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Location of the Carbohydrates Present in the HK-ATPase Vesicles Isolated from Hog Gastric Mucosa†

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ABSTRACT: The glycosylation of H^+K^+-ATPase vesicles isolated from hog gastric mucosa was investigated by various methods. Following protein separation on sodium dodecyl sulfate reducing gels and transfer to poly(vinyl difluoride) membranes, binding of concanavalin A was confined to the 94-kDa band which corresponds to the catalytic subunit. In contrast, wheat germ agglutinin binding occurred in a region below the 94-kDa subunit, corresponding to the 60–85-kDa region, and also to protein just above the catalytic subunit. Treatment with glycopeptidase F removed most of the concanavalin A staining and also the wheat germ agglutinin staining found below the 94-kDa region, but spared the higher molecular weight wheat germ agglutinin reactive material. During the deglycosylation experiments a protein of 35-kDa was produced. Sequencing analysis of V8 protease generated peptide fragments of the 35-kDa protein show at least 30% homology with the Na^+K^+-ATPase β-subunits. Labeling of the carbohydrates by galactosyltransferase and [^{3}H]uridine diphosphate–galactose showed that the sites of labeling were extracellular and were confined to the wheat germ agglutinin staining regions. Two molecular weight regions, below the 94-kDa region, of 60 and 85 kDa were identified. Electron microscopy using postembedding staining techniques showed that both concanavalin A and wheat germ agglutinin staining occurred on the extracellular face of the gastric vesicles. It is concluded that there are three classes of glycosylated proteins in hog gastric vesicles: (1) the 94-kDa protein containing a core (simple) oligosaccharide, with no or few *N*-acetylglucosamine residues available for either wheat germ agglutinin binding or galactose transfer; (2) the 60–85-kDa region containing two protein bands that have complex N-linked oligosaccharides; and (3) a protein of higher molecular weight than the catalytic unit which contains O-linked complex oligosaccharides. All of these glycoproteins appear to be located on the extracellular face of the gastric vesicles.

The gastric HK-ATPase of mammalian parietal cells is isolated as a vesicular preparation that retains the ability to transport protons in exchange for K^+ upon the addition of ATP (Sachs et al., 1974). These vesicles are largely (94%) inside out (cytosol on the outside) and impermeable to ions such as K^+ and Cl^- (Saccomani et al., 1977). By use of these vesicles, studies on the structure of the HK-ATPase in relation to its orientation in the lipid bilayer can be carried out. The hydrolysis of ATP is accomplished by a protein that has an apparent molecular weight of 94 000 on reducing acrylamide gels. This protein has been shown by various techniques to be the catalytic unit of the HK-ATPase. For example, the 94-kDa protein when isolated on sodium dodecyl sulfate (SDS) gels is labeled by [γ-^{32}P]ATP (Helmich et al., 1985), and also [^{3}H]omeprazole, an inhibitor of the proton pump, covalently binds the 94-kDa protein specifically (Wallmark et al., 1984). The cDNA sequence of the HK-ATPase catalytic unit has been obtained from rat and hog and has about 60% homology

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to the NaK-ATPase catalytic or α-subunit (Shull & Lingrel, 1986; Maeda et al., 1988).

Recently, it has been shown that a tryptic fragment of the rat HK-ATPase binds to the lectin concanavalin A (ConA) (Tai et al., 1989). Sequence information from this tryptic fragment showed the presence of a potential glycosylation consensus sequence starting at amino acid Asn 492 in the primary sequence of rat HK-ATPase. This tryptic fragment also included the fluoroisothiocyanate (FITC) binding site at Lys 516. This lysine is thought to be in the cytosolic domain of the protein, since its reaction with FITC is prevented by ATP (Jackson et al., 1983). Treatment of this ConA binding peptide fragment with *N*-glycanase abolished its affinity for ConA binding. This indicates that the carbohydrate moiety is N-linked to the protein. The membrane sidedness of the N-linked carbohydrate attached to the 94-kDa protein was determined during this investigation.

The mechanism for N-linked glycosylation of proteins depends on the transfer of sugar residues by phosphoryldolichol oligosaccharides. The saccharide is added to the growing carbohydrate chain in the lumen of the rough endoplasmic reticulum or the Golgi membrane but not the mitochondrial or plasma membranes. The processing of sugars then forms either simple (high mannose), complex (hybrid), or mixed types of carbohydrate units (Baynes et al., 1973). In all cases known, N-linked glycosylation specifies an extracellular directed orientation of the asparagine residue that is linked to the primary *N*-acetylglucosamine residue.

Earlier work has shown that hog gastric vesicles contained a considerable amount of carbohydrate (Spenney et al., 1974). By direct analysis, values of up to 30% carbohydrate content were obtained from hog vesicles, and electron microscopy showed the presence of a fuzzy coat around the vesicle membranes. Periodate–Schiff (PAS) staining of SDS gels showed PAS reactive material overlaying the 94-kDa band and extending below it to approximately 60 kDa.

It has been shown (Chow et al., 1989) that the 94-kDa protein can be retained on wheat germ agglutinin (WGA) affinity columns due to its association with a heavily glycosylated protein of 60–85 kDa. The Coomassie and silver stains of the proteins in the 60–85-kDa region are weak, but treatment with endoglycosidase F resulted in the formation of a protein band at about 35 kDa, a molecular weight similar to that of the core protein of the β-subunit of the NaK-ATPase (Kyte, 1981).

In this work, we have shown the presence of at least two glycoproteins by staining SDS gels with WGA and ConA. ConA reacted with the 94-kDa protein, while the region from 60 to 85 kDa, the β-subunit, stained intensely with WGA. Electron microscopy of postembedded vesicles (Newman et al., 1983) indicated that the sugar sites on both proteins were oriented toward the luminal face of the vesicles, which corresponds to the extracellular face of the HK-ATPase. These data were extended by labeling the vesicles with [^{3}H]UDP-galactose and galactosyltransferase (Torres & Hart, 1984) and deglycosylation experiments with glycopeptidase F. Also, amino acid sequences were obtained from peptide fragments isolated from the 35-kDa protein that is generated from the 60–85-kDa protein after glycopeptidase F treatment. The amino acid sequences obtained had at least 30% homology to the NaK-ATPase β-subunit, indicating that this protein is a β-subunit for the HK-ATPase.

Experimental Procedures

Materials. All biotinylated lectins were purchased from Sigma as were the globulin-free BSA, 4-chloro-1-naphthol, hydrogen peroxide, Trizma, and galactosyltransferase. The avidin–horseradish peroxidase (HRP), prestained standards, biotinylated standards, SDS, and glycine were purchased from Bio-Rad. [^{3}H]UDP-galactose and En^{3}Hance were purchased from NEN Research Products. Poly(vinyl difluoride) (PVDF) membranes were obtained from Millipore. G-50 resin and *Staphylococcus aureus* V8 protease were purchased from Pierce along with the nonionic detergents Nonidet P-40 (NP-40) and octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$). Glycopeptidase F was obtained from Boehringer Mannheim Chemicals. The colloidal gold lectins was purchased from EY-Labs. Gel and autoradiographs were scanned by a LKB 2202 ultroscan.

All vesicles used in this study were purified inside-out vesicles isolated from hog stomachs and stored in 10 mM Pipes/Tris and 30% (w/v) sucrose, pH 6.8, buffer at −80 °C (Saccomani et al., 1977).

ATPase Activity. The ATPase activity of vesicles used in this study was 110–120 μmol of P_i/(mg·h) in the presence of NH_4Cl or nigericin. The Mg^+, K^+-ATPase activities were 20–30 μmol of P_i/(mg·h), and Mg^+-ATPase activity was 15–20 μmol of P_i/(mg·h), indicating that the vesicles were sealed and intact.

Acrylamide Gels and Gel Transfer to PVDF Membranes. All the SDS–acrylamide slab gels utilized in this study were prepared and used according to the method of Laemmli (1970) except for the Tricine gels (Schagger & von Jagow, 1987) used to separate the low molecular weight peptide fragments generated by V8 protease digestion of the 35-kDa protein for amino acid sequencing. The Laemmli gels were either 10% or 10–15% gradient gels. The gels were transblotted to PVDF membranes by using either the Sartoblot semidry apparatus, 300 mA for 3 h, or the Trans-Blot cell from Bio-Rad at 150 mA overnight. The transblot buffer was 125 mM Tris, 5 mM glycine, and 20% methanol at pH 8.3.

Membranes were washed two times with distilled water and then stained and destained to observe the protein content with Coomassie blue. A picture was taken of the Coomassie-stained PVDF membrane and then washed with 100% methanol until the Coomassie was completely removed from the membrane. The PVDF membranes were washed with distilled water and rinsed with Tris-buffered saline (TBS), 50 mM Tris-HCl, and 200 mM NaCl, pH 7.4, to equilibrate them for Western blots.

Lectin Binding to PVDF Membranes. Lectin blots were carried out with biotinylated lectin–avidin horseradish peroxidase complex similar to the procedure outlined by Hawkes (1982). The major difference in our procedure is the use of PVDF membranes rather than nitrocellulose for the solid support media. After blotting, the lectin biotinylated–avidin HRP binding was observed by reacting the bound avidin–HRP with 4-chloro-1-naphthol and hydrogen peroxide in TBS. Controls were carried out in the presence of 0.5 M methyl mannoside or 0.5 M *N*-acetylglucosamine during ConA and WGA binding, respectively.

[^{3}H]UDP-galactose Labeling. Twenty microliters of G1 vesicles (5 mg/mL) was treated with $C_{12}E_8$ or left intact in 10 mM Hepes buffer at pH 7.3 with 5 mM galactose and 0.2 M sucrose; samples were vortexed and sonicated. Samples were incubated for 15 min at room temperature. Two to four microcuries of [^{3}H]UDP-galactose that was evaporated under nitrogen and resuspended in 30 μL of 25 mM 5′-AMP was added to the sample. The reaction was started by the addition of 0.4–0.6 unit of autogalactosylated galactosyltransferase (Holt & Hart, 1986). The reaction was carried out at 11 °C for 20 min. The samples were then applied to a column (2.5

in. × 0.25 in.) of G-50 resin; the resin was equilibrated with 10 mM Hepes, pH 7.3, containing 0.2 M sucrose. The loaded columns were centrifuged at 1000 rpm for 5 min. The eluent was placed on a second column and centrifuged again. The columns were rinsed with 50 μL of buffer which was added to the eluent. The samples were vortexed and sonicated with SDS sample buffer and loaded onto 10% acrylamide gels. Control samples were vesicles with [^{3}H]UDP-galactose alone and galactosyltransferase and [^{3}H]UDP-galactose to determine the amount of nonspecific labeling.

Glycopeptidase F Deglycosylation Procedure. Deglycosylation of N-linked carbohydrates was carried out on permeabilized vesicles (Tarentino, 1985). Samples of 100 μg (5 mg/mL) of protein were denatured in 0.2% SDS and 0.1 M β-mercaptoethanol and then vortexed. Sodium phosphate buffer (0.55 M), pH 8.6, was added to give a final concentration of 225 mM. NP-40 was added to a 7-fold excess over the final 0.1% SDS concentration. The sample was divided in half, and 6 units of glycopeptidase F was added to one portion and 225 mM phosphate buffer was added to the other half as a control. Incubation was for 17 h at 37 °C.

Sequence of the Putative β-Subunit. To gain sequence information from this deglycosylated protein, vesicles were subjected to glycopeptidase F treatment and run on a 10–15% Laemmli gel, and the 35-kDa band was sliced out and subjected to proteolytic cleavage by V8 protease. This slice was incubated with 10 μL (1 mg/mL) of V8 protease and 20 μL of Tris buffer, pH 8.2, for 24 h at room temperature, and then another 10 μL of protease was added for a total digestion time of 48 h (Cleveland, 1983). Electrophoresis buffer was added to the slice, and the mixture was homogenized and then loaded on a Tricine gel (10–21% gradient gel) (Schagger & von Jagow, 1987). The Tricine gel was aged for at least 24 h and preelectrophoresed for 20 min at 20 mA in the presence of 0.1 mM thioglycolate in the upper running buffer to help prevent N-terminal blockage of the newly cleaved peptide fragments. The fragments were transferred to PVDF membranes similar to the transfers of samples for Western blots. The only difference was the presence of 5 mM dithiothreitol in the transfer buffer. The peptide bands of interest were subjected to amino acid sequencing on an Applied Biosystems gas-phase sequenator.

TEM Lectin/Gold Procedure. Lectin/colloidal gold postembedding labeling was performed on G1 gastric vesicles which were fixed in 4% paraformaldehyde, dehydrated in graded steps of ethanol, embedded with L. R. White resin, polymerized at 60 °C for 48 h, and thin sectioned with a diamond knife (Newman et al., 1983). Thin sections were placed on unsupported Ni grids, incubated in 1:10 lectin/gold 10-nm particles (E-Y Labs), washed, fixed in 2% glutaraldehyde/PBS, washed, viewed, and then photographed in a Zeiss 109 TEM. Controls for ConA/gold consisted of native ConA incubation, to block ConA sites, then ConA/gold incubation (Gillett & Longhurst, 1982; Newman et al., 1982).

Criteria for counting lectin gold particles were as follows: (1) gold particles that were only attached to the lipid bilayer were counted; (2) if particles were directly on top of the membrane, assignments were not made; (3) particles not attached to a membrane were excluded.

Results

Location of Glycoproteins on SDS Gels. The protein distribution as determined by Coomassie staining of PAGE gels was compared to the ConA and WGA binding. Figure 1 shows staining of a PVDF membrane gel transfer with three different protein concentrations. The 94-kDa protein comprises

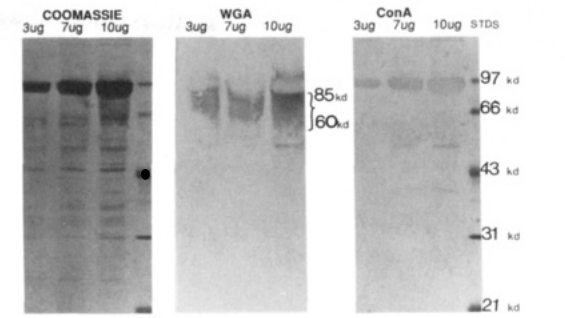


FIGURE 1: Comparison of the Coomassie, WGA, and ConA binding to sealed vesicles. This concentration range of G1 vesicles enables selective ConA binding to the 94-kDa protein. The WGA does not appear to bind the 94-kDa protein but does bind to protein in the 60–85-kDa region and directly above the 94-kDa protein.

the majority of Coomassie blue staining, accounting for about 70% of the total protein. There is only light Coomassie staining in the 60–85-kDa range. At low protein concentration, the ConA staining of the 94-kDa band is apparent. However, at higher protein concentrations the ConA staining is decreased. Even though ConA binding does not produce intense staining, it was found to be specific. WGA staining is considerably more intense than ConA staining. The biotinylated WGA lectin does not appear to bind to the 94-kDa HK-ATPase but binds very strongly to the 60–85-kDa region and also to protein directly above the 94-kDa protein. This experiment indicated that we could differentiate the 94-kDa protein from other proteins found in G1 vesicles by lectin binding. Concanavalin A binds to mannose groups within the carbohydrate moiety and also to inositol phosphate. WGA binds to free peripheral *N*-acetylglucosamine residues found in complex and hybrid oligosaccharides and also to sialic acid. The carbohydrate composition of G1 vesicle preparation indicates that no inositol phosphate or sialic acid is present (Spenney et al., 1974).

The 94-kDa protein must contain at least a simple-type oligosaccharide since it is detected by ConA. Most likely it is a high mannose chain since it does not bind WGA, indicating the absence of free *N*-acetylglucosamine residues. In contrast, the 60–85-kDa region and the region above 94 kDa contain free *N*-acetylglucosamine groups due to the intense staining with WGA.

Labeling with [^{3}H]UDP-galactose. Vesicles, either intact or $C_{12}E_8$ permeabilized, were incubated with radioactive UDP-galactose and galactosyltransferase as shown in Figure 2. Three major regions of radioactivity are detected in the gel by autoradiography. Two of the areas prominently labeled are found in the 60–85-kDa region and do not stain strongly with Coomassie. Also, these two labeled areas were not well-defined with WGA staining. The third region of incorporation appears above the 94-kDa band; the higher molecular weight band could be an aggregate of the 94- or 85-kDa bands. The labeling which occurs directly above the 94-kDa protein may be the same protein that stains with WGA in this molecular weight region. The amount of radioactivity in all of these regions increased after permeabilization of the vesicles. The concentration of $C_{12}E_8$ used in these experiments was not enough to solubilize the enzyme or disrupt the conformation of the HK-ATPase since it was still able to maintain ATPase activity under these conditions (Rabon & Bassilian, 1988). In the presence of $C_{12}E_8$, the incorporation of label into all three regions increased more than 5-fold as determined by optical density scanning of the autoradiograph and the corresponding gel.

The proteins with complex saccharides that contain free *N*-acetylglucosamine groups allowed WGA binding and la-

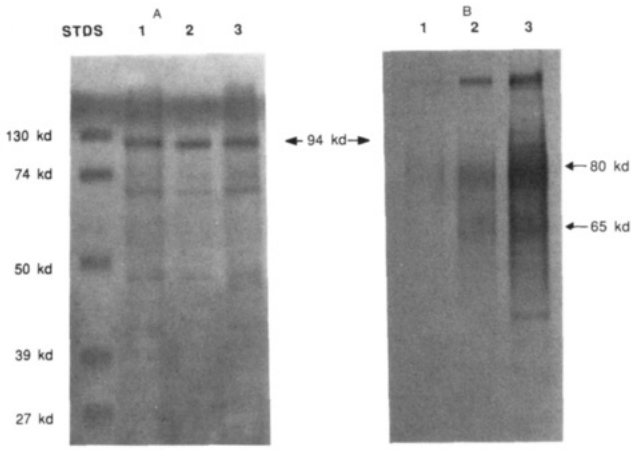


FIGURE 2: Coomassie (A) stain and autoradiograph (B) of intact vesicles and permeabilized vesicles labeled with [^{3}H]UDP-galactose separated on a 10% gradient gel. Samples 1–3 were treated with [^{3}H]UDP-galactose; samples 2 and 3 also contained galactosyltransferase. (Lane 1) Control, vesicles treated with $C_{12}E_8$. (Lane 2) Intact vesicles (inside out). (Lane 3) Vesicles permeabilized with $C_{12}E_8$. Sample 1 shows that very little nonspecific labeling occurs during this experiment. Also, a control of only [^{3}H]UDP-galactose and galactosyltransferase was subjected to electrophoresis, but no radioactivity was observed.

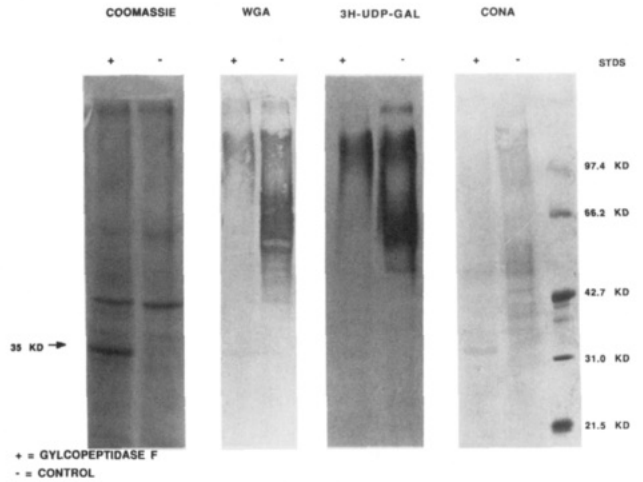


FIGURE 3: Coomassie stain, WGA, ConA binding, and [^{3}H]UDP-galactose-labeled permeabilized vesicles after glycopeptidase F treatment. The samples were separated on a 10–15% Laemmli gel and then transferred to PVDF membranes for staining and blotting or the gel was treated with En^{3}hance and subjected to autoradiography. The control samples were G1 vesicles (inside out) exposed to 0.3% NP-40 for 17 h at 37 °C without glycopeptidase F (−). The glycopeptidase F treated samples were identical with the control sample with the addition of glycopeptidase F (+).

beling by galactosyltransferase. These proteins appear to be oriented so that the carbohydrate faces the vesicle interior, the extracellular face of the membrane. There appear to be three molecular weight regions that contain this type of oligosaccharide, and none of them correlate with the HK-ATPase catalytic subunit.

The catalytic subunit does not appear to be labeled by galactosyltransferase. This would indicate that the carbohydrate unit associated with the 94-kDa protein is of the simple type. This would explain the lack of WGA staining to the 94-kDa protein.

Deglycosylation. Following glycopeptidase F treatment of permeabilized vesicles, a new protein band of 35 kDa was produced. Figure 3 shows deglycosylated and control samples after staining with Coomassie, WGA, and ConA and also the autoradiograph of deglycosylated [^{3}H]galactose-labeled samples. In the conditions in which deglycosylation was carried out, the 94-kDa HK-ATPase aggregates, so that no direct evidence of deglycosylation can be obtained for this protein. In neither of the control samples is the 35-kDa protein detected, indicating that it is not produced by proteolytic cleavage of the 94-kDa protein but a new deglycosylated protein core. Scanning the Coomassie stain associated with the 35-kDa protein and comparing it to the amount of 94-kDa protein that is found in the same sample volume revealed an approximate protein ratio of 35/94 kDa of 1.2:1.0. By the WGA staining pattern it seems that this protein originates from the 60–85-kDa region. There still remains some WGA staining in the treated sample, but the ConA binding is almost completely lost. This supports the results of Tai et al. (1989), who concluded that the carbohydrate moieties found in G1 vesicles which bind ConA are mostly N-linked. The remaining WGA binding may be due to the presence of O-linked carbohydrate moieties in the vesicle preparation or incomplete treatment with glycopeptidase F.

During the [^{3}H]UDP-galactose labeling experiments we discovered the presence of three heavily glycosylated proteins besides the high molecular weight aggregate. These proteins do not stain strongly with Coomassie in the 60–85-kDa region and are not separated very well with biotinylated WGA binding. After glycopeptidase F treatment of permeabilized labeled vesicles, the label associated with the 60–85-kDa region was completely eliminated with the formation of the core protein at 35 kDa. This would indicate that the 65- and 80-kDa proteins may have the same amino acid sequence and only differ in the amount of glycosylation.

Another interesting observation made during the glycopeptidase F experiment was the appearance of a broad labeled band at a molecular weight above 130 kDa but below the large protein aggregate. This is seen in both the control sample and the glycopeptidase F treated sample, indicating that this glycoprotein aggregated during treatment at 37 °C for 17 h. This labeling is not seen in samples exposed to just the galactosyltransferase experimental procedure as seen in Figure 2. This new protein aggregate is not affected by glycopeptidase F and appears to be O-linked, indicating that there are at least three different proteins in G1 hog vesicles: (1) the catalytic subunit; (2) the 35-kDa putative β-subunit which originates from the 60–85-kDa region; and (3) a protein that contains a large amount of O-linked carbohydrate.

Sequence of the Putative β-Subunit. Sequencing from the N terminus of the 35-kDa protein was attempted but appeared to be blocked. The 35-kDa protein band was cut out of a 10–15% Laemmli gel and subjected to V8 protease digestion. Figure 4 shows the low molecular weight peptide pattern obtained on a Tricine gel. Three peptides were sequenced; peptides A and B contained the same N-terminal sequence, and peptide C had a unique N-terminal sequence. The sequences that were obtained had at least 30% homology with the NaK-ATPase β_1- and β_2-subunit isoforms found in rat as shown in Table I (Young et al., 1987; Martin-Vasallo et al., 1989). This suggests that the 35-kDa protein could be a β-subunit for the HK-ATPase.

Electron Microscopy. Lectin colloidal/gold binding to postembedded vesicles was used to determine the sidedness of glycosylation for both the catalytic and putative β-subunit of HK-ATPase. Figure 5 shows that the ConA/colloidal gold particles bind mainly on the interior of the vesicle and that this binding is specific. In Figure 6, the electron micrograph shows binding with WGA/colloidal gold particles to vesicles. As with ConA binding, the majority of the particles are found on the interior of the vesicles. Figure 7 shows the bar graph

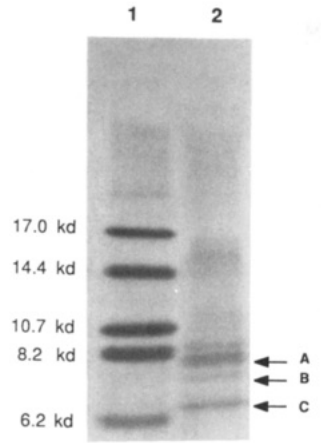
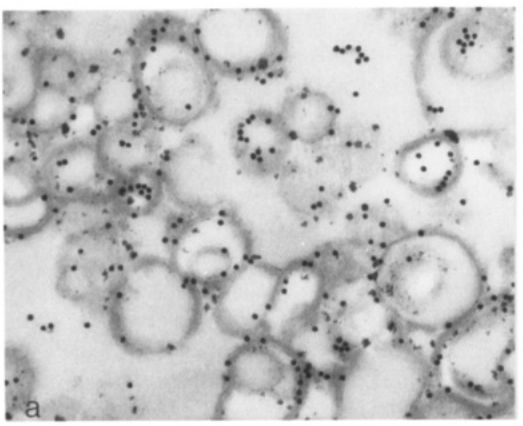


FIGURE 4: Peptide fragment pattern of the 35-kDa protein after extensive V8 protease digestion (lane 2). The digested gel slice was homogenized and run on a 10–21% gradient Tricine gel and then transferred to a PVDF membrane. The three peptide fragments that were sequenced have the following approximate molecular weights: (A) 8000; (B) 7500; (C) 6500. Lane 1 shows the Sigma SDS-17 low molecular weight standards used to identify the molecular weights of these peptide fragments.



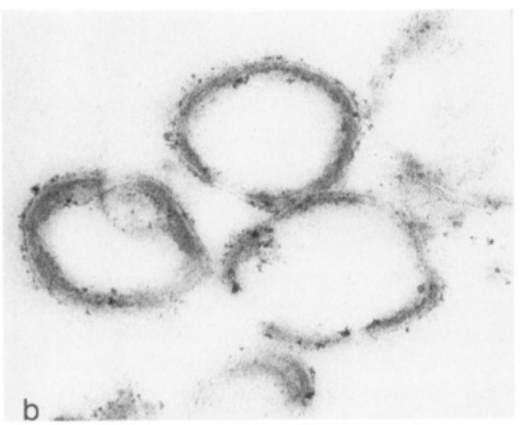


FIGURE 5: (a) Electron micrograph of hog gastric G1 vesicles (inside out) labeled with ConA/colloidal gold 5-nm particles by postembedding method. Greater than 70% observed labeling occurred on inner membrane (gastric luminal surface). (58 645×). (b) ConA control. G1 vesicles incubated in ConA, then ConA/gold 5-nm particles, postembedding method. Virtually no vesicles (less than 1%) were labeled. (92 800×).

of counted particles for both ConA and WGA binding; respectively, 75% and 85% of the gold particles were found on the interior of the membrane. These data suggest that the glycosylation sites on the 94-kDa protein are luminally located as are the glycosylation sites of the possible β-subunit which binds only WGA.

Table I: Amino Acid Sequence of Peptides Isolated from the Putative HK-ATPase β-Subunit[a]

NaK β1	229	E Y F G M G A F Y G F P L Q Y Y P Y Y G K L L Q P K Y L Q P L L
PEPTIDE A/B		E Y F P A D G T Y S L H Y F P Y Y G K K A Q P H Y S N P L V
NaK β2	215	I M F P A N G N I D L M Y F P Y Y G K K F H V N Y T Q P L V
NaK β1	172	G K P C I I I K L N R M L G F K P K
PEPTIDE C		X K X X F I I K M N H I V K F L
NaK β2	174	G Q P C V F I K M N R V I N F Y A

[a] Sequence homology of the peptides generated from V8 protease digestion of the 35-kDa protein to the NaK-ATPase β-subunit sequences from Young et al. (1987) and Martin-Vasallo et al. (1989).

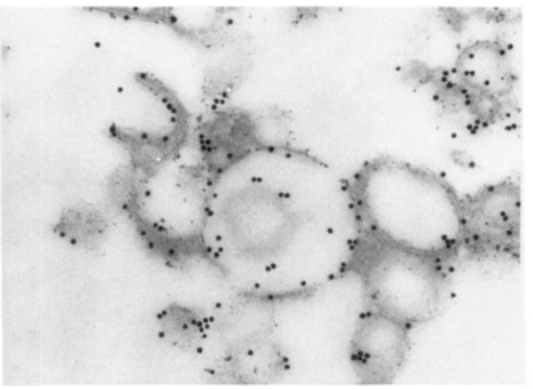


FIGURE 6: G1 vesicles labeled with WGA/colloidal gold 10-nm particles by postembedding method. Greater than 80% observed labeling occurred on inner membrane. (54 207×).

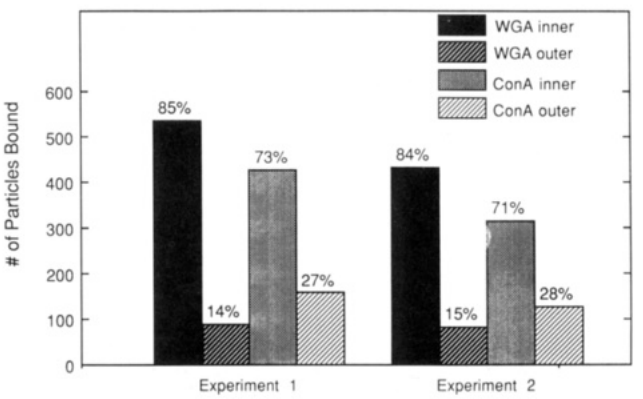


FIGURE 7: Electron microscope morphometric wheat germ agglutinin and ConA/gold particle labeling. Percentages of membrane-bound particles are shown from two experiments. Luminal (inner vesicular surface) lectin binding is favored.

Discussion

The characterization of carbohydrates associated with purified hog gastric vesicles has allowed a reevaluation of the protein heterogeneity in the molecular weight region of the catalytic subunit of the HK-ATPase and the gastric vesicles in general. It appears that for the rat and hog HK-ATPase, the 94-kDa subunit is indeed glycosylated and the oligosaccharide is N-linked to the protein. The amino acid sequence obtained from a ConA binding tryptic fragment from HK-ATPase indicated that there is only one N-linked glycosylation consensus sequence, Asn-Ser-Thr, amino acids 492–495, found in the isolated tryptic fragment from rat, and amino acids 493–496 found in hog (Hall et al., unpublished results). This region is predicted by hydropathy profiles to be in the cytosolic domain of the HK-ATPase. It has been shown with hog preparations that Lys 497 binds pyridoxal phosphate and is

protected by ATP (Tamura et al., 1989). This indicates that the structural conformation of this part of the enzyme is affected by ATP binding. On the basis of the sidedness of ConA/colloidal gold reactivity, the majority of the oligosaccharide is found on the interior of vesicles. It appears that the studies on glycosylation of the catalytic subunit and substrate protected binding studies of the HK-ATPase are in contradiction. Perhaps a considerable fraction of the putative cytosolic domain interacts with the hydrophobic membrane spanning peptides, enabling extracellular glycosylation to occur only four amino acids away from cytosolic labeling.

Preliminary reconstruction of proteins, such as NaK-ATPase and the Ca-ATPase, suggests that more of the protein is intramembranal than predicted from hydropathy plots (Hebert et al., 1988). This study supports the suggestion that there may be more interaction between membrane spanning peptides with predicted hydrophilic domains of the HK-ATPase than first believed.

The catalytic subunit contains a minor amount of carbohydrate compared to the three molecular weight regions that bind to WGA. This group of glycosylated proteins must account for the fuzzy coat observed in EM and for the carbohydrate found on direct analysis. [^{3}H]UDP-galactose labeling of the vesicles was increased 5-fold by permeabilization of the vesicles with nonionic detergent. From the distribution of counts on the autoradiograph, two molecular weight bands were detected at 65 and 80 kDa. This suggests that these oligosaccharides are also present on the luminal surface. The location of these carbohydrate moieties was reconfirmed in the WGA/colloidal gold EM experiments. Following deglycosylation, the 60- and 85-kDa proteins disappear and a resultant protein appears at 35 kDa.

The 35-kDa core protein and its presence in HK-ATPase vesicles association with the catalytic subunit have been shown in this paper and elsewhere (Okamoto et al., 1989). Other observations that strengthen this possibility of close association between the β-subunit and the catalytic unit are the following. For example, target molecular weight analysis using electron irradiation has shown that the molecular weight of the protein migrating at 94 kDa is about 140 kDa when determined by irradiation. A similar molecular weight by irradiation was observed for protein phosphorylation by [γ-^{32}P]ATP (Rabon et al., 1988). This could be interpreted as evidence for close association between the catalytic subunit and a protein of about 40 kDa.

Table I shows the sequence analysis of the N-terminal sequences of peptide fragments generated by V8 protease digestion of the 35-kDa protein. There appears to be at least 30% amino acid sequence homology with the β_1-subunit and 45% homology with the β_2-subunit isoform of the NaK-ATPase. From the sequence obtained, there appears to be more homology with β_2 than β_1, but the cDNA sequence is needed for a complete comparison of the different β-subunits. This evidence suggests the presence of a HK-ATPase β-subunit and that it originates from a glycoprotein in the 60–85-kDa range. However, it appears that plasma membrane insertion of the HK-ATPase can occur in cultured cells transfected with the cDNA derived from rat HK-ATPase (Caplan et al., 1989). Thus, in contrast to the NaK-ATPase, the β-subunit of the HK-ATPase is not necessary for plasma membrane insertion. To prove the function of the β-subunit, cloning and expression of both the catalytic unit and the β-subunit are required.

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Registry No. ATPase, 9000-83-3.

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