 μ M SCH 28080. In (B)–(F), H,K-ATPase specific activity (expressed as a percentage of the specific activity in the absence of SCH 28080) was measured in the presence of 0.5 mM KCl. Each data point represents the difference in specific activities in the presence and the absence of the stated SCH 28080 concentration. In all graphs, data points and error bars represent the mean $\pm$ S.E.M. for triplicate activity measurements under each stated condition from at least three independent infections.

ment with proteinase K of Sf9 microsomes expressing $\alpha_{ft3}\beta$ eliminated the 94 kDa flu tag signal (Figure 5A, lanes 1 and 2), indicating the exposure of this flu tag on the outside surface of Sf9 microsomal membranes. However, treatment with proteinase K of Sf9 microsomes expressing $\alpha_{wt}\beta_{ft}$ had no effect on the molecular mass of partly glycosylated HK β (Figure 5A, lanes 4 and 5). Solubilization of microsomes in Triton X-100 eliminated the partly glycosylated protease-protected products (Figure 5A, lane 6), confirming that protection was conferred by the microsomal membrane barrier and not by inherent peptide resistance to proteinase K. These results, as well as proteinase K digestion of a cytoplasmic flu tag at HK α Gly⁶⁷⁷ and retention of a luminal flu tag at the HK β C-terminus, confirm the cytoplasmic-side-out orientation of Sf9 microsomes. Interestingly, proteinase K treatment eliminated the 34 kDa HK β core peptide (Figure 5A, lanes

4 and 5), indicating that the C-terminus of nascent β subunit is initially exposed on the cytoplasmic side of Sf9 microsomes. Treatment with proteinase K of Sf9 microsomes expressing $\alpha_{ft1}\beta$ eliminated the 94 kDa flu tag signal, indicating the cytoplasmic orientation of the Leu⁸⁴⁴ flu tag (Figure 5B, lanes 1 and 2). In contrast, treatment with proteinase K of Sf9 microsomes expressing $\alpha_{ft2}\beta$ resulted in the transfer of the flu tag signal from 94 kDa (Figure 5B, lane 4) to 30, 17.5 and 11 kDa (lane 5), indicating the presence of the α_{ft2} flu tag on three nesting peptides associated with the inside, or extracytoplasmic surface, of Sf9 microsomal membranes. None of the three bands was immuno-reactive with HK α C2 antibody (results not shown). In the presence of Triton X-100, all bands were eliminated by proteinase K (Figure 5B, lane 6). These results place the Pro⁸⁹⁸ flu tag on the luminal face of Sf9 microsomes.

Figure 5 Membrane orientation of flu tags incorporated into HK α and HK β subunits is detected by protease protection assays

Sf9 cells were infected for 72 h with rBVs; isolated microsomal membranes were incubated with proteinase K (pK, 250 μ g/ml) for 1 h in the presence and the absence of Triton X-100 (TX-100). Membrane proteins were separated by SDS/PAGE on 4–20% (w/v) polyacrylamide gradient gels (**A**) or 16% (w/v) polyacrylamide/tricine gels (**B**), then transferred to PVDF and probed with flu tag antibody. (**A**) Lanes 1–3, Sf9 membranes expressing HK $\alpha_{fl3}\beta$; lanes 4–6, Sf9 membranes expressing HK $\alpha_{wt}\beta_{fl}$. (**B**) Lanes 1–3, Sf9 membranes expressing HK $\alpha_{fl1}\beta$; lanes 4–6, Sf9 membranes expressing HK $\alpha_{fl2}\beta$ (50 μ g/ml proteinase K). Molecular mass assignments are shown at right.

Immunolabelling of infected Sf9 cells

As protease protection assays provided evidence for the membrane topology of H,K-ATPase flu tags only in the context of Sf9 microsomes, we sought confirmation of this topology at the cellular level by immunocytochemical labelling of intact and permeabilized Sf9 cells. Figure 6 shows immunomicrographs of Sf9 cells infected for 48 h with $\alpha_{wt}\beta$ rBVs, then labelled with HK α C-terminal-specific antibody (Figures 6a and 6b), and with HK β C-terminal-specific antibody (Figures 6c and 6d). Intact cells were not labelled by HK α C2 (Figure 6a); however, treatment of the cells with saponin resulted in intense cellular labelling with HK α C2 (Figure 6b), indicating the cytoplasmic orientation of the HK α C-terminus, as demonstrated previously [18]. The distribution of HK α C2 label was pan-cellular, with more intense labelling at the cell periphery, which is consistent with the expression of HK α in intracellular membrane compartments and in the plasma membrane. In contrast, intact cells were labelled efficiently by HK β C3 (Figure 6c) and saponin treatment did not yield more intense labelling (Figure 6d), a result consistent with the extracellular exposure of HK β C-terminus [7].

The validity of flu tags as probes of H,K-ATPase membrane

Figure 6 Membrane orientation of wild-type HK α and HK β C-termini is detected by immunocytochemistry

Sf9 cells were infected with HK $\alpha_{wt}\beta$ rBVs for 48 h, then labelled with HK α - and HK β -specific antibodies: (**a**, **c**) without 0.1% saponin; (**b**, **d**) with 0.1% saponin. (**a**, **b**) Labelling with HK α C-terminal antibody (HK α C2); (**c**, **d**) labelling with HK β C-terminal antibody (HK β C3). Scale bars, 10 μ m.

Figure 7 Membrane orientation of flu tag in a cytoplasmic HK α site or a luminal HK β site is detected by immunocytochemistry

Sf9 cells were infected with rBVs for 48 h then labelled with flu tag antibody: (**a**, **c**, **e**) without 0.1% saponin; (**b**, **d**, **f**) with 0.1% saponin. (**a**, **b**) Cells infected with HK $\alpha_{fl3}\beta$; (**c**, **d**) cells infected with HK $\alpha_{wt}\beta_{fl}$; (**e**, **f**) cells infected with HK β_{fl} . Scale bars, 10 μ m.

Figure 8 Membrane orientation of HK α flu tags at Leu⁸⁴⁴ and Pro⁸⁹⁸ is detected by immunocytochemistry

Sf9 cells were infected with rBVs for 48 h then labelled with flu tag antibody: (a, c, e) without 0.1% saponin; (b, d, f) with 0.1% saponin. (a, b) Cells infected with $\alpha_{n3}\beta$; (c, d) cells infected with HK $\alpha_{n1}\beta$; (e, f) cells infected with HK $\alpha_{n2}\beta$. Scale bars, 10 μ m.

topology was assessed by inserting flu tags into a known cytoplasmic region of HK α and into the extracytoplasmic HK β C-terminus. Figure 7 shows immunomicrographs of Sf9 cells infected for 48 h with $\alpha_{n3}\beta$ rBVs (Figures 7a and 7b), with $\alpha_{wt}\beta_{n1}$ rBVs (Figures 7c and 7d) or with β_{n1} rBVs (Figures 7e and 7f) and then immunolabelled with flu tag mAb. Sf9 cells expressing $\alpha_{n3}\beta$ were labelled only when permeabilized by saponin (Figure 7b), indicating the cytoplasmic exposure of Gly⁶⁶⁷. In contrast, intact Sf9 cells expressing $\alpha_{wt}\beta_{n1}$ or β_{n1} alone showed distinct, intense labelling at the cellular periphery (Figures 7c and 7e), indicating the extracytoplasmic exposure of the HK β C-terminus. After permeabilization, most cells still showed surface labelling; in addition, the antibody labelled numerous intracellular punctate sites (Figures 7d and 7f). These results show that Sf9 cells express two subsets of HK β : one processed through the Golgi apparatus and successfully directed to the plasma membrane in the absence of HK α (Figure 7e), and a second detained in intracellular vesicles, perhaps as a result of incomplete glycosylation as shown in immunoblot analyses (Figure 3).

The topology of HK α flu tags at Leu⁸⁴⁴ and Pro⁸⁹⁸ was assessed in experiments shown in Figure 8. Sf9 cells were infected for 48 h with $\alpha_{n1}\beta$ rBVs (Figures 8a and 8b), α_{n2} alone (Figures 8c and 8d) or $\alpha_{n2}\beta$ (Figures 8e and 8f), and then immunolabelled with the flu tag mAb. Without saponin, cells expressing the HK α Leu⁸⁴⁴ flu tag were unlabelled (Figure 8a). Treatment with saponin revealed intense cellular and peripheral flu tag

Table 2 MALDI-MS of gastric H,K-ATPase tryptic peptides

Measured mass (atomic mass units)	Predicted HK α sequence
2338	Ala ⁴ –Lys ²⁵
1056	Gly ²⁰⁵ –Arg ²¹⁴
1094	Asp ⁵¹⁰ –Lys ⁵¹⁸
1375	Asp ⁶⁸² –Arg ⁶⁹³
3867	Ala ⁷³⁸ –Arg ⁷⁷⁶
1662	Ala ⁸³⁷ –Lys ⁸⁵⁰
2807	Phe ⁹⁹⁶ –Lys ¹⁰¹⁷

fluorescence (Figure 8b), a result consistent with protease protection assays showing extravesicular topology of this flu tag, showing the cytoplasmic exposure of Leu⁸⁴⁴. Cells expressing α_{n2} alone gave similar results: no labelling in the absence of saponin (Figure 8c), and intracellular and peripheral labelling after permeabilization (Figure 8d). In contrast, when α_{n2} was co-expressed with HK β , approx. 15% of cells were labelled by HA.11 before permeabilization (Figure 8e); after permeabilization virtually all of the cells were labelled (Figure 8f). These results confirm the extracellular exposure of HK α Pro⁸⁹⁸ deduced from protease protection assays, and indicate that the co-expression and interaction of HK β with HK α is necessary for the extracellular expression of this region of HK α . The relatively infrequent labelling of intact cells expressing $\alpha_{n2}\beta$ might reflect extracellular epitope masking by HK β or the proximity of Pro⁸⁹⁸ to the bilayer. These interpretations are consistent with the observed increase in labelling frequency after permeabilization with saponin, which might improve antibody access to the epitope by disturbing bilayer structure and affecting HK α and HK β interactions.

Further information on HK α topology was acquired from MALDI-MS analysis of the 100 000 g supernatant of native H,K-ATPase microsomal vesicles subjected to limited hydrolysis with trypsin. Seven peaks in the mass spectrum (results not shown) could be identified reproducibly as being HK α -derived (Table 2) on the basis of the correlation of peak mass with the known primary structure and tryptic cleavage sites of pig gastric H,K-ATPase. Two of the peptides, Ala⁸³⁷–Lys⁸⁵⁰ and Phe⁹⁹⁶–Lys¹⁰¹⁷, originate in the C-terminal one-third of HK α . Because the H,K-ATPase-enriched gastric microsomes used in this assay are oriented cytoplasmic side out [17], this result not only verifies the cytoplasmic exposure of Leu⁸⁴⁴ but also places a significant constraint on the location of the putative H9/H10 Tm domain.

DISCUSSION

The C-terminal domain of P-type ATPase α subunits is active in cation binding and occlusion [21,29], conformational changes [5] and interactions with the β subunit [16,30]; topology determination is therefore crucial to understanding the structural basis of these functions. Differing interpretations of hydropathy plots, and several lines of evidence from chemical modification, translation *in vitro* and immunochemical studies, have led to the formulation of secondary structure models of HK α that predict either eight or ten Tm domains (Figure 1). To resolve these two models, we used epitope insertion to establish the membrane topology of Leu⁸⁴⁴, Pro⁸⁹⁸ and Phe⁹⁹⁶ in the C-terminal region of HK α . Epitope tags have not previously been applied to the H,K-

Figure 9 Proposed model of secondary structure in the C-terminal one-third of pig HK α

ATPase; in the closely related Na,K-ATPase α subunit, the topology of several residues has been deduced by epitope insertion [14,15,31].

Our choice of the three HK α residues as topological markers was driven by differing predictions of their membrane sidedness in the two models, and by their location in hydrophilic sequences flanked by putative Tm domains, indicating probable exposure on either the cytoplasmic or the luminal face of secretory membranes. Clearly, membrane sidedness of flu tags on heterologously expressed HK α and HK β is a relevant topological tool only if the subunits acquire membrane topologies reflective of native folding of HK $\alpha\beta$ heterodimers in parietal cell apical membranes. In this study, the criterion for such neo-native folding was the demonstration of K-stimulated, SCH 28080-sensitive ATPase activity in Sf9 membrane fractions.

The insect cell expression system, in which wild-type or mutated cDNA species are expressed after cell infection with rBVs, has specific advantages for the study of membrane protein topology. These include comparatively high levels of expression, at least partial glycosylation of expressed proteins, targeting of these proteins to intracellular and cell-surface membrane compartments, and the capacity for the concurrent expression of two or more polypeptides under independent promoter control [20]. Above all, the insect cell system permits the functional expression of multimeric membrane proteins. Interactions between the α and β subunits of Na,K-ATPase, and the kinetic properties of Na,K-ATPase isoenzymes, have been studied in insect cells, with heterodimer function measured by ouabain-sensitive ATPase activity (reviewed in [32]). In addition, the expression of wild-type [22,33,34] and mutated [29] rat gastric H,K-ATPase has been studied in insect cells, with the functional capacity of HK $\alpha\beta$ heterodimers being assessed by SCH 28080-sensitive phosphorylation of the HK α .

In this study, infection of Sf9 cells with rBVs encoding HK α and HK β cDNA species resulted in the efficient expression of

both subunits. The fact that flu-tagged HK $\alpha\beta$ retained catalytic activity indicates that heterodimers acquired neo-native conformation in the foreign milieu of Sf9 intracellular and plasma membranes. In addition, tryptic hydrolysis of Sf9 membranes expressing HK $\alpha\beta$ subunits yielded the same patterns of epitopic peptides as those of native H,K-ATPase, providing further evidence of neo-native conformation [35]. Therefore HK $\alpha\beta$ flu tag immunoreactivity with respect to Sf9 cell membranes permits topological deductions to be made about the native H,K-ATPase α subunit. Flu tag topology mapping was validated by α_{113} tag labelling (at cytoplasmic Gly⁶⁷⁷) only in permeabilized Sf9 cells and by β_{11} tag labelling (at the extracellular HK β C-terminus) of intact Sf9 cells. The latter observation is also direct evidence for the targeting of HK β to Sf9 cell plasma membranes. Because Sf9 membranes showed H,K-ATPase activity (which requires HK $\alpha\beta$ heterodimers), at least some HK α moieties were also targeted to Sf9 cell plasma membranes, although it is possible that all H,K-ATPase activity was associated with HK $\alpha\beta$ heterodimers trapped in Sf9 intracellular membrane vesicles, much like native H,K-ATPase localized in cytoplasmic tubulovesicles in gastric parietal cells [25].

The significant findings of this study were: (1) the HK α_{113} flu tag was digested by proteinase K in cytoplasmic-side-out Sf9 microsomes and immunolabelled only in permeabilized Sf9 cells, which is consistent with a cytoplasmic topology for Leu⁸⁴⁴; (2) the HK α_{113} flu tag was protected from proteinase K digestion in cytoplasmic-side-out Sf9 microsomes and was immunolabelled in intact Sf9 cells, which is consistent with a luminal topology for Pro⁸⁹⁸; (3) the α_{112} protease-protected epitopic peptide map (Figure 5B) defines the length of the H7 and H8 Tm domains and their extracellular loop; and (4) MALDI-MS data placed Phe⁹⁹⁶ on the cytoplasmic face of the membrane. Clearly, these findings place constraints on the topology of neighbouring domains of HK α ; the revised six-Tm model of HK α C-terminal secondary structure shown in Figure 9 accommodates these constraints.

The placement in this revised model of Tm domains H7 to H10 was deduced from protease protection assays of Sf9 membranes expressing the Pro⁸⁹⁸ flu tag (α_{n2}) and from mass spectrometric detection of a cytoplasmic tryptic peptide extending from Phe⁹⁹⁶ to Lys¹⁰¹⁷. Although Leu⁸⁴⁴ and Pro⁸⁹⁸ are separated by 54 residues, enough for two Tm domains, their assignment to opposite sides of the membrane allows only one Tm domain (H7) between them, most probably extending from Pro⁸⁵⁸ to Ala⁸⁸⁴. Digestion with proteinase K would be impeded at Pro⁸⁵⁸, providing a common N-terminal residue for the three protected epitopic peptides shown in Figure 5(B). Including the nine-residue flu tag, the 17.5 kDa peptide thus extends to approx. residue 1006, which is consistent both with its non-reactivity with HK α C-terminal antibody and with at least 28 residues being exposed cytoplasmically at the HK α C-terminus. The 11 kDa peptide extends to approx. residue 947, the limit of cytoplasmic protease digestion from the C-terminal end of the peptide, and therefore represents the H7 Tm domain, an extra-cytoplasmic loop of 41 residues from Gln⁸⁸⁵ to Gln⁹²⁵, and the H8 Tm domain from residues 926 to 946.

Although intact Sf 9 cells expressing the Pro⁸⁹⁸ flu tag were labelled with flu tag antibody, relatively few cells were immunoreactive (Figure 8e). We interpret this finding in terms of the Pro⁸⁹⁸ flu tag's lying close to the membrane surface, possibly occluded by HK β . Such a juxtamembranous location of amino acids in this region of P-type ATPases is not without precedent. On the basis of site-directed mutagenic replacement effects on Na⁺ transport kinetics, four amino acids (Asp⁸⁸⁴-Asp⁸⁸⁵-Arg⁸⁸⁶-Trp⁸⁸⁷) in an extracellular loop of rat Na,K-ATPase α subunit between Tm domains H7 and H8 were proposed to participate in Na⁺ pumping [36]. Furthermore, the inability of one of the mutants to bind ouabain was explained by invoking partitioning of the extracellular loop into the plane of the membrane, positioning these residues to accept and/or release Na⁺ ions originating in the cytoplasm. In voltage-dependent Na⁺ and K⁺ channels, pore-forming loops formed by hydrophilic extracellular domains of channel subunits are thought to invaginate into the membrane bilayer, where they are stabilized by subunit Tm domains [37]. The same structural motif has been invoked for the 'hour-glass' membrane topology of aquaporin [38]. In this context, it is interesting that the equivalent four amino acid sequence in HK α is Glu⁹⁰¹-Asn⁹⁰²-His⁹⁰³-His⁹⁰⁴. In that H₃O⁺ is the H,K-ATPase-transported equivalent of Na⁺ in the Na,K-ATPase [39], the pair of corresponding histidine residues in the HK α sequence, if partitioned into the bilayer, is clearly well-positioned to act as an acceptor/donor pair for hydrated protons in the H,K-ATPase catalytic cycle.

A luminal orientation for Pro⁸⁹⁸ is compatible with recent evidence for the extracellular topology of residues immediately C-terminal to Pro⁸⁹⁸. A detergent-solubilized pig H,K-ATPase α subunit tryptic peptide retained on a wheatgerm agglutinin column by virtue of the affinity of HK β for wheatgerm agglutinin was identified as extending from Leu⁸⁵⁵ to Arg⁹²² [30]. Subsequent yeast two-hybrid analysis of putatively interacting rabbit HK α and HK β domains identified an HK α -binding sequence (Arg⁸⁹⁸ to Arg⁹²²), and two different HK β -binding sequences, both in the putative extracellular domain of HK β [30]. These results place rabbit HK α Arg⁸⁹⁸ to Arg⁹²² on the luminal side of the secretory membrane. Corroborating evidence comes from mechanistic studies of the inhibition of H,K-ATPase by omeprazole [40]. Omeprazole-derived membrane-impermeant cationic sulphenamides covalently label luminally oriented thiols, making the reagent a useful probe of HK α topology. The inhibitory and binding capacities of four cationic sulphenamides were measured in vesicular pig H,K-ATPase under active

acid-transporting conditions [40]. Inhibition was correlated with initial sulphenamide binding to Cys⁸¹³; after longer incubation, three of the sulphenamides also bound to Cys⁸⁹³ and the fourth (pantoprazole) showed no labelling of Cys⁸⁹³. The results are consistent with a luminally oriented Cys⁸⁹³ relatively inaccessible to cationic sulphenamides compared with Cys⁸¹³. Delayed labelling of Cys⁸⁹³ might indicate that conformational changes induced by Cys⁸¹³ labelling eventually allow cationic sulphenamides access to hitherto-occluded Cys⁸⁹³.

In a previous study, HK α residues 888–907 were proposed to encompass the epitope for the cytoplasmically immunoreactive mAb HK 12.18 [10]. In that study, a 20-mer synthetic peptide based on residues 888–907 was found to bind to HK 12.18 by direct ELISA, to displace the antibody from microsomal H,K-ATPase in competitive ELISAs, and to block the immunostaining of gastric parietal cells. The results were interpreted as showing the cytoplasmic exposure of residues 888–907, an untenable conclusion given the present results assigning Pro⁸⁹⁸ to the luminal side of the secretory membrane. An alternative primary HK 12.18 epitope has been proposed more recently on the basis of the expression of rabbit HK α cDNA restriction fragments in *E. coli*, although other results in the same study indicated a secondary HK 12.18 epitope located between residues 853 and 946 [26].

In the revised secondary structure model (Figure 9), our assignment to the cytoplasm of HK α residues Arg⁹⁴⁷-Lys⁹⁴⁸-Thr⁹⁴⁹-Arg⁹⁵⁰-Arg⁹⁵¹ and of Arg⁹⁹⁵ conforms to the 'positive inside rule', based on the prevalence of positively charged residues in the cytoplasmic domains of membrane proteins [41]. A more recent statistical analysis of 37 complex multispanning membrane proteins also showed a high probability of cytoplasmic exposure of Arg and Lys [42]. Nevertheless, despite the presence of eight tryptic cleavage sites C-terminal to Lys⁹⁵⁰, our mass spectrometric analysis of tryptic peptides released from cytoplasmic-side-out microsomes enriched in native H,K-ATPase revealed only one peptide (with the predicted sequence Phe⁹⁹⁶-Lys¹⁰¹⁷). These considerations impose a final topological constraint on the C-terminal region of HK α ; the 43 residues between Arg⁹⁵¹ and Arg⁹⁹⁵ constitute a Tm helical pair (H9 and H10 Tm domains), with the remaining residues (Arg⁹⁵¹-Tyr¹⁰³³) representing the HK α cytoplasmic C-terminus.

In summary, this study in a functional H,K-ATPase model establishes the cytoplasmic orientation of HK α Leu⁸⁴⁴ and Phe⁹⁹⁶, and the luminal orientation of HK α Pro⁸⁹⁸, placing constraints on possible models of the secondary structure of HK α . The results are most consistent with a six-Tm-domain organization in the C-terminal one-third of H,K-ATPase α subunit. The revised model increases the structural resolution in this functionally significant domain of the enzyme, and presents further hypotheses of topology that are testable by epitope mapping.

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