Biochimica et Biophysica Acta, 1149 (1993) 151–165

BBAMEM 76003

Antibody epitope mapping of the gastric H^+/K^+-ATPase

Frederic Mercier [a,b], Denis Bayle [c], Marie Besancon [a,b], Ted Joys [d], Jai Moo Shin [a,b], Miguel J.M. Lewin [c], Christian Prinz [a,b], Michael A. Reuben [a,b], Annik Soumarmon [b], Helen Wong [a,b], John H. Walsh [a,b] and George Sachs [a,b]

[a] *Department of Physiology, UCLA and Wadsworth VA, Los Angeles, CA (USA),* [b] *Department of Medicine, UCLA and Wadsworth VA, Los Angeles, CA (USA),* [c] *U 10, Inserm, Hôpital Bichat, Paris (France) and* [d] *Texas Tech University Health Science Center, School of Medicine, Lubbock, TX (USA)*

(Received 22 December 1992)

Key words: ATPase, H^+/K^+-; Acid secretion; Epitope; Topology; Baculovirus

Several antibodies against the gastric H^+/K^+-ATPase were analysed for the topological and sequence location of their epitopes. Topological mapping was done by comparing indirect immunofluorescent staining in intact and permeabilised rat parietal cells. Epitope definition was by Western analysis of intact and of trypsin or V8-proteinase-fragmented hog gastric ATPase combined with N-terminal sequencing of the fragments; by Western analysis of fragments of rabbit α subunit expressed in *Escherichia coli*; by analysis of rabbit α and β subunits expressed in baculovirus-transfected SF 9 cells and by ELISA assay of synthetic octamers of one region of the hog α subunit. It was confirmed that the monoclonal antibody, mAb 95–111, recognised a cytoplasmic region between M4 and M5, close to the ATP-binding domain. The major epitope for monoclonal antibody mAb 12–18 was also in this region, but a second epitope was confirmed to be present in the M7/M8 region. The monoclonal antibody, mAb 146–14, was shown to recognise an extracytoplasmic epitope dependent on intact disulfide bonds, present in the rat and the rabbit, but absent in the hog β subunit, due to non-conservative amino-acid substitutions. This antibody also recognised an epitope present in the α subunit of the H^+/K^+-ATPase at the M7 extracytoplasmic interface, perhaps indicating structural association of these two regions. The polyclonal antibody, pAb39, raised against the C-terminal portion of the enzyme, reacted only with the cytoplasmic surface of the H^+/K^+-ATPase, showing that the α subunit of the enzyme has an even number of membrane spanning segments.

Introduction

Ion pumps of the phosphorylating type, such as the Na^+/K^+-ATPase, the Ca-ATPases and the H^+/K^+-ATPase all contain a large catalytic or α subunit composed of about 1030 amino acids. The Na^+/K^+-ATPase and the H^+/K^+-ATPase also have a smaller β subunit of about 290 amino acids that is glycosylated on its extracytoplasmic face. Analysis of the membrane topology of these enzymes depends on sites of chemical labelling, hydropathy predictions for membrane spanning α helices, as well as on antibody mapping and mutagenesis of functional sites. Comparing amino-acid sequences of the larger, catalytic, subunit shows 75% homology between the H^+/K^+-ATPase and the Na^+/K^+-ATPase and 15% homology between the H^+/K^+-ATPase and the sr Ca-ATPase, but a close similarity in hydropathy profile for all three enzymes. These data are covered in recent reviews on these enzymes [1–3].

The H^+/K^+-ATPase, the gastric-acid pump, can be isolated, like the Ca-ATPase of sarcoplasmic reticulum, in the form of intact, cytoplasmic side out vesicles. Separation and N-terminal sequencing of membrane embedded tryptic peptides and determination of the sites of labelling of the H^+/K^+-ATPase by extracytoplasmic inhibitors provided direct biochemical evidence for eight membrane spanning segments, four in the first and four in the last third of the α subunit of those enzyme [4,5]. The first four correspond to the hydropathy prediction, the last four do not, although similar to four of the segments in this region predicted for the Ca-ATPase [6]. Although no biochemical evidence was found for the additional pair of membrane segments predicted by hydropathy in the last 70 amino acids, the total number of membrane spanning α helices may be 10 as suggested for the Ca-ATPase [6] rather than the eight of the H^+/K^+-ATPase for which there is currently physical evidence. Most of the β subunit is extracytoplasmic and the six cysteines in the

Coorespondence to: G. Sachs, Department of Physiology, UCLA and Wadsworth VA, Building 113, Room 324, Los Angeles, CA 90073, USA.

extracytoplasmic domain are disulfide-linked, as for the β subunit of the Na^+/K^+-ATPase [7,8].

As part of the determination of the topology of the H^+/K^+-ATPase, mapping of antibody epitopes should be a useful adjunct to biochemical methods. For example antibodies were used to determine the sidedness of the M7/M8 loop of the Ca-ATPase [9,10] and the C-terminal segment of the Na^+/K^+-ATPase [11], which had been a controversial issue.

Two types of antibody against the H^+/K^+-ATPase were used in this study. One was a rabbit polyclonal antibody generated against a synthetic peptide fragment corresponding to positions 1011–1020 at the C-terminal sequence (pAb39) of the α subunit. The others were monoclonals. They had been generated either against the separated α subunit (mAb 12–18) [12], hog gastric membranes (mAb 95–111) [27] or against intact rat parietal cells (mAb 146–14) [14]. All of the monoclonals had been shown to recognise the ATPase in parietal cells, but had been developed before the presence of a β subunit was recognised. Only mAb 95–111 is inhibitory to the H^+/K^+-ATPase [15].

We determined sidedness of the antibody epitopes by indirect immunofluorescent reaction with intact or detergent-treated parietal cells. The epitopes of the monoclonal antibodies were assigned to either the α or β subunit by Western blotting of native or baculovirus expressed enzyme subunits. The location of the epitopes in the primary sequence was determined by Western blotting of fragments of the α subunit generated by enzymic digestion or by expression of cDNA restriction fragments of the α subunit of the enzyme in *Escherichia coli*. For one antibody we also used ELISA reaction with sequentially synthesised octamers to define one of its epitopes [16].

The epitope for antibody mAb 95–111 was shown to have a cytoplasmic location, and to be in the same region defined by Western blotting of fragments expressed in *E. coli* [13]. The monoclonal antibody mAb 12–18 appeared to recognise a region of the α subunit, in the cytoplasmic region between M4 and M5 as shown by Western blotting of V8 proteinase fragments and of fragments expressed in *E. coli* but an additional site was found in the C-terminal one-third of the enzyme in accordance with previously published synthetic peptide displacement experiments [12]. The monoclonal antibody, mAb 146–14, which had been shown to label the extracytoplasmic domain of the enzyme in parietal cells [14,17], was shown to recognise primarily the β subunits of rat and of rabbit but not the β subunit of hog enzyme. Reaction was inhibited by dithiothreitol treatment. In addition, this antibody recognised an epitope in the α subunit in all species after SDS treatment. The polyclonal antibody raised against the C-terminal moiety of the H^+/K^+-ATPase reacted exclusively with the cytoplasmic surface of the H^+/K^+-ATPase, showing that there was an even number of membrane-spanning segments in the α subunit of the acid pump.

Materials and Methods

General methods

Preparation of gastric vesicles. Gastric vesicles from hog gastric mucosa were prepared as previously described by differential and density gradient centrifugation [18]. The fraction floating on top of 7.5% Ficoll, 0.25 M sucrose (G1) was used in these experiments. It has been shown that these vesicles are oriented > 95% with the cytoplasmic surface facing out [18]. For rabbit and rat gastric membranes, a post-10 000 $\times g$, microsomal 100 000 $\times g$ pellet was used.

Enzyme digestion of gastric vesicles. This was done as described elsewhere [4,5] using either tryptic digestion of intact vesicles or V8 proteinase digestion of isolated solubilised α subunit [4], and is briefly described.

I: Tryptic digestion in the presence of ligands. H^+/K^+-ATPase (approx. 2 mg/ml) was treated with Sigma type XIII trypsin (17 700 U/mg) at a trypsin-to-protein ratio of 1:40. The incubation was carried out at 37°C for 30 min in the presence of 250 mM sucrose, 50 mM Tris-HCl (pH 6.8), 0.2 mM Na_2ATP, 25 μM SCH28080 and 1 mM EGTA. The reaction was stopped by the addition of trypsin inhibitor (8-fold excess over trypsin) the membranes were centrifuged and resuspended to their initial concentration [4]. The membranes were dissolved in sample buffer as detailed below for electrophoresis. This method provides two large fragments of the enzyme of 68 and 33 kDa, the former beginning at position 47 in the primary sequence, the latter at position 671. The β subunit is not visibly hydrolysed under these conditions. This allows Western blot analysis of the general region of the epitopes in terms of the first two-thirds or the last one-third of the enzyme.

II: Tryptic digestion in the absence of ligands. The-ATPase (approx. 2 mg/ml) was digested for 10 min by trypsin as above but in the absence of ATP or SCH28080, at a ratio trypsin-to-protein of 1:40. After digestion, iodoacetamide (100 mM) was added and the membranes freed of trypsin and soluble peptide fragments by centrifugation for 8 min in an airfuge at 20 psi. The membranes were then dissolved in 0.4% CHAPS in the continuing presence of iodoacetamide, delipidated by precipitation with 2 volumes methanol on ice, and the pellet dissolved in SDS as below.

III: V8 Proteinase digest. As previously described [5], FPLC was used to separate the α and the β subunits in the solubilised enzyme, and then the α subunit was digested, followed by SDS gel separation and sequencing. 3 mg of protein were dissolved in 2% SDS and loaded on a FPLC superose-6 column. The α subunit was eluted with 50 mM Tris-HCl (pH 7.8), 0.05% SDS

at 0.2 ml per min and concentrated in a speed vacuum lyophiliser. 600 μg of the subunit was digested by 40 μg V8 proteinase at 37°C for 4 h, methanol was added (2:1) and the suspension precipitated by centrifugation. The pellet was resuspended in 200 μl 20 mM Tris-HCl (pH 7), solubilised in electrophoresis sample buffer and separated on a tricine gradient gel as below. Protein measurements were made using the Lowry method [19].

SDS gel electrophoresis.

I: Tricine gradient gels. The suspended membranes or methanol precipitate of the membrane residue were combined with 20% volume of sample buffer, (0.3 M Tris, 10% SDS, 30% sucrose and 0.025% Bromophenol blue) and the solution placed on top of a 10% (34:1 acrylamide/methylene bisacrylamide) to 21% (17:1 acrylamide/methylene bisacrylamide) 1.5 mm gradient slab gel using the tricine buffer method of Schagger and von Jagow [20]. Gels were run in the cold room (4°C) for 18 h at 90 V constant voltage, along with a lane for prestained (Sigma 106–18 kDa) molecular mass standards and/or CNBr fragments of horse myoglobin (Sigma, 17–2.5 kDa). In every case duplicate lanes were run to provide sufficient material for Western analysis and for simultaneous sequencing.

Standard curves of ln (molecular mass) as a function of relative mobility were used to estimate the molecular mass of the peptide products of digestion. The accuracy of the molecular mass determination appeared to be within 10%, based on predicted tryptic or V8 proteinase cleavage sites within the primary sequence of catalytic subunit of the enzyme. The peptides were transferred electrophoretically to PVDF membranes (Millipore) for 18–24 h in the cold room (4°C) in a tank transfer apparatus at 120 mA constant current, in a transfer buffer of 0.15 M glycine, 0.02 M Tris and 20% methanol. A sandwich of three sheets of Whatman 3 mm filter paper was placed on either side of the gel which had a pre-wetted PVDF membrane on the anode side. After transfer, the blots were rinsed twice in distilled water and stained with Coomassie blue in 10% glacial acetic acid and 45% methanol. Figs. 3, 4 and 6 represent this method of separation.

II: Laemmli gels. For the experiments where sequencing was unnecessary (Figs. 8 and 10) standard Laemmli gels were run using 10% acrylamide as the separation gel [21].

Sequencing. Stained bands were sequenced using a gas-phase sequencer at the UCLA microsequencing facility using the Applied Biosystems 475A system consisting of a 470A sequencer, a 120A phenylhydantoin analyser and a 900A data module. The quantity of a given peptide was obtained by averaging the elevation above background for the amino acid in each cycle above background for cycles 2 to 7. For unambiguous identification, it was necessary to load between 80 to 100 μg protein per lane, and to sequence from two lanes [4,5].

Antibodies. The production and characterisation of the monoclonal antibody 146–14 (mAb 146–14) has been described elsewhere [14,17]. The extracytoplasmic location of its epitope had been demonstrated by post embedding immunogold labelling on ultra-thin frozen sections of gastric tissue using an anti-actin antibody as a control. The monoclonal antibody mAb 12–18 (mAb 12–18) has also been described previously and was supplied by Dr. A. Smolka [12]. Antibody mAb 95–111 was prepared as previously described [13,15,27].

The polyclonal antibody 39 (pAb39) was produced in rabbits against the synthetic oligopeptide YDEIRKLGVR which corresponds to the sequence 1011–1020 of the H^+/K^+-ATPase α subunit [22].

The peptide was synthesised and purified by Dr. Joe Reeves at UCLA/CURE. It was conjugated to Keyhole Limpet hemocyanin and the conjugate was used to immunise rabbits as an emulsion with equal parts of Freund's adjuvant. The rabbit IgG was purified over a protein A sepharose CL-4B column by elution with acetic acid. The titre of pAb39, as determined by liquid phase radioimmunoassay using iodinated peptide, was 1:20000. The ID_{50} for this antibody at this dilution was 100 pM. In data not shown, this antibody reacts on Western blots exclusively with the α subunit of the H^+/K^+-ATPase, and its epitope was rapidly destroyed by trypsin digestion.

The polyclonal pAb HC was a gift from B. Lupo (Centre CNRS-INSERM de Pharmacologie, Montpellier, France) and is a immunoaffinity-purified polyclonal antibody raised against a synthetic peptide representing the sequence between positions 497 and 520 of the α subunit. Antibody mAb β which is an anti β antibody was a generous gift of Dr. Matsuda, Japan.

Epitope mapping

Western blot analysis. For immunoblotting, the Protoblot Western blot AP System (Promega) was used with antibodies mAb 146–14 (5 μg/ml), mAb 12–18 (3 μg/ml) and mAb 95–111 (2 μg/ml) and with pAb39 (at a dilution of 1:500) and pAb HC.(dilution 1:50) as first antibodies, alkaline-phosphatase-conjugated anti-rabbit or anti-mouse IgG (both 1:7000 dilution) as secondary antibodies and Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate for staining of the bound secondary antibody.

One duplicate lane of the electrotransferred Coomassie stained PVDF lanes was used for microsequencing and the other was used for Western analysis. This lane was destained in 70% methanol/10% glacial acetic acid and then rinsed in TBS T20 (Tris buffer saline Tween-20). Non-specific sites were blocked by incubation for 15 min in presence of TBS T20 and 1% BSA. Incubations were performed with the different

primary antibodies for 45 min followed by three washes in TBS T20 before additional incubation for 45 min with the secondary antibodies. After three washes in TBS T20, the substrates for the conjugated alkaline phosphatase were added and the staining stopped after 5 min by several washes with water. It was necessary to carry out a series of timed incubations for each antibody to determine conditions of specific staining, since it was found that this was critical in obtaining low backgrounds on the PVDF membranes.

Epitope mapping using overlapping synthetic octamers. A chemically-based method of mapping linear epitopes is the Geysen technique [16], in which overlapping octapeptides corresponding to the protein sequence are synthesized as immobilised epitopes on polypropylene pegs (Cambridge Research Biochemicals, Norwich, UK) and antigenic activity of the immobilised octamers for a given antibody measured by ELISA.

The ELISA reactions on the pegs were carried out with the antibody mAb 146–14, as for Western analysis. The sequence 853–902 was selected for analysis based on the likely location of the epitope for mAb 146–14, derived from sequence data. This region was analysed by synthesising a series of overlapping octamers in which the first peg had the peptide 853–860, the second 854–861 and so forth until the last octamer synthesised was 895–902.

Epitope mapping of antibodies by expression in E. coli of H^+/K^+-ATPase cDNA restriction fragments. The H^+/K^+-ATPase α subunit of rabbit cDNA was fragmented by different restriction enzymes to allow expression of selected regions of the protein. The separated fragments were ligated into the PuEx plasmids; transformed bacteria were grown and fusion proteins expressed as previously detailed [13]. The bacteria were pelleted, lysed in SDS and loaded on to 10% Laemmli polyacrylamide gels ($3 \cdot 10^6$ bacteria per track).

Detection of antibody binding. After transfer of proteins to nitrocellulose by electrotransfer, the reactivity of mAbs 95–111, 12–18 and 146–14 was determined by Western blotting. Incubations were performed using mAb 12–18 (ascites fluid, 1:6000 dilution) [^{125}I]mAb 95–111 (100000 cpm/ml) and mAb 146–14 (2 μg/ml) in phosphate-buffered saline, 0.2% Tween-20 (pH 8.0) for 2 h at room temperature. For non-radioactive antibodies, development of color was performed by incubating with anti-mouse antibodies at a dilution of 1:7000 as described above. For the radioactive antibodies used in the cDNA method, autoradiography of the dried gel was performed after 4 rinses, following incubation with the iodinated antibody [13].

Mapping using expressed subunits in SF9 cells.

I: Transfection of baculovirus with α or β H^+/K^+-ATPase rabbit cDNA. Rabbit stomach cDNA clones for the α [23] and β [7] subunits were subcloned into the baculovirus transfer plasmid pVL1393 (Invitrogen) the α clone H^+/K^+ α32 and the β clone H^+/K^+ β49–10 were prepared as *Eco*RI cut inserts and separately ligated into *Eco*RI cut pVL1393. Selected constructs for each subunit were confirmed to be in proper orientation by restriction mapping [24]. The baculovirus pVL1393 H^+/K^+-ATPase α or β constructs were separately transfected into *Spodoptera frugiperda* (SF9) insect cells along with isolated wild-type baculovirus DNA using standard calcium phosphate precipitation. Virus stock was harvested from 25-cm^2 tissue culture flasks after 48 h of infection. Dilutions of viral stock were used to plate out on to a lawn of SF9 cells and areas showing non-occluded plaques were selected and rescreened until plaque pure.

II: Preparation of baculovirus-expressed protein. SF9 cells were cultured in EX-CELL 401 + L-glutamine (JRH Biosciences) with 10% fetal calf serum and 1% penicillin/streptomycin and infected with α or β subunit containing vectors. Infected cells were incubated at 27°C in spinner flasks for 72 h before harvesting by centrifugation at 2000 rpm for 5 min. The cells in the pellet were then used for immunofluorescence after permeabilisation by Triton X-100, or homogenised as below for membrane preparation and Western blotting after SDS gel separation.

III: Preparation of membranes from SF9 cells. SF9 cells were resuspended in 5 mM Pipes-Tris (pH 6.8), 0.5 mM PMSF, 1 mM EGTA before being disrupted by homogenisation in a Teflon glass homogeniser at 100 rpm. The homogenate was centrifuged for 10 min at $1000 \times g$, the supernatant was saved, and the pellet was resuspended in the same buffer supplemented with 5 mM $MgCl_2$ and homogenised again. After another centrifugation for 3 min at $1000 \times g$, the supernatant was added to the previous one and both were sedimented at $100000 \times g$ for 30 min. This pellet was separated on Laemmli gels as above, with or without dithiothreitol (DTT).

Topology of epitopes

This was determined using intact or permeabilised parietal cells.

Preparation of parietal cells. This was done as has been described in detail elsewhere [25]. Briefly, a cell suspension was generated from rat stomach using the everted sac method with pronase digestion. The crude cell suspension was then fractionated by elutriation in an elutriator rotor (JE-6, Beckman Instruments). This procedure separates cells on the basis of varying sedimentation velocities, which are proportional to the square of the radius of different cell types. Since parietal cells are the largest cell found in fundic epithelium the method was found to produce a highly enriched preparation of this cell type known to be the only one to contain the H^+/K^+-ATPase. The rotor was run in a

J-21C centrifuge (Beckman Instruments). A Masterflex pump (No. 7568) was used to load the crude cell suspension at defined flow rates into the separation chamber (standard chamber). At a flow rate of 18 ml/min and a rotor velocity of 2500 rpm, $2 \cdot 10^8$ cells were loaded into the elutriator. After washing with 100 ml medium (20 ml/min, 2400 rpm), small and medium sized cells were collected at 40 ml/min and a speed of 2000 rpm and were discarded. At 60 ml/min and 2000 rpm, a cell fraction having an average cell size of more than 16 μ, the parietal cell rich fraction, was collected. The cell yield in this fraction was 1.8×10^8 cells per preparation and consisted of $> 90\%$ parietal cells with 90% Trypan blue exclusion.

Sidedness of epitopes in parietal cells. Determination of the sidedness of an epitope that is part of a plasma membrane enzyme is often done by comparing immunofluorescence in intact and detergent permeabilised cells. Although the H^+/K^+-ATPase of the parietal cell is an apical plasma membrane enzyme, it is located in cytoplasmic membranous vesicles in the resting state and in microvilli of an infolding of the apical membrane, the secretory canaliculus, during secretion [26]. Therefore, independent of epitope location, permeabilisation invariably enhances or changes distribution of fluorescence, since more enzyme becomes accessible to the antibody. We found that it was essential to carry out initial reaction in cells which were prepared fresh and not fixed. Fixation was only carried out prior to the secondary antibody step. Further, only if there were reaction at this step, could we conclude that the epitope was extracytoplasmic and only if there were essentially no reaction in the absence of detergent could we conclude that the epitope was cytoplasmic, given the specialised location of the H^+/K^+-ATPase.

Isolated rat gastric cells were attached directly on to microscope slides coated with Probe-On (Fisher Biotech) and processed for indirect immunofluorescence microscopy without any fixation in order to preserve membrane integrity. The cells were washed with phosphate-buffered saline and the primary antibodies diluted in phosphate-buffered saline containing 0.2% gelatin were incubated for 1 h with the cells on

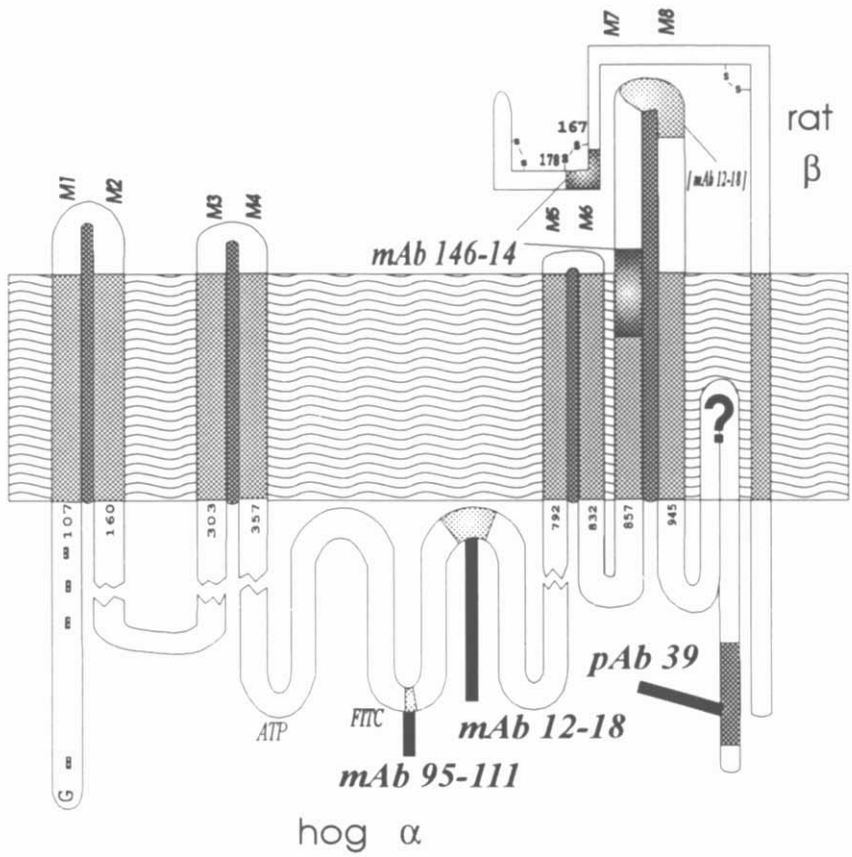


Fig. 1. A working model of the membrane topology of the α subunit of the hog H^+/K^+-ATPase, established by biochemical methods [4]. The last hydrophobic region has not been assigned a transmembrane topology based on biochemical analysis, but may well be containing a transmembrane segment pair. Most of the topological methods used the hog enzyme because of its ready availability and purity.The location of the epitopes for mAb 95–111, mAb 12–18, mAb 146–14 and pAb39 are shown on this diagram of the α subunit. Also shown is a model of the rat β subunit, since this subunit of rat, but not hog, reacts strongly with mAb 146–14.

the slide followed by three washes. To avoid unacceptable cell loss and damage, the gastric cells were then fixed with 3.7% formaldehyde after incubation with the first antibody. The cells were then incubated for 1 h with the secondary anti-rabbit (for polyclonal antibody) or anti-mouse antibody (for monoclonal antibodies). The slides were washed three times in TBS containing 0.2% gelatin. The cells were mounted in 90% glycerol/10% 10 × PBS.

For each antibody, the same staining was carried out on cells previously permeabilized with 0.2% Triton X-100. The secondary antibodies used were FITC-conjugated sheep anti-mouse or anti-rabbit IgG (Sigma). Cells were observed and photographed on a Zeiss microscope at a magnification of 400 × .

The same protocol was used for immunofluorescence analysis of the SF9 cells expressing rabbit α or β subunits, using Triton X-100 as a permeabilizing agent. Intact SF9 cells were not used in these experiments, since only the overall reaction with antibody was of interest.

Materials

All reagents were analytical grade or better. Enzymes were obtained from Boehringer-Mannheim and Sigma and secondary antibodies from Promega.

Results

The topology and sites of the epitopes mapped are shown in summary in Fig. 1, which represents the α subunit of the hog, rabbit or rat H^+/K^+-ATPase, as well as the β subunit of the rat. The rat β subunit is the one best recognised by mAb 146–14, whereas the hog β subunit is not recognised by this antibody. The eight membrane-spanning segments of the α subunit for which we have obtained biochemical evidence, and the location of tryptic cleavage sites in intact cytoplasmic-side-out vesicles are shown in this figure. The last pair of potential membrane-spanning segments is shown, provisionally, as membrane embedded, rather than membrane spanning in the absence of direct biochemical evidence for this pair [4]. The β subunit is shown as having a single membrane-spanning segment.

Antibody mAb 95–111

Previous studies on this antibody had concluded that it reacted with an epitope contained within the sequence between amino acids at position 529–561 of the rabbit sequence. This was demonstrated by expression of this peptide fragment in *E. coli* containing the cDNA sequence for this fragment, derived from the cDNA for the α subunit of rabbit enzyme [13]. This is just beyond the binding site for FITC at Lys-518 in the rabbit cDNA sequence. Moreover, this antibody was shown to inhibit the H^+/K^+-ATPase in a K competitive fashion, although the activating K^+ site is extracytoplasmic [5]. The binding site for the antibody was cytoplasmic, since the antibody was able to precipitate 70% of the H^+/K^+-ATPase from a microsomal fraction [15].

Intact parietal cells showed no immunofluorescence when reacted with this antibody and permeabilisation prior to the reaction was obligatory for immunoreactivity (Fig. 2A,B). Again, this is compatible with cytoplasmic location of the epitope of mAb 95–111.

Western blotting of the limited digestion pattern showed reactivity with the large 69 kDa and not the smaller 33 kDa pattern consistent with the cDNA defined epitope, as shown in Fig. 3, lane 3. In the less restricted tryptic digestion pattern, antibody 95–111 showed a reaction pattern very similar to the reaction pattern with antibody pAb HC, which was raised against the amino acids between 499 and 522 (Fig. 3, lane 4 in the limited digest). The continuing similarity of the patterns following digestion with trypsin in the absence of ligands (data not shown) suggests that the epitope for mAb 95–111 is close to, but not identical with, the epitope for pAb HC. This provides evidence that the cDNA method gives data that correspond to the Western blot analysis of peptide fragments of the α subunit.

The location therefore of the epitope for mAb 95–111 is shown on Fig. 1 as being in the cytoplasmic

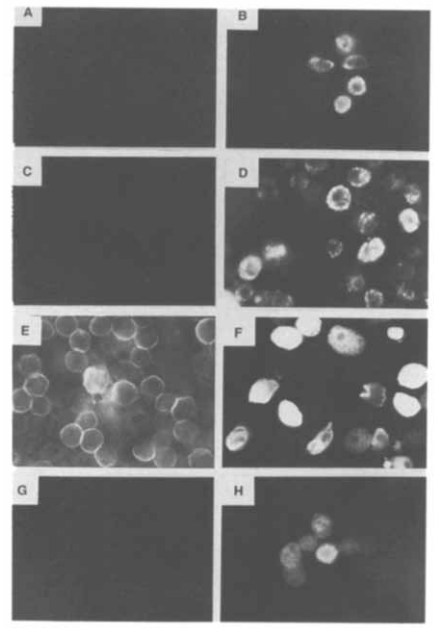


Fig. 2. The topology of the epitopes using intact or permeabilised rat parietal cells as determined by indirect immunofluorescence. A and B are intact and permeabilised cells stained with mAb 95–111. C and D are intact and permeabilised cells stained with mAb 12–18. E and F intact and permeabilised cells stained with mAb 146–14. G and H are intact and permeabilised cells stained with pAb39.

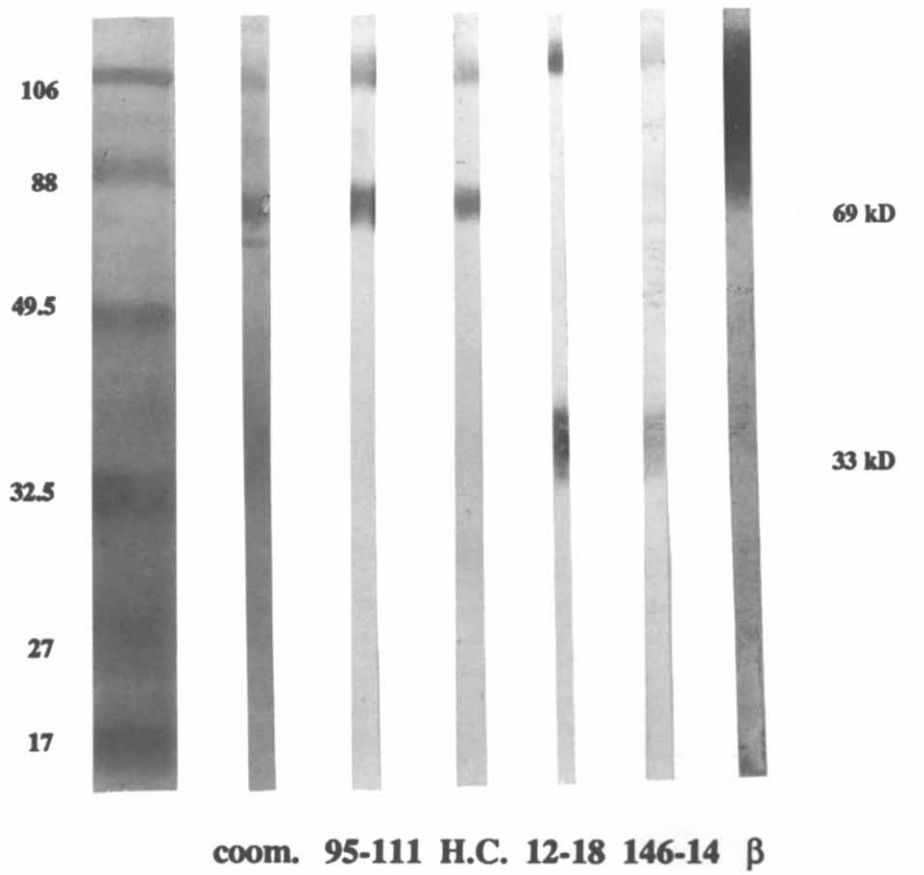


Fig. 3. Location of the antibody epitopes using limited digestion conditions with trypsin at a trypsin-to-protein ratio of 1:40. The intact, cytoplasmic side out hog gastric vesicles were digested in the presence of SCH28080 and ATP as detailed in Materials and Methods and the products separated on a tricine gradient slab gel followed by electrotransfer to PVDF membranes. The first lane shows the molecular mass standards, the second lane the Coomassie stained gel. Lane 3 shows the reaction with mAb 95–111, lane 4 reaction with pAb HC, lane 5 the reaction of mAb 12–18, lane 6 reaction of mAb 146–14 and lane 7 reaction with anti-β antibody.

domain and in the loop between M4 and M5, at the positions determined by the cDNA method.

Antibody mAb 12–18

Fig. 2C,D shows that permeabilisation of parietal cells was necessary to show any immunofluorescence with mAb 12–18. Hence, the epitope defined by these particular studies is on the cytoplasmic side of the H^+/K^+-ATPase.

The mAb 12–18 recognised the 33 kDa, and not the 68 kDa fragment generated by trypsin (Fig. 3, lane 5), hence the epitope was located between positions 670 and 960 on the α subunit of the enzyme. As illustrated below, two epitopes were found for this antibody, but both are included in this fragment of the enzyme.

When the V8 digest of the isolated hog α subunit was blotted by mAb 12–18, it reacted with a fragment of 11 kDa, which had a single sequence beginning at position 584 and probably terminating based on the presence of a carboxylic amino acid at position 689. (Fig. 4).

Western blotting of the expressed fragments of the rabbit α subunit in *E. coli* showed a positive result with a clone containing a sequence spanning amino

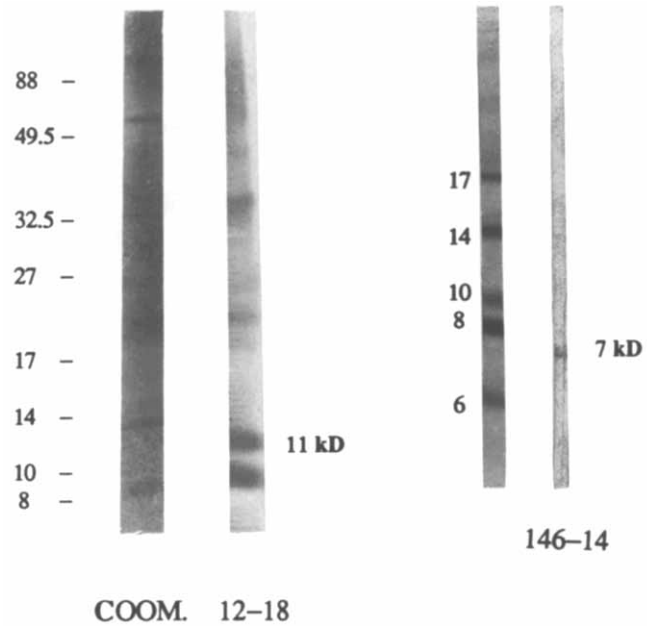
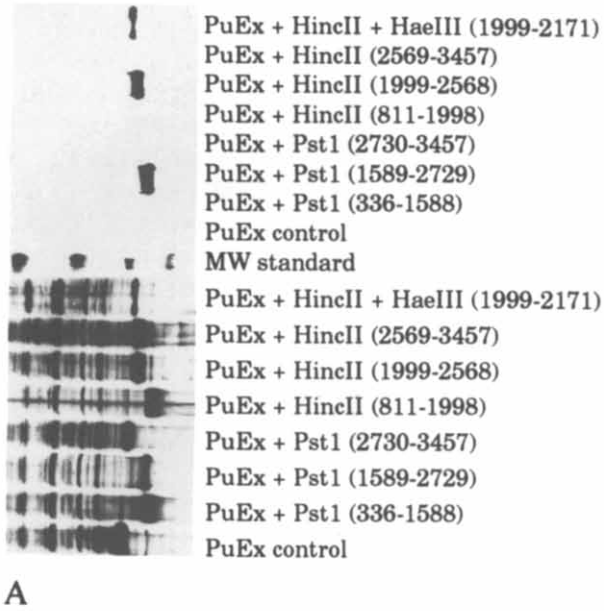


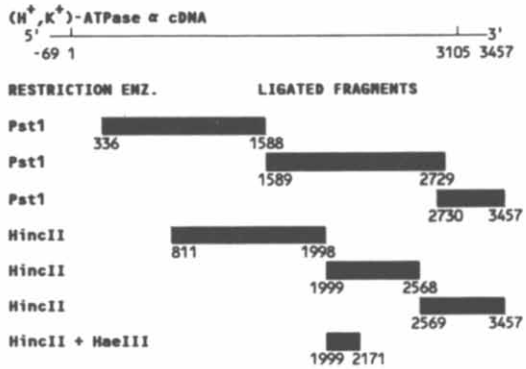
Fig. 4. Characterisation of mAb 12–18 and 146–14 epitopes using a V8 proteinase digest of the separated hog α subunit. The digest was performed as detailed in Materials and Methods. On the right is the Coomassie stained molecular mass standard lane, lane 2 shows the Western blot using mAb 12–18 and lane 3 low range molecular mass standards and lane 4 the Western blot using mAb 146–14.

acids from position 665 to 723. (Fig. 5A,B). In combination with the results of the V8 proteinase digest, this places this epitope for mAb 12–18 between position



A

MAPPING OF HK 12-18 EPITOPE



B

Fig. 5. Characterisation of the mAb 12–18 epitope using expression of restriction fragments of rabbit α cDNA in *E. coli*. In B, the restriction fragments produced by *Pst*1 and *Hin*cII expressed in *E. coli* are shown, along with an additional fragment generated by the combination of *Hin*cII and *Hae*III. The fragments represent the α subunit from position 112 to 1035. In A, on the left is shown the Western blot results using mAb 12–18, placing the epitope between positions 1999 to 2171, namely amino-acid positions 666 and 723. The Coomassie stain is shown on the left of (A). The number is derived from the cDNA sequence of the α subunit of rabbit, beginning with the initiation methionine in the amino-acid sequence.

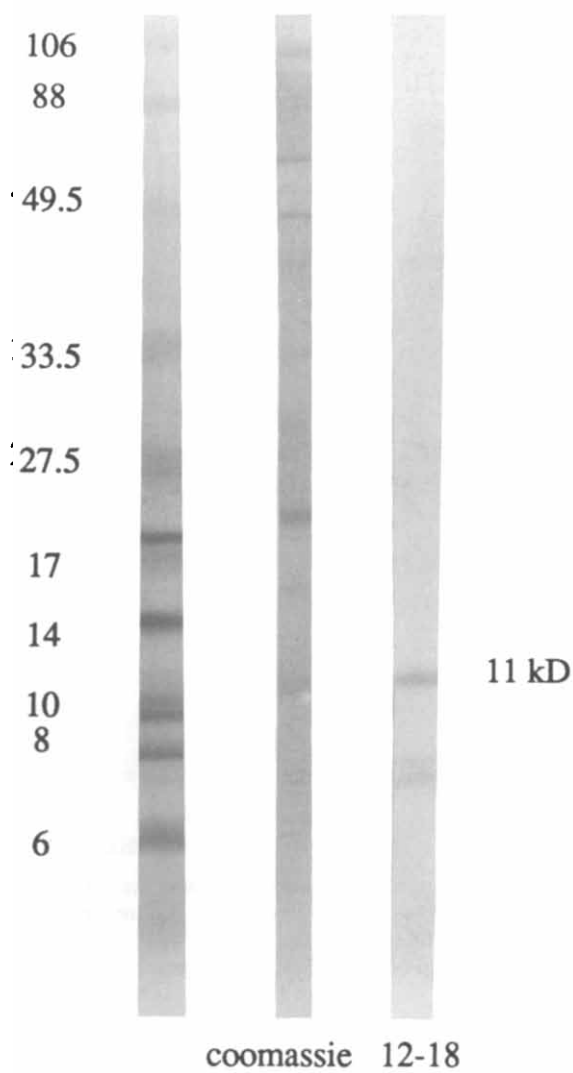


Fig. 6. Characterisation of the mAb 12–18 epitope using tryptic digestion at a 1:4 trypsin-to-protein ratio. Lane 1 shows the Coomassie stained standard for molecular mass determination, lane 2 the Coomassie stain of the digest, and lane 3 (left) the Western blot result using mAb 12–18.

665 and 689 as shown in Fig. 1. This epitope is on the cytoplasmic side of the enzyme's α subunit.

However, it has been shown that a synthetic peptide containing a sequence between positions 880 and 906 of the α subunit of hog can displace completely mAb 12–18 binding to intact hog gastric vesicles [12]. Further, when tryptic digestion is performed at a ratio of 1/4 trypsin to protein in the absence of ligands, a fragment of 11.2 kDa with a single sequence stretching from positions 853 to probably 946 reacts with mAb 12–18 (Fig. 6, lane 2). Apparently, this antibody can recognise an alternative sequence in this region that is present in the hog enzyme.

Antibody mAb 146–14

This antibody was generated by injection of a suspension of rat parietal cells into mice, hence it is

possible that either the α or β subunit contain the epitope recognised by this antibody. Indeed in the original publication where Western blotting was performed in the absence of reducing agents, there was reaction with a 60–90 kDa region in rat enzyme, suggestive of recognition of β but not α subunit [14,17]. At the time of publication of these papers, the β subunit had not been described. As will be seen below, reaction with the α subunit was not detected, because the reaction with the β subunit was so intense that the reaction was stopped before the staining of the larger subunit could be seen.

This antibody was the only one studied to react with intact rat parietal cells by immunofluorescent staining as shown in Fig. 2E. Although permeabilisation increased reaction (Fig. 2F), this is due to the intracellular location of most of the enzyme, as explained above. Hence, as previously established by immunogold methods, the epitope for this antibody in rat parietal cells is extracytoplasmic. It was also shown that mAb 146 did not recognise any of the expressed cDNA fragments with Western analysis of clones encompassing positions 112 to 1035 of the rabbit α subunit.

When this antibody was used for Western blot analysis of hog gastric H^+/K^+-ATPase, in more than 20 experiments, there was never any recognition of the 60–90 kDa region occupied by the β subunit, in contrast to rat enzyme. With hog ATPase only the α subunit reacted as shown in Fig. 3, lane 6 (compare the position of the β subunit in lane 7 of this figure). In these experiments, no reducing agent was present in the gels. It can be seen that the mAb 146 recognised the smaller 33 kDa fragment and not the 68 kDa fragment placing its epitope in the C-terminal one-third of the hog α subunit. Western analysis of the V8 proteinase digest of the hog α subunit showed that a fragment at 7 kDa reacted with mAb 146–14 (Fig. 4, lane 4). This sequence begins at position 838 in the hog

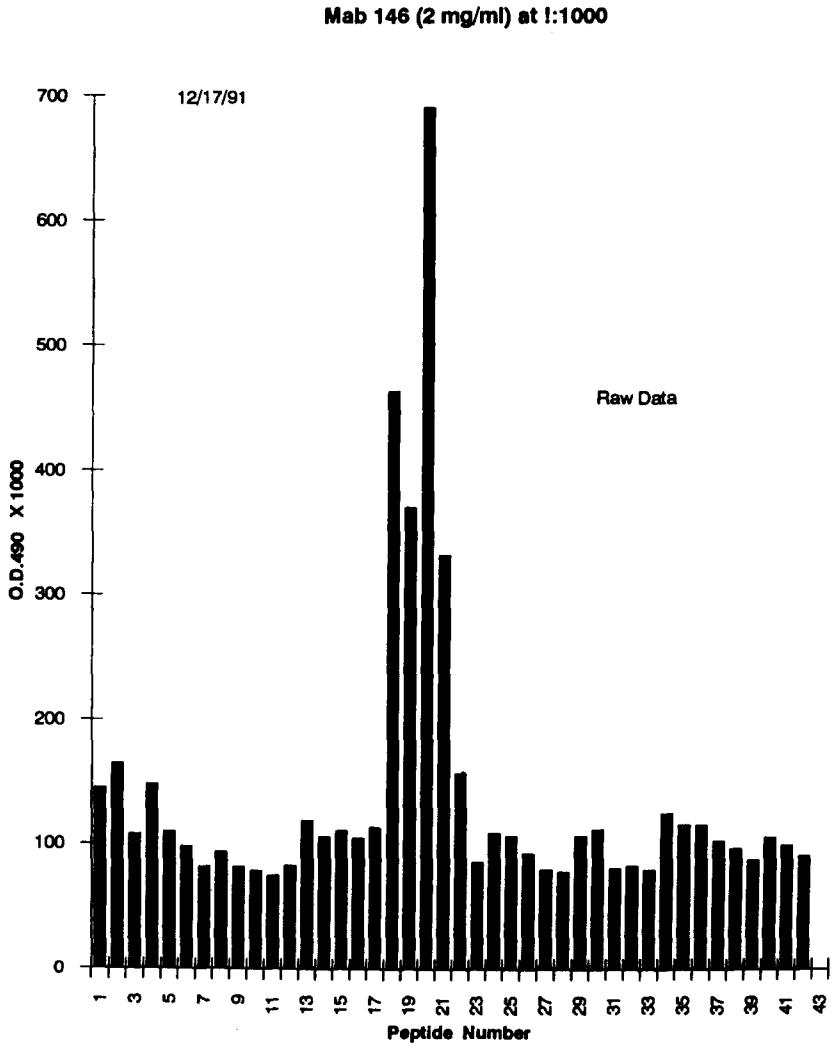


Fig. 7. Mapping of the epitope of mAb 146–14 using synthetic octamers. Sequential octamers were synthesised, and ELISA reaction with mAb 146–14 showed positive fluorescence only for peptides 18–21, spanning positions 870 to 880 in the hog sequence. Peptide 1 is LVNEPLAA beginning at position 853 in the hog sequence, and peptide 43 is LRPQWENH, ending at position 902.

sequence and is predicted to end at position 901. In data not shown, this antibody also recognised a tryptic fragment beginning at position 854 and presumably extending to position 920.

Accordingly, the region between 854 and 903 was analysed by the octamer technique [16]. As shown in Fig. 7, mAb 146–14 recognised a series of overlapping octamers QSFAGFTD (870–877), SFAGFTDY (871–878), FAGFTDYF (872–879) and AGFTDYFT (873–880). The octamers preceding (IQSFAGFA) and following (GFTDYFTA) these sequences showed only background fluorescence. The epitope sequence AGFTD is predicted to be present in the M7 transmembrane segment close to the extracytoplasmic surface, and is probably the epitope recognised in the hog α subunit, as shown in Fig. 1. This location is still in agreement with the presumed extracytoplasmic location of the epitope recognised by mAb 146–14.

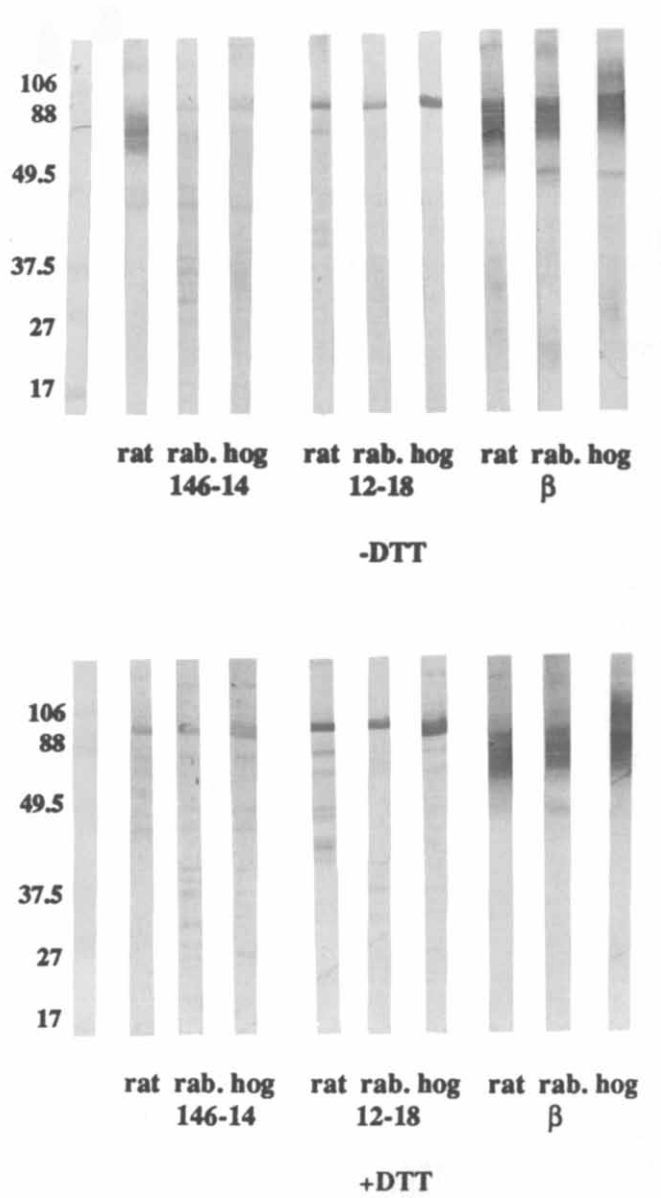


Fig. 8. Western blot analysis of isolated rat, rabbit and hog microsomes. The top PVDF membrane was derived from a run in the absence of DTT, the bottom in the presence of DTT using the Laemmli gel separation. The first three lanes show the results of blotting with mAb 146–14, the second three lanes the results of blotting with mAb 12–18 and the last three, the results of blotting with anti-β antibody. mAb 146–14 recognises the β subunit strongly in the rat, weakly in the rabbit and not at all in hog. The addition of DTT essentially abolishes this recognition. The anti-β antibody recognises the β subunit in the absence and the presence of DTT.

However, when Western analysis of intact rat, rabbit and hog enzyme was carried out in the absence of DTT, reaction was seen again in the region of the β subunit of rat and rabbit, as well as with the α subunit of all three species. DTT abolished all or most of the reactivity with the β subunit but did not affect reaction with the α subunit (Fig. 8). These data again show that mAb 146–14 recognises rat, but not hog, β subunit, as well as the α subunits of rat, rabbit and hog. The recognition of the β subunit appears to depend on the presence of disulfide bonds, and there is no clear homology between the linear epitope, AGFTD, present in the α subunit and any sequence in the β subunit. Further substantiation of this conclusion required separate expression of α and β subunits and expression of the subunits was performed in baculovirus-transfected SF9 cells.

Hence, mAb 146–14 was used to probe baculovirus-transfected SF9 cells that contained either the α or β subunit from rabbit. An immunofluorescence experiment is shown in Fig. 9. There is good expression in these cells of both subunits. Evidently, mAb 146–14 recognises the β subunit expressed in this system, not the α subunit, whereas mAb 12–18 recognises the α and not the β subunit.

Western blotting of the SF9 transfected microsomes run on SDS gels now shows that mAb 146–14 reacts with both the α and the β subunit once solubilised by SDS (Fig. 10 (−DTT), lanes 1 and 5). The β subunit is seen at 35 kDa, the unglycosylated core protein, with another broader band between 45 and 50 kDa (Fig. 10, lane 5). These two bands are also recognised by the β subunit antibody mAb β (Fig. 10, lane 5), which recognises the β subunit of hog H^+/K^+-ATPase (Fig. 3, lane 7). Further, in the presence of DTT as for native rabbit enzyme, the reactivity with the β subunit largely disappears, with maintained recognition of the α subunit (Fig. 10 (+DTT), lane 5).

The monoclonal antibody mAb 146–14, therefore, recognises two epitopes, one on the β subunit, the other on the α subunit. The epitope on the β subunit is dependent on the presence of intact disulfide bridges in the extracytoplasmic domain of the β subunit. Hence, in rat and rabbit cells, the epitope is placed in the extracytoplasmic portion of the α, β heterodimer, accounting for the fluorescence and immunogold results [14,17]. Apparently, the crucial disulfide is also formed

in the SF9 cells, accounting for the immunofluorescent reaction in these cells. This epitope, however, must be altered in hog β subunit so that no reaction is found even in the absence of reducing reagent. The epitope found for the α subunit is not available to the antibody in the SF9 cells, perhaps explained by its location close to the hydrophobic sector of the bilayer.

Polyclonal antibody pAb39

Fig. 2G demonstrates that no staining was observed on the rat parietal cells in the absence of detergent, but staining after detergent treatment (Fig. 2H), suggesting a cytoplasmic location for the sequence used to generate this antibody. These data confirm the cytoplasmic location of the C-terminal region of the α subunit of the gastric H^+/K^+-ATPase [30]. The location of the epitope for pAb39 is shown in Fig. 1.

Discussion

Analysis of antibody epitopes and their location is frequently used in determining secondary structure and functional regions of proteins [27]. Often, polyclonal or monoclonal antibodies are available generated against impure or pure proteins, of unknown epitope, but useful in detection of the protein of interest. As more sequences become available from cloning methods antibodies are generated against specific sequences for detection of protein, as well as for determining secondary structure. Further, an increasing number of

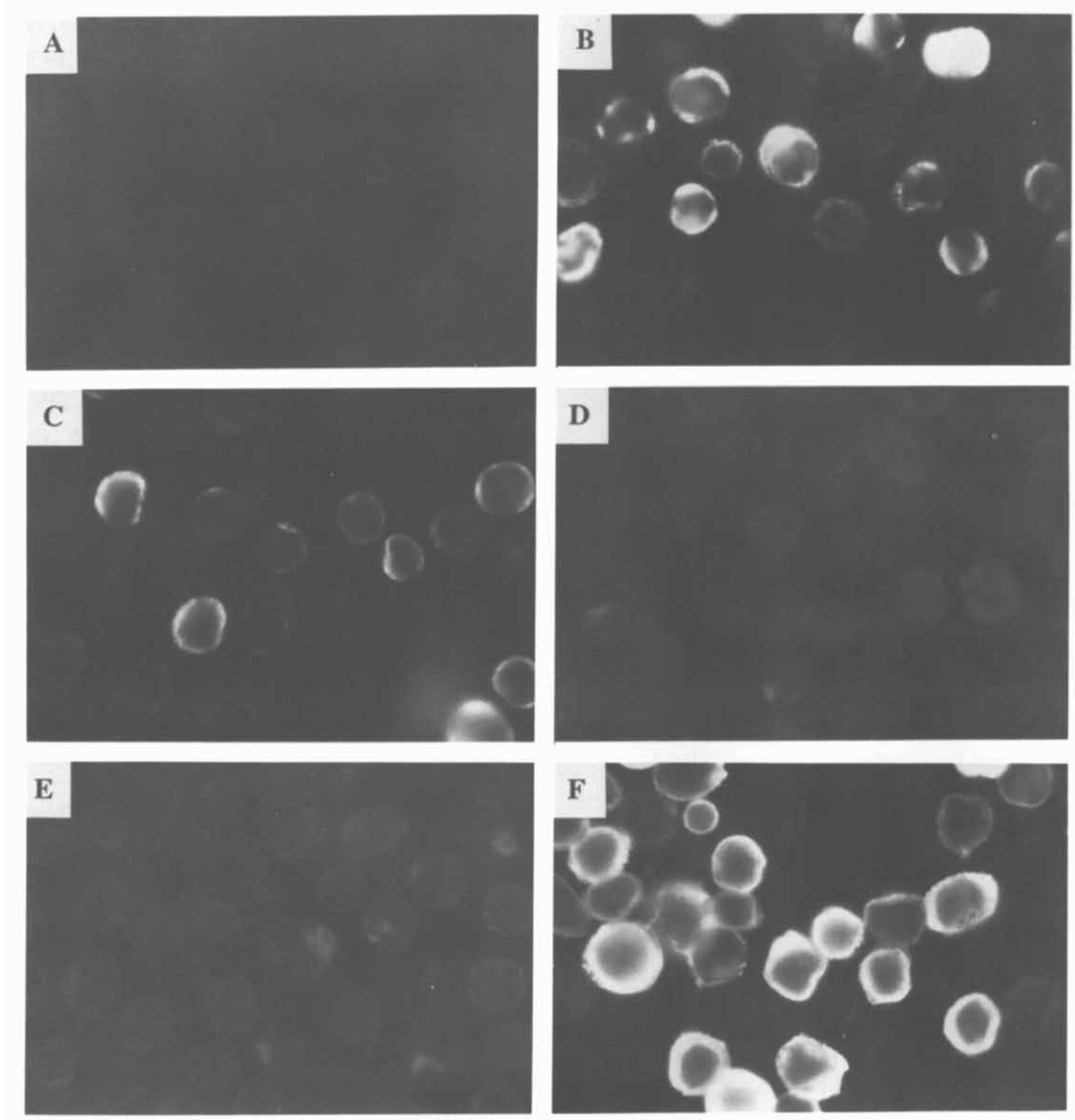


Fig. 9. Immunofluorescent labelling of SF9 cells expressing either α or β subunit of rabbit H^+/K^+-ATPase. Permeabilised SF9 cells were stained with antibody as described in the text. A, C and E were cells transfected with α cDNA, whereas B, D and F represent cells transfected with β cDNA. A and B were stained with mAb 146–14, C and D with mAb 12–18, and E and F with anti-β antibody.

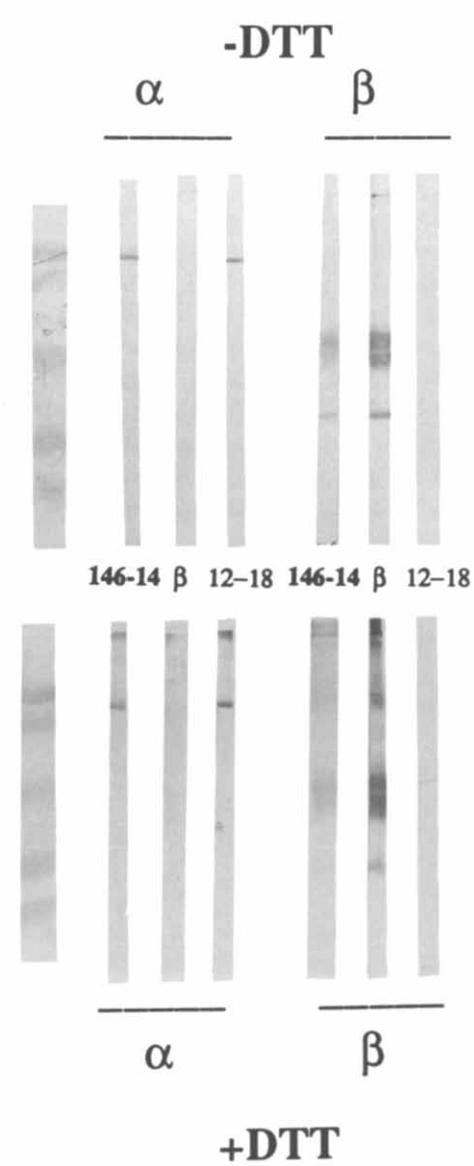


Fig. 10. Western blotting of microsomes from SF9 cells expressing either α or β subunit. The result of blotting microsomes for SF9 cells expressing the α subunit are shown on the left, for those expressing β subunit on the right. The results above are in the absence of DTT, below in the presence of DTT. It can be seen that both mAb 146–14 and mAb 12–18 recognise the α subunit. mAb 146–14 recognises the β subunit well in the absence but not in the presence of DTT, whereas the β antibody recognises only the β subunit.

protein sequences will become available as part of the genome project, and antibody techniques will surely be used to find novel proteins. The above data analysed antibodies of both types, those raised against the H^+/K^+-ATPase before sequence was known, of either the α or β subunit, and one raised against a sequence close to the C-terminal end of the α subunit.

It is often thought that especially monoclonal antibodies are absolutely specific for a single linear epitope composed of about 5 amino acids. As shown here, those raised against the H^+/K^+-ATPase often recognise more than one region of this heterodimeric enzyme, even different subunits.

In one instance, mAb 95–111, considerable information had already been generated. The epitope had been shown to between positions 529 and 561 of the rabbit α subunit by cDNA expression of restriction fragments of the gene [13], the sidedness of the epitope had been determined by immunoprecipitation [27] and K^+-competitive inhibition of the H^+/K^+-ATPase by this antibody has also been shown [15]. This antibody was used to validate the techniques employed in this study.

The hog enzyme is readily obtained relatively pure, and in the form of intact cytoplasmic side out vesicles [18], similar to the sr vesicles of the Ca-ATPase [9,10]. In the latter case, ELISA assays using intact or detergent-treated vesicles have been applied to determine sidedness of epitopes. In our hands, the use of detergent gave variable results, as did direct absorption studies due to non-specific adherence of the antibody to the ELISA well. Alcohol disruption was more consistent, but mAb 146–14, clearly reactive for an extracytoplasmic domain of the β (and α) subunit gave a very weak ELISA reaction in vesicles, as if the epitope was not being exposed by any of the treatments employed. Since this was the only antibody we had that reacts with the vesicle interior, no reagent was available to validate this particular technique for the H^+/K^+-ATPase.

It is possible, however, to prepare highly-enriched preparations of rat or rabbit parietal cells in the laboratory, that are intact and functional in terms of receptor responses, such as carbachol stimulation of $[Ca^{2+}]_i$ or in terms of pH_i regulation. The method chosen for determining the sidedness of the epitope of mAb 95–111 was to plate the cells on to precoated slides, in the absence of any fixative, without or with detergent. The cells were then incubated with primary antibody and then after fixation (which was necessary to retain cells on the slide) reacted with a secondary fluorescently-tagged antibody. Again, this method showed that the epitope for mAb 95–111 did not react unless the cells were permeabilised.

Tryptic or V8 proteinase digestion followed by tricine gradient gel separation produces well-defined bands of peptides, many of which have been sequenced [4,5]. Western analysis of these bands gave data consistent with the cDNA method used to define the epitope region of mAb 95–111 on the α subunit of the H^+/K^+-ATPase.

With these techniques mAb 12–18 was analysed. Cellular immunofluorescence showed a cytoplasmic location of the epitope. Further, it was shown that the major epitope recognised was between amino acids at position 665 and 689 of the hog gastric H^+/K^+-ATPase. The precise location of this epitope was derived from a

combination of sequence analysis and tryptic and V8 proteinase digestion and from cDNA restriction fragment expression followed by Western analysis. It is often difficult to generate the small cDNA fragments necessary to define an epitope to as narrow a region as can be derived from overlapping peptide sequence analysis of proteolytic fragments but in this case the combination of these techniques allowed a small segment of the protein to be identified as the epitope.

However, we obtained the surprising result that, in analysis of tryptic fragments, that there had to be an epitope elsewhere in the hog α subunit recognised by mAb 12–18. This had already been indicated by the ability of a synthetic peptide from position 880 to position 906 to displace completely mAb 12–18 from binding to gastric vesicles [12]. In our case, a peptide of unique sequence between 853 and 946 was recognised in hog enzyme, but not recognised in rabbit protein expressed from cDNA fragments in *E. coli*. This antibody did not recognise the V8 fragment of hog enzyme which starts as position 838 and probably extends to Glu-901. The epitope is therefore after this amino acid. The displacement of this antibody from hog vesicles by a synthetic peptide extending to position 906 places this epitope between position 901 and 906. The sequence ENHHLQDL (901–908) of hog enzyme is replaced by EDHHLQDL in the rabbit cDNA sequence perhaps explaining the lack of recognition of this region in the *E. coli* expression method.

The location of this region of the enzyme based on the model of Fig. 1, which is derived from direct experiments, is in the extracytoplasmic domain of the enzyme between M7 and M8. This secondary site however does not interfere with the topological assignment, which appears to be dominated by the cytoplasmic epitope located between M4 and M5. The conclusion, therefore, from the displacement studies, that the epitope for mAb 12–18 was cytoplasmic was correct, only the relevant epitope was wrongly identified [12]. mAb 12–18, therefore, recognises two regions of the α subunit of the H^+/K^+-ATPase of hog enzyme, on opposite sides of the membrane based on a model derived from tryptic digestion of intact cytoplasmic side-out vesicles [4].

The analysis of mAb 146–14 proved to be more complicated. As stated above, this antibody was generated against a cell suspension and then selected by parietal cell staining and Western blotting [14]. We confirmed that the location of the major epitope of this antibody was extracytoplasmic by immunofluorescent methods [17]. Western analysis using rat membranes had suggested that it was an anti-β antibody, but in the case of Western analysis of hog gastric ATPase, only reactivity with intact α subunit, or fragments of α subunit was found. Western analysis of these fragments placed the epitope of this antibody in hog enzyme between positions 853 and 901. Accordingly, we used successive octamers to show that the sequence AGFTD, positions 873–877, reacted strongly with mAb 146–14. The location of this sequence is projected to be at the extracytoplasmic boundary between lipid and phospholipid head groups in the M7 segment of the α subunit of hog enzyme. It is therefore possible that staining of intact cells expressing only the α subunit would give negative results, because of lack of accessibility of this epitope to the antibody. Again in the absence of DTT in the gel, mAb 146–14 recognised the rat and rabbit β subunit but did not recognise this subunit under reducing conditions, a situation also found in the natural parietal cell antibodies present in the serum of pernicious anemia patients [29]. To establish further the recognition of both subunits by this antibody, we expressed these individually in SF9 cells.

In the case of rabbit α and β subunit expressed in SF9 cells, this antibody stained only the cells expressing the β subunit, which can be explained by the accessibility of the epitope of the β subunit, and the membranous location of the epitope found in the α subunit. This was confirmed when Western analysis was performed on SDS-solubilised SF9 membranes when both epitopes should become accessible to antibody. Here, both subunits were recognised by mAb 146–14 in the absence of DTT and only the α subunit was recognised in the presence of DTT.

Evidently, this monoclonal antibody recognises an epitope in the β subunit that depends on integrity of a disulfide bond in the extracytoplasmic domain, and an epitope in the α subunit that does not depend on secondary structure. Comparing sequences of hog enzyme that does not contain the epitope in the β subunit to rat and rabbit β subunit, and the position of disulfides in the subunit, it is possible to conclude tentatively that the epitope recognised in rat and rabbit β subunit is in the region between Cys-162 and Cys-178, and is present in the disulfide-determined secondary structure of the this region of the β subunit. In this region between positions 162 and 178, Leu-165 and Val-166 in rat are replaced by arginine and proline in the hog sequence, whereas Val-166 is replaced by threonine in the rabbit. The replacements in the hog subunit are non-conservative, explaining the absence of reactivity of the β subunit of the hog. The requirement for an intact disulfide bond for recognition of the β subunit shows further that this epitope is presented to the immune system in its native disulfide form, not in its reduced form. Since rat reacts and hog does not, the site of reaction of mAb 146–14 with β subunit of the rat is illustrated in the scheme of Fig. 1.

Additionally, the mAb β antibody recognised the 6 kDa fragment of the hog β subunit beginning at Ala-236. This peptide fragment of the C-terminal domain of this subunit was produced by trypsinolysis of leaky,

not intact, gastric vesicles, followed by electrophoresis in the presence of DTT. (Shin, J.M and Sachs, G., unpublished observations). This suggests extracytoplasmic location of the C-terminal domain of the β subunit.

The polyclonal antibody, pAb39, recognised the synthetic peptide against which it was generated, between positions 1011 and 1020 of hog enzyme. Further, by cell assay, this epitope was cytoplasmic. Hence the C-terminal region of the α subunit is cytoplasmic, as previously shown by iodination [30]. The N-terminal region is also cytoplasmic, showing that there is an even number of membrane spanning in the α subunit of the gastric H^+/K^+-ATPase, as in the Ca ATPase of sarcoplasmic reticulum and plasma membrane [6,31]. A similar finding has been made for the Na^+/K^+-ATPase using sequence specific antibodies [11].

The epitopes defined above for the three monoclonal antibodies show an interesting distribution. mAb 95–111 was the only one found with a single unique epitope. This antibody is inhibitory to the enzyme and is K^+-competitive, which may indicate that the epitope is involved in the conformational changes in the cytoplasmic domain induced by K^+-binding in the extracytoplasmic or membrane domain. This epitope may be relatively close to the cytoplasmic surface of the bilayer, and is within or close to the ATP-binding domain.

The mAb 12–18 appears to recognise two epitopes. The cytoplasmic epitope dominates in staining reactions, suggesting that it might be the better recognised. The synthetic peptide containing the extracytoplasmic epitope, shown to be present in the extracytoplasmic loop between membrane spanning segments 7 and 8 however can displace completely binding of this antibody to intact, cytoplasmic-side-out vesicles [12]. The epitopes are not predicted to be close together, since they are separated by the bilayer itself. Perhaps a homology is recognised, not evident from primary sequence, or there is some membrane insertion of the loop between M7 and M8, allowing a closer approach than expected. The second epitope recognised by mAb 12–18 is similar in location to the antibodies used to define the topology of this loop in the Ca-ATPase [9,10].

In the case of mAb 146–14, two epitopes were defined, one in the β, the other in the α subunit. The former was determined by the presence of intact disulfide bonds and, therefore, is not in the linear sequence of this subunit. The latter is present in the linear sequence of the α subunit predicted to be close to the membrane interface. Conformational interactions have been shown between the two subunits, dependent on the presence of disulfide bonds [32,33] and perhaps the presence of two epitopes recognised by this antibody can be interpreted as showing close contact between the M7 interface region and the region enclosed by the second disulfide bridge of the β subunit.

Acknowledgements

Sequencing was performed by Dr. Audree Fowler at UCLA, supported by BRS grant No. 1S10RR0554-01. This study was supported by USV SMI and NIH grants DK 40615, 41301 and 14752 and AHA-GLAA grant 970 GI-2.

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