Glycosylation is essential for biosynthesis of functional gastric H^+,K^+ -ATPase in insect cells

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The role of N-linked glycosylation in the functional properties of gastric H^+,K^+ -ATPase has been examined with tunicamycin and 1-deoxymannojirimycin, inhibitors of glycoprotein biosynthesis and glycoprotein processing respectively. Tunicamycin completely abolished both K^+ -stimulated and 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2a]pyridine (SCH 28080)-sensitive ATPase activity and SCH 28080-sensitive phosphorylation capacity. The expression level of both H^+,K^+ -ATPase subunits remained unaffected. 1-Deoxymannojirimycin clearly affected the structure of the N-linked oligosaccharide

moieties without affecting specific phosphorylation capacity. Purification of the functional recombinant enzyme from non-functional H^+,K^+ -ATPase subunits coincided with purification of glycosylated β -subunits and not of non-glycosylated β -subunits. Transport of the H^+,K^+ -ATPase β -subunit to the plasma membrane but not its ability to assemble with the α -subunit depended on N-glycosylation events. We conclude that the acquisition, but not the exact structure, of N-linked oligosaccharide moieties, is essential for biosynthesis of functional gastric H^+,K^+ -ATPase in insect cells.

INTRODUCTION

H^+,K^+ -ATPase, or the gastric proton pump, is an intrinsic membrane protein. Like Na^+,K^+ -ATPase and Ca^{2+} -ATPase, it belongs to the class of the E1–E2, or P-type family of, transport ATPases. The enzyme consists of two protein subunits (designated α and β) and is therefore related more to Na^+,K^+ -ATPase than to Ca^{2+} -ATPase. Both H^+,K^+ -ATPase subunits have been cloned and their primary structures have been elucidated from several species [1]. A major feature of the H^+,K^+ -ATPase β -subunit is the presence of seven consensus sequences for N-linked glycosylation (six for the pig enzyme) that are all co-translationally glycosylated [2]. Until now, the functional role of N-linked glycosylation for heterodimeric P-type ATPases has been examined for Na^+,K^+ -ATPase only. In several reports it has been demonstrated that N-glycosylation is not essential for Na^+,K^+ -ATPase activity [3–5]. Because H^+,K^+ -ATPase and Na^+,K^+ -ATPase are structurally and functionally very similar, it has been assumed that N-glycosylation is also not essential for H^+,K^+ -ATPase activity [6]. This, however, has not been tested yet. Although H^+,K^+ -ATPase can be completely deglycosylated *in vitro* by peptide N-glycosylase F, complete deglycosylation generally requires the use of detergents [7]. Because H^+,K^+ -ATPase activity is highly sensitive to inactivation by detergents, the use of peptide N-glycosylase F is almost inevitably impaired, with a loss of catalytic activity of H^+,K^+ -ATPase. Therefore it is virtually impossible to correlate deglycosylation studies *in vitro* with the activity of the gastric proton pump. We succeeded in applying the baculovirus expression system to obtain catalytically active rat gastric H^+,K^+ -ATPase from an insect cell source [8]. This enabled us to study the characteristics of H^+,K^+ -ATPase in a model system *in vitro*. We have used glycoprotein biosynthesis and processing inhibitors to study the role of N-glycosylation for

functional properties of H^+,K^+ -ATPase. Our results clearly show that the presence, but not the exact composition, of N-linked oligosaccharide units is essential to obtain a catalytically active recombinant H^+,K^+ -ATPase.

MATERIALS AND METHODS

Cells and viruses

Sf9 cells (ATCC CRL-1711) were maintained as described previously [8]. For production of H^+,K^+ -ATPase, Sf9 cells were grown to 1.5×10^6 cells/ml in spinner flasks (Bellco, Vineland, NJ, U.S.A.), pelleted by centrifugation for 10 min at 100 *g* at ambient temperature and infected with DLZ α AS β viruses encoding both H^+,K^+ -ATPase subunits and a β -galactosidase marker cassette [8] at 10^7 cells/ml with a multiplicity of infection of 3. After 1 h of infection at 27 °C, cells were transferred to 100 ml of fresh culture medium supplemented with 1% (v/v) ethanol and incubated at 27 °C with various concentrations of the glycoprotein biosynthesis inhibitor tunicamycin or the glycoprotein processing inhibitor 1-deoxymannojirimycin (dMAN) (both from Boehringer, Mannheim, Germany) as indicated. Ethanol was added to the culture medium after infecting the cells, because this leads to synthesis of higher levels of functional H^+,K^+ -ATPase [9]. Occasionally, cells were infected as monolayer cultures in tissue culture flasks.

Preparation of Sf9 membranes

Infected cells at 3 days after infection were centrifuged at 2000 *g* for 5 min at ambient temperature. The cell pellet was frozen at –20 °C until further processing, which was done by resuspending the cell pellets at 10^7 cells/ml in ice-cold homogenization buffer

Abbreviations used: dMAN, 1-deoxymannojirimycin; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2a]pyridine.

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[25 mM Hepes/Tris (pH 7.0)/10 % (w/v) sucrose/2 mM EDTA/5 μ g/ml leupeptin]. Membranes were disrupted by sonication with three 15 s pulses with a probe sonicator (5 mm diameter) set at 65–70 W (Branson Power Company, Denbury, CT, U.S.A.) with subsequent cooling on ice. After centrifugation for 30 min at 10000 *g* and 4 °C, the supernatant was centrifuged for 60 min at 100000 *g* and 4 °C. Pelleted membranes from this step were resuspended in 0.2 times the initial volume of storage buffer [25 mM Hepes/Tris (pH 7.0)/10 % (w/v) sucrose/2 mM EDTA]. Functional H^+,K^+ -ATPase in this crude membrane preparation could be enriched from contaminating non-specific (auto) phosphorylating enzymes and from non-functional H^+,K^+ -ATPase subunits by centrifuging the membranes for 1 h at 100000 *g* and 4 °C over a layer of 25 % (w/v) sucrose in 25 mM Hepes/Tris (pH 7.0)/2 mM EDTA on a cushion of 38 % (w/v) sucrose in the same buffer. The 25–38 % (w/v) sucrose interface was collected, diluted to 10 % (w/v) sucrose in the same buffer and pelleted by centrifugation for 1 h at 100000 *g* and 4 °C. The membrane pellets obtained were resuspended in storage buffer and all fractions were stored at –20 °C.

Phosphorylation assay

The functional H^+,K^+ -ATPase content of the membrane fractions was determined by phosphorylation with [γ - 32 P]ATP (Amersham, Little Chalfont, Bucks., U.K.) as follows: 10–50 μ g of membrane proteins was preincubated in 50 μ l of 25 mM Tris/acetic acid (pH 6.0)/1 mM $MgCl_2$ for 1 h on ice. Next, 10 μ l of 0.6 μ M [γ - 32 P]ATP (diluted in unlabelled ATP to approx. 50–100 Ci/mmol) was added and the reaction proceeded for 10 s on ice. The reaction was stopped by adding 5 ml of ice-cold stopping solution [100 mM phosphorous acid/5 % (w/v) trichloroacetic acid]. Phosphorylated proteins were separated from free label by filtration on 0.8 μ m filters (type ME-27; Schleicher and Schull, Dassel, Germany) and repeated washing with stopping solution. Filters were analysed by liquid-scintillation analysis. The specific phosphorylation capacity of H^+,K^+ -ATPase was determined as the difference between the phosphorylation capacity in the absence and presence of 100 μ M 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2-*a*]pyridine (SCH 28080) as a specific H^+,K^+ -ATPase inhibitor [10], which was included in the preincubation mixture. Each sample was assayed in triplicate. The specific phosphorylation capacity of H^+,K^+ -ATPase in crude membrane fractions from untreated cultures was generally 3–6 times higher than the amount of non-specific phosphorylation, with a standard error of less than 3 %.

ATPase activity assay

The ATPase activity of the H^+,K^+ -ATPase produced in the membrane fractions was measured as the SCH 28080-sensitive liberation of inorganic phosphate from [γ - 32 P]ATP as follows: 1–5 μ g of membrane proteins was incubated in 100 μ l of 100 mM Tris/acetic acid (pH 7.0)/1 mM $MgCl_2$ /1 mM $NaNO_3$ /1.5 mM KCl/0.1 mM EGTA/10 μ M [γ - 32 P]ATP (diluted in unlabelled ATP to approx. 200 mCi/mmol), either with or without 100 μ M SCH 28080 at 37 °C for a maximum of 30 min, each performed in triplicate. Then the reaction mixture was placed on ice and mixed with 900 μ l of 10 % (w/v) activated charcoal (type SX-1; Norit, Amersfoort, The Netherlands)/5.5 % (w/v) trichloroacetic acid. After centrifugation for 30 s in a Microfuge, the radioactivity in 200 μ l of the clear supernatant containing the liberated inorganic phosphate was counted. In the absence of either or both $MgCl_2$ and KCl, no SCH 28080-sensitive ATPase activity was detected (results not shown). A background of non-specific ATPase activity (SCH 28080-insensitive) of 100–150 nmol/h per

mg was usually observed under these conditions. The specific ATPase activity of H^+,K^+ -ATPase in crude membrane fractions from untreated cultures was generally approx. 40–80 nmol/h per mg with a standard error of less than 3 %. Total ATP hydrolysis never exceeded 35 % of the ATP present in the reaction mixtures.

β -Galactosidase assay

The β -galactosidase content of infected cells was determined as described previously [9].

Confocal laser scan microscopy

Sf9 cells were grown on sterile microscope coverslips in complete growth medium and infected with a multiplicity of infection of 3 for 1 h at 27 °C. After infection, cells were incubated at 27 °C for 48 h in complete growth medium with additions as indicated. Cells were washed three times for 5 min with PBS, pH 7.4, followed by fixation in 1 % (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. Further processing was done by permeabilization at –20 °C in 100 % methanol for 5 min. Next, the coverslips were dried at room temperature and non-specific binding sites for antibodies were blocked by incubation for 30–60 min in PBS/0.05 % polyoxyethylene sorbitan monolaurate (Tween-20)/1 % (w/v) gelatin/2 % (v/v) fetal calf serum with gentle rocking. After being washed with PBS/0.05 % Tween-20 three times for 5 min each, the cells were incubated with subunit-specific antibodies for 30–60 min in PBS/0.05 % Tween-20/1 % (w/v) gelatin/2 % (v/v) fetal bovine serum. Free antibodies were removed by washing the cells as above. Next the cells were incubated with fluorescently labelled secondary antibodies (Dako, Glostrup, Denmark) for 1 h in PBS/0.05 % Tween-20/1 % (w/v) gelatin/2 % (v/v) fetal bovine serum. From this moment onwards, the coverslips were kept in the dark as much as possible. After washing as above, the cells were desalted by a short wash with Milli-Q water (Millipore, Bedford, MA, U.S.A.), dehydrated with methanol, dried and mounted in 10 % (w/v) Mowiol (Hoechst, Amsterdam, The Netherlands), 2.5 % (w/v) NaN_3 and 25 % (v/v) glycerol in 0.1 M Tris/HCl, pH 8.5. They were examined on a Bio-Rad MRC1000 confocal microscope. Images were averaged over eight scans.

Protein analysis and subunit quantification

Protein was determined with the modified Lowry method described by Peterson [11], with BSA as a standard. The H^+,K^+ -ATPase subunit content of the membrane fractions was determined with a previously described quantitative ELISA [12].

Immunoblotting and immunoprecipitation

Immunoblotting was performed with subunit-specific antibodies as described previously [8]. Occasionally, staining of specific proteins on immunoblots was done with a chemiluminescence detection kit in accordance with the instructions of the manufacturer (Tropix, Bedford, MA, U.S.A.). Immunoprecipitation was performed as follows: 200 μ g of crude Sf9 membrane proteins were solubilized in 0.2 ml of buffer containing 1 % (w/v) octa(ethylene glycol) monododecyl ether, 0.01 % SDS, 0.05 M Tris/HCl, pH 8.8, and 0.15 M NaCl. Monoclonal antibody 5B6 (5 μ g) against the H^+,K^+ -ATPase α -subunit [13] was added, and the entire mixture was incubated at 4 °C for 2 h. After centrifugation at 10000 *g* for 2 min, the supernatant was incubated with 50 μ l of Protein A immobilized on agarose (KemEnTec, Copenhagen, Denmark) and incubated for 3 h at 4 °C with

constant agitation. The immunoprecipitates were collected by centrifugation for 1 min at 1000 *g*, washed three times for 5 min in the same buffer as described above and solubilized in SDS/PAGE sample buffer. Identification of H⁺,K⁺-ATPase subunits in the precipitates was established by immunoblotting. When either the antibody or the Protein A-agarose was omitted from the immunoprecipitation procedure, essentially no α - or β -subunits were found in the precipitates (results not shown).

RESULTS

Effect of inhibitors of glycoprotein biosynthesis and processing on the glycosylation and activities of H⁺,K⁺-ATPase produced *in vitro*

In the absence of inhibitors, infection of Sf9 cells with DLZ α As β virus leads to the synthesis of substantial amounts of functional H⁺,K⁺-ATPase. Functional aspects of this H⁺,K⁺-ATPase produced *in vitro* can be demonstrated as an SCH 28080-sensitive ATP-dependent autophosphorylation capacity as well as an Mg²⁺-dependent and K⁺-stimulated ATPase activity. These activities are absent from non-infected cells or cells harbouring only one of the two H⁺,K⁺-ATPase subunits [8,9]. Addition of more than 100 ng/ml tunicamycin to the culture medium resulted in a highly reproducible dose-dependent decrease in the amount of functional H⁺,K⁺-ATPase synthesized. This decrease in H⁺,K⁺-ATPase activity was correlated with a simultaneous decrease of the amount of glycosylated β -subunits as revealed by immunoblotting (Figure 1B). Treatment with tunicamycin had no visible effect on the H⁺,K⁺-ATPase α -subunit (Figure 1A). At tunicamycin concentrations above 1 μ g/ml, only non-glycosylated β -subunits were detected by Western blotting and no significant H⁺,K⁺-ATPase activity could be measured. In

Figure 1 Effect of tunicamycin on the glycosylation and activity of H⁺,K⁺-ATPase

In (C) the phosphorylation capacity (○, ●) and the ATPase activity (□, ■) of H⁺,K⁺-ATPase (●, ■) and endogenous (auto)phosphorylating and ATP-hydrolysing activity (○, □) in crude membrane fractions are plotted against the tunicamycin concentration in the culture medium. Values represent means $\pm$ S.E.M. for three to nine experiments; 100% activities are: phosphorylation capacity, 2.16 ± 0.28 pmol/mg for H⁺,K⁺-ATPase and 0.75 ± 0.04 for endogenous (auto)phosphorylating enzymes; ATPase activity, 64 ± 8 nmol/h per mg for H⁺,K⁺-ATPase and 115 ± 8 nmol/h per mg for endogenous enzymes. (A, B) immunoblots of α -subunit (A) or β -subunit (B). Each lane contained 2.0 μ g of crude membrane protein. H⁺,K⁺-ATPase subunits were revealed with specific antibodies against either H⁺,K⁺-ATPase α -subunit (HKB) [29] or β -subunit (2G11) [2]. The horizontal positions of the lanes in (A) and (B) correspond to the tunicamycin concentrations beneath, in (C).

Figure 2 Effect of dMAN on the glycosylation pattern of the H⁺,K⁺-ATPase β -subunit

Each lane contained 5.0 μ g of total protein. The β -subunit was revealed with monoclonal antibody 2G11. Abbreviations: β_{HM} , β -subunits with high-mannose precursors (approx. 51 kDa); β_C , β -subunit core protein (34 kDa). The smearing of the band of β -subunit core protein was caused by the intense staining of this subunit on the Western blot.

contrast with this inhibitory effect on the catalytic activities of H⁺,K⁺-ATPase, the level of endogenous (auto)phosphorylating enzymes (SCH 28080-insensitive) was hardly affected by tunicamycin, demonstrating that the inhibitory action is not a general effect of tunicamycin on the activities of the (auto)phosphorylating enzymes in Sf9 cells. Similarly to its effect on the steady-state phosphorylation capacity, tunicamycin inhibited the K⁺-stimulated ATPase activity of the recombinant enzyme. To exclude direct effects of tunicamycin on H⁺,K⁺-ATPase activity, a control experiment was performed in which pig gastric H⁺,K⁺-ATPase (1 mg/ml) was incubated at 20 °C for 20 h at pH 7.0 with different concentrations of tunicamycin, whereupon the phosphorylation capacity was determined. No significant effects were found after preincubation with tunicamycin concentrations up to 10 μ g/ml (results not shown). These results strongly suggest that N-glycosylation is somehow essential for H⁺,K⁺-ATPase activity.

Tunicamycin eliminates the formation of all N-linked oligosaccharides on proteins. Therefore, when using tunicamycin, we cannot establish whether the acquisition of oligosaccharides alone is sufficient for H⁺,K⁺-ATPase activity, or whether processing of the oligosaccharide precursors into complex glycosylated forms (as in native H⁺,K⁺-ATPase) is also required. Despite the fact that insect cells are poorly equipped with the enzymes to perform complex-type N-glycosylation [14], some recombinant baculovirus-infected insect-cell-expressed proteins do indeed carry complex-type oligosaccharides [15]. To assess whether or not the structure of the oligosaccharides on the H⁺,K⁺-ATPase subunits could play a role in the catalytic activity of H⁺,K⁺-ATPase, the effect of dMAN was studied. dMAN is a specific inhibitor of the α -mannosidase I-catalysed trimming of the high-mannose oligosaccharide precursor Man₉GlcNAc₂Asn to Man₆GlcNAc₂Asn. The latter form can be trimmed to Man₃GlcNAc₂Asn by Golgi-type α -mannosidase II and can subsequently be extended into complex-type glycosylated forms. Therefore, in the presence of dMAN, processing of the core glycosylated Glc₃Man₉GlcNAc₂Asn is arrested at the Man₉GlcNAc₂Asn stage, eliminating the ability of Sf9 cells to perform complex-type glycosylation.

First we studied the effect of addition of dMAN to the culture medium on the glycosylation pattern of the H⁺,K⁺-ATPase β -subunit. In the absence of dMAN, glycosylated β -subunits appear on the Western blot as a smear of proteins with an apparent molecular mass of approx. 40–50 kDa. In the presence of at least 3 mM dMAN, the smear of the glycosylated β -subunits synthesized *in vitro* is decreased to two immunoreactive protein bands with apparent molecular masses of approx. 51 and 48 kDa, the upper band being predominant (Figure 2). This phenomenon was independent of the duration of the infectious process and

Figure 3 Glycosylated β -subunits co-purify with functional H^+,K^+ -ATPase

The H^+,K^+ -ATPase α -subunit (A) or β -subunit (B) in different Sf9 membrane fractions was revealed with subunit-specific antibodies (see the legend to Figure 1). Abbreviations: β_c , β -subunit core protein (34 kDa); β_{gly} , glycosylated β -subunits (40–50 kDa); CM, crude membranes; IF, 25–38% (w/v) sucrose interface; P, 38% (w/v) sucrose pellet. Each lane contained 1.0 μ g of protein. The activity of the resulting fraction is shown in (C) as the steady-state phosphorylation capacity; numbers in parentheses represent the units of phosphorylation capacity in each of the fractions.

was observed at 2, 3 and 4 days after infection (results not shown). The molecular mass of the upper band closely corresponds to the expected molecular mass of a core-glycosylated β -subunit with seven N-linked oligosaccharide precursors [2]. The lower band might represent either a proteolytic breakdown product or a β -subunit with only six oligosaccharide precursors (as the result of one site's being less accessible), although other explanations cannot be excluded. The lower-molecular-mass fractions that appear in the absence of dMAN are most probably β -subunits with heterogeneously trimmed high-mannose oligosaccharide units. This effect of dMAN is consistent with the established action of this compound [16]. Next, the effect of dMAN on the activity of the recombinant expressed H^+,K^+ -ATPase was measured. If processing of the oligosaccharide moieties on the H^+,K^+ -ATPase subunits is essential for its catalytic activities, inhibiting this processing with dMAN should have an all-or-none effect on H^+,K^+ -ATPase activity. This proved not to be so, because the steady-state phosphorylation capacity of an H^+,K^+ -ATPase preparation synthesized in the presence of 3 mM dMAN in the culture medium (a concentration that strongly inhibits mannosidase I activity; Figure 2) was identical with a control preparation (results not shown). Thus only the presence and not the exact structure of the oligosaccharide moieties is essential for H^+,K^+ -ATPase activity.

Glycosylated β -subunits co-purify with functional H^+,K^+ -ATPase

Additional results demonstrating the need of H^+,K^+ -ATPase to be N-glycosylated to perform catalytic activities was obtained from purification experiments. Only a relatively small fraction of the H^+,K^+ -ATPase subunits synthesized *in vitro* possessed catalytic activity [8,9]. Functional H^+,K^+ -ATPase subunits in the standard crude membrane preparation could be separated from non-functional H^+,K^+ -ATPase subunits by using a discontinuous sucrose density gradient as described in the Materials and methods section. The extent of glycosylation of the obtained fractions was examined on a Western blot (Figure 3). Compared with a crude membrane preparation, the purified H^+,K^+ -ATPase fraction contained more glycosylated and almost no non-

Figure 4 N-glycosylation is essential for cell-surface expression of the H^+,K^+ -ATPase β -subunit

Confocal image analysis of H^+,K^+ -ATPase subunits in infected Sf9 cells. H^+,K^+ -ATPase subunits were revealed with subunit-specific antibodies (see the legend to Figure 1). Antibody binding was revealed with fluorescein isothiocyanate-labelled goat anti-mouse antibodies to monoclonal sera and tetramethyl rhodamine isothiocyanate-labelled pig anti-rabbit antibodies to polyclonal sera. (A) H^+,K^+ -ATPase α -subunit; (B) H^+,K^+ -ATPase β -subunit; (C) as (B), but with 5 μ g/ml tunicamycin in the culture medium.

glycosylated β -subunits. Because Sf9 cells are a relatively poorly studied cell type, there are no known marker enzymes from (sub)cellular organelles to specify the intracellular location of the functional H^+,K^+ -ATPase subunits. The non-glycosylated β -subunits were more abundant in the pellet fraction, in which only little H^+,K^+ -ATPase activity was found. This supports our conclusion that glycosylation is essential for H^+,K^+ -ATPase activity.

N-Glycosylation is essential for targeting the β -subunit to the plasma membrane

The intracellular distribution of the H^+,K^+ -ATPase subunits in recombinant-baculovirus-infected Sf9 cells was examined by confocal image processing. As shown in Figure 4(A), the H^+,K^+ -ATPase α -subunit is found exclusively in intracellular membranous structures. No detectable levels of the α -subunit were found in the plasma membrane, which is consistent with observations by others [17]. This means that the catalytically active H^+,K^+ -ATPase fraction also originates from an intracellular source.

The H^+,K^+ -ATPase β -subunit (Figure 4B) is partly targeted to the plasma membrane and partly localized in intracellular membranous structures. In the presence of 5 μ g/ml tunicamycin, the non-glycosylated β -subunit is localized entirely in intracellular membranous structures (Figure 4C). Apparently, proper processing of the H^+,K^+ -ATPase β -subunit to the plasma membrane depends on the presence of N-linked oligosaccharides on this subunit, although other explanations cannot be excluded. These results are in contrast with observations on the Na^+,K^+ -ATPase β -subunit by others [3–5]. It was demonstrated in several different cell types that the Na^+,K^+ -ATPase β -subunit does not need to be N-glycosylated to be transported to the plasma membrane. However, such a study has not yet been reported for the Na^+,K^+ -ATPase expressed in insect cells. Because the H^+,K^+ -ATPase α -subunit is detected exclusively in intracellular membranous structures, processing of the H^+,K^+ -ATPase β -subunit to the plasma membrane does not seem to be essential for synthesis of a functional H^+,K^+ -ATPase in insect cells.

N-Glycosylation is not essential for subunit assembly

In order to express H^+,K^+ -ATPase functionally, we showed the need for both H^+,K^+ -ATPase subunits to be co-expressed in individual Sf9 cells [8]. This means that both H^+,K^+ -ATPase subunits must be engaged in the formation of a heteroduplex molecule, or a higher-order structure, to establish catalytic

Figure 5 N-glycosylation is not essential for H⁺,K⁺-ATPase subunit assembly

Immunoprecipitation was performed as described in the Materials and methods section with an antibody against the H⁺,K⁺-ATPase α -subunit. Presence of the H⁺,K⁺-ATPase β -subunit in the precipitates was analysed by Western blotting. Lane 1, untreated Sf9 cells; lane 2, tunicamycin-treated Sf9 cells; lane 3, Sf9 cells expressing only the H⁺,K⁺-ATPase β -subunit. Abbreviations: β_c , β -subunit core protein (34 kDa); β_{gly} , glycosylated β -subunits (40–50 kDa); 5B6, monoclonal antibody used for precipitation of the H⁺,K⁺-ATPase α -subunit, which is itself stained during the protocol (molecular mass of heavy chain 55 kDa).

activity. This kind of protein interaction has often been studied with immunoprecipitation procedures. We therefore used this method to study the role of N-glycosylation in the assembly of H⁺,K⁺-ATPase subunits. The result is shown in Figure 5. In immunoprecipitates from untreated cultures, both glycosylated and non-glycosylated H⁺,K⁺-ATPase β -subunits were precipitated with the anti- α -subunit antibody (Figure 5, lane 1). This means that both forms of the β -subunit must be engaged with the α -subunit in a detergent-resistant complex and are therefore tightly associated. No glycosylated β -subunits were produced in tunicamycin-treated cultures. As a result, the quantity of non-glycosylated β -subunits was increased relative to untreated cultures. This effect was also visible in immunoprecipitates from tunicamycin-treated cultures (Figure 5; compare lane 2 with lane 1). In control precipitates from cells expressing only the H⁺,K⁺-ATPase β -subunit, essentially no β -subunits were precipitated with the anti- α -subunit antibody (Figure 5, lane 3).

DISCUSSION

The present study shows the effects of inhibitors of glycoprotein biosynthesis and processing (tunicamycin and dMAN respectively) on the activity of recombinant H⁺,K⁺-ATPase expressed in insect cells. There might be some limitations on the use of tunicamycin, however, because in several reports it has been noted that the inhibition of N-glycosylation by tunicamycin in expression studies *in vitro* resulted in the concomitant inhibition of protein synthesis [5,18,19]. This of course complicates the interpretation of the results of such experiments. Several lines of evidence, however, show that this is not so in the present study. First, Western blot analysis reveals no apparent decrease in the total amount of either H⁺,K⁺-ATPase α - or β -subunits (Figure 1). Secondly, the level of H⁺,K⁺-ATPase subunits in the membrane preparations has been quantified with ELISA techniques and no decrease in the amount of H⁺,K⁺-ATPase subunits was found (results not shown). In addition, the levels of endogenous (auto)phosphorylating enzymes and ATPases are hardly affected by tunicamycin in concentrations up to 10 μ g/ml. The same is true for the β -galactosidase activity that is used as a reporter enzyme (results not shown). It therefore seems that the inhibitory effect of tunicamycin on protein accumulation is cell-type-specific and might also be protein-specific. Apparently, adding tunicamycin to the growth medium does not affect the viability of the

insect cells. Because the level of H⁺,K⁺-ATPase subunit synthesis was unaffected by tunicamycin, the results of our experiments can be interpreted as they are. Hence our results clearly show that the acquisition of N-linked oligosaccharide moieties is essential for obtaining a catalytically active H⁺,K⁺-ATPase in insect cells.

The question of which mechanism of N-glycosylation is essential for catalytic activity of H⁺,K⁺-ATPase remains to be answered. An obvious explanation is that the sugar moieties are essential for subunit stabilization. Such a role has also been proposed for a variety of other (membrane) glycoproteins [20–22]. Another possibility is that an essential conformational transition of the H⁺,K⁺-ATPase subunits is obtained only after subunit glycosylation. Such a glycosylation-dependent folding event has also been reported for other proteins [23]. This option is supported by the fact that transport of the β -subunit to the plasma membrane depends on N-glycosylation events as well. However, this latter criterion does not discriminate for the functional properties of the recombinant H⁺,K⁺-ATPase in Sf9 cells because the functional enzyme is retrieved from intracellular membranous structures. In addition, another structurally related property of the β -subunit, assembly with the α -subunit into a heterodimer complex, does not depend on the presence of N-linked oligosaccharides on the β -subunit. This could mean that subunit assembly takes place before the subunits are to be glycosylated, or that the sugar moieties are otherwise engaged in the formation of a catalytically active H⁺,K⁺-ATPase. Therefore a much more interesting speculation might be that (some of) the sugar residues present in all precursor, intermediate and fully complex glycosylated forms (Man₃GlcNAc₂ N-linked to an Asn residue) on the H⁺,K⁺-ATPase subunits are themselves involved in the catalytic cycle of H⁺,K⁺-ATPase, but neither of the above-mentioned possibilities can be excluded by our results.

It would be interesting to examine whether the difference in dependence on N-glycosylation events between H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase could be related to some other differences between the two. For instance, for reasons as yet unknown, H⁺,K⁺-ATPase is much more susceptible to inactivation by detergent than Na⁺,K⁺-ATPase. The latter enzyme is usually purified by detergent extraction [24], but the use of detergents with H⁺,K⁺-ATPase almost always leads to loss of catalytic activity of most of the preparation [25].

The role of N-glycosylation for H⁺,K⁺-ATPase activity has not been studied before. Therefore no results about the role of N-glycosylation for H⁺,K⁺-ATPase activity are available. In this light, it is interesting to note that in highly purified native H⁺,K⁺-ATPase samples from gastric parietal cells, only glycosylated β -subunits are present. In fact, extensive glycosylation was one of the main reasons for the late discovery of the H⁺,K⁺-ATPase β -subunit [26]. This supports our conclusion that N-glycosylation is essential for gastric H⁺,K⁺-ATPase activity. In turn, because expression of H⁺,K⁺-ATPase subunits with the baculovirus expression system results in the synthesis of glycosylated as well as non-glycosylated β -subunits, our findings might also account for the presence of substantial amounts of non-functional H⁺,K⁺-ATPase subunits when using this system.

It should be noted that the use of tunicamycin to eliminate the formation of N-linked oligosaccharide structures does not allow us to discriminate between its effect on N-linked glycosylation events on either the α - or β -subunit of H⁺,K⁺-ATPase. Although we have used glycosylation patterns of the H⁺,K⁺-ATPase β -subunit to demonstrate the action of the inhibitors used in this study, the inhibitory action of tunicamycin on the catalytic properties of gastric H⁺,K⁺-ATPase cannot be ascribed automatically and exclusively to its effects on this H⁺,K⁺-ATPase

subunit. In fact, the presence of a cytosolically located N-linked sugar moiety, has been reported for the H^+,K^+ -ATPase α -subunit [26,27] and also for the Na^+,K^+ -ATPase α -subunit [28]. Although we have not yet established whether or not the recombinant-baculovirus-expressed H^+,K^+ -ATPase α -subunit is N-glycosylated, the possibility that a cytosolically located N-linked sugar moiety on the α -subunit could be involved in the catalytic cycle of H^+,K^+ -ATPase has also to be considered seriously. This latter option challenges our current understanding of how this proton pump functions.

We thank Dr. Michael Caplan and Dr. John Forte for their gift of antibodies and Dr. Ger Strous for his critical reading of the manuscript. This work was sponsored by the Netherlands Foundation for Scientific Research, division of medical sciences (NWO-GMW), under grant number 902-22-086.

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