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An intrinsic membrane glycoprotein with cytosolically oriented N-linked sugars

(Na$^+$ pump/Na$^+$,K$^+$-ATPase/N-glycosylation/protein glycosylation/glycoprotein topography)

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ABSTRACT **We demonstrate that the Na$^+$-pump α-subunit polypeptide is glycosylated by using bovine milk galactosyltransferase, a specific enzyme which attaches galactose to terminal *N*-acetylglucosamine residues. The galactose acceptor sites are available for glycosylation only after permeabilization of right-side-out vesicles prepared from kidney outer medulla; therefore, the oligosaccharide moieties are facing the cytoplasm of the cell. We further show that the oligosaccharides are bound to asparagine residues of the α-subunit polypeptide, since the protein–carbohydrate linkage is hydrolyzed by peptide-N glycosidase F (an enzyme specific for N-linked sugars). Thus, the Na$^+$-pump α subunit is a glycoprotein with its N-linked oligosaccharide moieties located at the cytosolic face of the cell membrane. Intrinsic membrane glycoproteins with such an oligosaccharide–protein linkage and cell membrane orientation have not been previously reported, to our knowledge.**

The sodium pump (Na$^+$,K$^+$-ATPase, EC 3.6.1.37) is responsible for the coupled active transport of Na$^+$ and K$^+$ across the plasma membrane of most eukaryotic cells (1, 2); it is composed of an α subunit and a heavily glycosylated β subunit (3, 4). The glycoprotein nature of the Na$^+$-pump β subunit has been widely recognized; however, the glycosylation status of the α subunit is controversial. There are as many papers reporting that the α subunit is glycosylated (5–9) as that it is not (10–18). Most of the papers reporting negative results used chemical methods to detect sugars that were probably insufficiently sensitive to detect glycosylation at the level reported in the present work. The methods used by us involve the attachment of a radioactive sugar to the glycoprotein and determination of the incorporated radioactivity.

The oligosaccharide chains of membrane glycoproteins have been almost exclusively found exposed to the extracellular environment, and current models of glycoprotein biosynthesis and post-translational processing account for this topography (19). Recently, evidence has been accumulating for the existence of membrane glycoproteins with carbohydrate moieties exposed at the cytoplasmic membrane surface (20–23). The common structural feature of these glycoproteins is a GlcNAc attached via an O linkage to serine or threonine residues of the peptide backbone. In contrast, many integral membrane proteins which are glycosylated have their sugar moieties N-linked to asparagine residues of the polypeptides. We now show that the α subunit of the Na$^+$ pump is glycosylated and that the N-linked carbohydrates are exposed to the cytosol.

EXPERIMENTAL PROCEDURES

Dog kidney Na$^+$,K$^+$-ATPase was prepared and assayed as described previously (24). The protein subunits were separated by polyacrylamide gel electrophoresis (PAGE) using the system described by Laemmli (25). Dog kidney outer medulla right-side-out vesicles were prepared and treated with saponin as reported by Kyte *et al.* (26). After treatment with saponin, the vesicles were separated from excess saponin by centrifugation. Permeable and intact vesicle preparations were labeled with either UDP-[4,5,-^{3}H]galactose (New England Nuclear) or UDP-[^{14}C]galactose (Amersham) and galactosyltransferase, as indicated below, and washed by centrifugation and resuspension. The protein was dissolved with SDS and separated by PAGE. The bands of Coomassie blue-stained α subunit were excised and dissolved in H_2O_2, and their radioactivities were determined. Controls performed in the presence of saponin showed that saponin does not interfere with the galactosyltransferase reaction. β-Elimination was performed as indicated by Capasso *et al.* (21). Controls in the absence of galactosyltransferase were always run in parallel, and the nonspecific radioactivity apparently bound was subtracted.

Autogalactosylation of Galactosyltransferase. Bovine milk galactosyltransferase (EC 2.4.1.22; Sigma) was allowed to autogalactosylate in the presence of 0.4 mM UDP-galactose (Sigma), 5 mM $MnCl_2$, 50 mM Tris·HCl at pH 7.2, 1 mM 2-mercaptoethanol, and 1% aprotinin, in a total volume of 250 μl. After 30 min at 37°C, an equal volume of glycerol was added. The galactosyltransferase was then stored in this solution (10 units/ml) at −20°C until used.

Radiolabeling of the Na$^+$,K$^+$-ATPase. Na$^+$,K$^+$-ATPase samples (about 0.1 mg) were resuspended in 40 mM galactose/1 mM 5′-AMP/0.2% aprotinin/5 mM $MnCl_2$/40 μM UDP-[^{3}H]-galactose (0.4–4 μCi; 1 Ci = 37 GBq)/5 mM Tris·HCl, pH 7.2/0.1 mM 2-mercaptoethanol/5% (vol/vol) glycerol/0.1-0.6 unit of galactosyltransferase. After incubation for 30 min at 37°C, the Na$^+$,K$^+$-ATPase was washed by centrifugation and resuspension with 50 mM Tris·HCl, pH 7.4.

Glycopeptidase Treatment. Purified Na$^+$-pump protein (about 80 μg in 15 μl) was dissolved with 15 μl of 1% SDS, then diluted with 25 μl of a buffer containing 200 mM EDTA and 4% (vol/vol) 2-mercaptoethanol, and taken to pH 7.5 with Trizma base, and 45 μl (0–10 units) of peptide N-glycosidase F (PNGase; EC 3.2.2.18) from *Flavobacterium meningosepticum* (Boehringer Mannheim N-glycosidase F) was added. The final SDS concentration was 0.15%. Digestion with PNGase was then performed at 37°C for 20–24 hr. Similar digestion results were obtained when 0.1% SDS and 0.5% Nonidet P-40 were used. In the latter case, the protein was separated by liquid chromatography rather than SDS/PAGE.

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Abbreviations: PNGase, peptide N-glycosidase F; ER, endoplasmic reticulum.

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RESULTS AND DISCUSSION

The Na^+-Pump α Subunit Is a Glycoprotein. [^{3}H]Galactose was transferred enzymatically from UDP-galactose to the purified Na^+,K^+-ATPase by incubation with bovine milk galactosyltransferase. This enzyme specifically catalyzes the addition of galactose to terminal GlcNAc residues (27). Both the α and β subunits of the Na^+ pump were labeled (Fig. 1). The β subunit contained 3- to 5-fold more radioactivity than the α subunit. That this is labeling of the Na^+,K^+-ATPase and not a minor contaminant was shown by immunoprecipitation of the radioactivity with a monoclonal antibody against the α subunit kindly provided by D. M. Fambrough (Johns Hopkins University). Right-side-out kidney vesicles were labeled with UDP-[^{3}H]galactose and galactosyltransferase. The protein immunoprecipitated by the antibody against the α subunit was radioactive (120 cpm; control 30 cpm). The amount of radioactivity which immunoprecipitated was markedly increased in vesicles treated with saponin prior to transferase action (443 cpm; control 59 cpm). Thus, the Na^+-pump α subunit is a glycoprotein whose oligosaccharide moieties possess terminal GlcNAc residues to which radiolabeled galactose binds. In a different experiment (data not shown) it was observed that, after electroblotting to a nitrocellulose membrane, the α subunit reacted with concanavalin A, a lectin that binds to α-mannosyl, α-glucosyl, and to a lesser extent GlcNAc residues (28).

Taking into account the specific radioactivity of the bound galactose and the phosphorylation capacity of the Na^+,K^+-ATPase preparation used in these experiments (24), our results suggest that about one radioactive galactose binds to the α subunit per phosphorylation site.

The Carbohydrate Moieties Are Facing the Cell Cytosol. The orientation of the oligosaccharides bound to the α subunit with respect to the cell membrane was studied by using right-side-out vesicles prepared from dog kidney microsomes. This is a biochemically and morphologically (by electron microscopy) characterized system (26, 29, 30) that has been used previously by several groups to determine the sidedness of reactive amino acids and ligand binding to the Na^+-pump protein. The vesicles are tightly sealed, and the cytoplasmic face of the membrane is inaccessible from the external medium unless the vesicles are made leaky. Our preparations contained at least 85% sealed right-side-out vesicles. This estimate was made by measuring the Na^+,K^+-ATPase activity before and after permeabilization of the vesicles by saponin. In the four experiments shown in Fig. 2, the median Na^+,K^+-ATPase activities were 0.2 and 1.7 μmol/min per mg of protein, before and after saponin treatment, respectively. Treatment with saponin opens the vesicles and results in free access of ATP and cations (Na^+, Mg^{2+}) to the catalytic sites on the Na^+,K^+-ATPase, which are known to be located at the cytosolic side of the cell membrane (2). Both intact and saponin-permeabilized vesicles were separately treated with UDP-[^{3}H]galactose and galactosyltransferase [which cannot cross the membrane (27)], washed by centrifugation and resuspension, and finally subjected to PAGE. The radioactivity bound to the α subunit for each condition was then determined. Permeabilization of the vesicles by saponin resulted in a 4- to 8-fold increase in incorporation of radioactivity (Fig. 2). This indicates that most of the galactose acceptor sites on the α subunit are facing the cytosolic side of the cell membrane. We also attempted to permeabilize the vesicles by physical means. This included sonication, freezing and thawing, and passage through a French press. None of these methods resulted in a major increase in either ATPase activity or [^{3}H]galactose incorporation. Under optimal sonication conditions, we were able to increase ATPase activity only 2-fold and [^{3}H]galactose incorporation about 1.8-fold. Previous attempts to permeabilize these vesicles by using similar physical approaches have also been unsuccessful (B. Forbush, personal communication). As an alternative to saponin we employed SDS (29), an anionic detergent. Treatment with SDS gave results similar to those with saponin (see Fig. 2).

It might be argued that the majority of the α subunits labeled with [^{3}H]galactose are not in vesicle membranes derived from the plasma membrane but in the membranes of

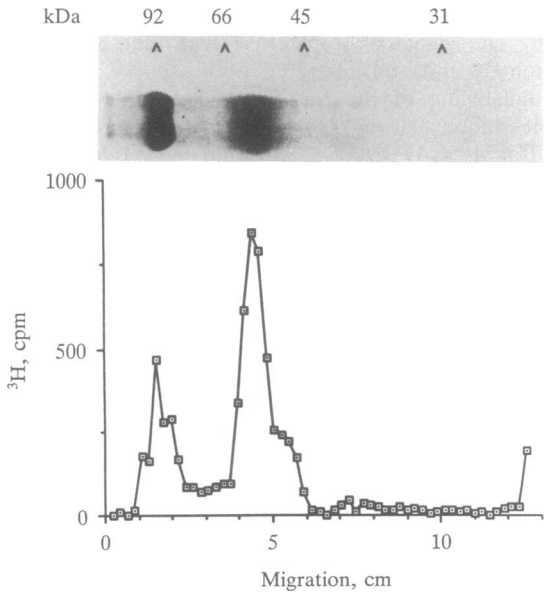

FIG. 1. Oligosaccharide labeling of the Na^+ pump α subunit. Purified Na^+,K^+-ATPase was labeled with UDP-[^{3}H]galactose and galactosyltransferase, and the protein subunits were separated by PAGE in a Laemmli 12% gel. After staining with Coomassie blue the gel was cut into small pieces and dissolved in H_2O_2, and the radioactivity was determined. The profile of radioactivity, the stained gel, and the positions of protein standards run in parallel are shown.

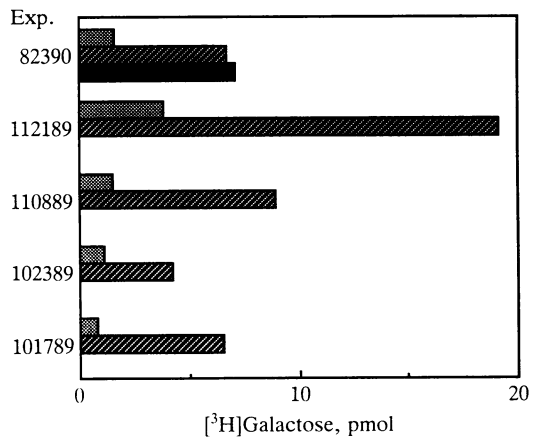

FIG. 2. Sidedness of the galactose-acceptor sites. Permeable (hatched bars) and intact (stippled bars) vesicle preparations were labeled with UDP-[^{3}H]galactose and galactosyltransferase. The results from parallel controls in the absence of galactosyltransferase were subtracted. The Na^+-pump subunits were separated by PAGE in a Laemmli 7.5% gel. The quantities of α-subunit protein in the gel as detected by the densitometer scanning of the intact, permeable, and control vesicles varied by less than 10%. The bands corresponding to the α subunit were excised and dissolved in H_2O_2, and their radioactivities were determined. The radioactivity content is expressed as pmol of galactose. The results of five independent experiments are shown. In experiment 82390, SDS (solid bar) was also employed to permeabilize the vesicles, as described previously (29).

either intracellular organelles or smaller membrane vesicles enclosed within the major vesicles during the preparation. There exists experimental evidence that suggests that this is not the case. Analysis of the vesicle preparation by electron microscopy did not show a significant amount of enclosed membranes inside the vesicles (30). Consistent with this, the exposure of latent Na^+,K^+-ATPase activity by treatment of the vesicles with saponin or deoxycholate showed a first-order dependence with respect to detergent concentration (see figure 2 in ref. 26) and not a two-phase process as would be expected if most of the Na^+,K^+-ATPase were contained in right-side-out smaller vesicles enclosed within the major vesicles. The possibility that most of the latent Na^+,K^+-ATPase is contained in membranes derived from organelles enclosed by the vesicles can be ruled out since the quantity of Na^+-pump molecules normally present in intracellular organelles of kidney cells is too low to account for the level of latent Na^+,K^+-ATPase measured by permeabilization of the right-side-out vesicles (31, 32). In our experiments there was a consistent correlation between permeabilization of the vesicles, the increase in Na^+,K^+-ATPase activity (which indicates accessibility of the ligands to the intracellular aspects of the Na^+-pump protein), and the increase in binding of [^{3}H]galactose to the α subunit. In conclusion, higher labeling after vesicle permeabilization necessarily means, under our conditions, labeling of the cytosolic aspects of the Na pump.

Oligosaccharides Are N-Linked to the α Subunit. The Na^+-pump protein was treated with PNGase to determine whether the oligosaccharides are N-linked to the protein. This enzyme specifically hydrolyzes the carbohydrate–peptide bond of N-linked sugars without affecting O-linked glycopeptides. Increasing concentrations of PNGase result in the progressive reduction of the apparent molecular weight of the β subunit (i.e., increase in mobility) in PAGE without affecting the mobility of the α subunit at all (Fig. 3 *Upper*). This latter observation might suggest that no cleavage of N-linked sugars from the α subunit had occurred. However, the autoradiogram of Fig. 3 *Lower* shows that the radioactivity bound to both subunits (α and β) was progressively lost at increasing concentrations of PNGase. The same result was obtained when the bands corresponding to the α subunit in a gel like that shown in Fig. 3 *Upper* were excised and their radioactivity was determined by scintillation counting. They had 201, 115, 82, 50, 57, and 16 cpm when 0, 1, 2, 4, 6, and 9 units of PNGase, respectively, were used. This is direct evidence, based upon the known specificity of PNGase, that most of the radiolabeled carbohydrate moieties of the α subunit are N-linked. The possibility that protease contamination of the PNGase preparation might account for the loss of label is made very unlikely by the following points. First, the PNGase treatment does not produce any appreciable change in the quantity of Coomassie blue-stained α subunit separated by gel electrophoresis (Fig. 3 *Upper*). Second, the PNGase used in our studies has been previously examined for protease or glycosidase contamination, and none was detected (33).

Another method often used to cleave the sugar-protein bond, which preferentially hydrolyzes O-linked oligosaccharides, is β-elimination. Application of this procedure (21) to the Na^+,K^+-ATPase did not release the radiolabeled carbohydrates bound to the Na^+-pump α subunit (data not shown). This is in accord with our observation (Fig. 3) that most of the radiolabeled oligosaccharide moieties bound to the α subunit are N-linked (and not O-linked) to the protein. Parallel runs carried out with α-fetuin as the substrate for β-elimination resulted in the liberation of small molecular weight hexoses, as would be expected for a glycoprotein containing O-linked oligosaccharides.

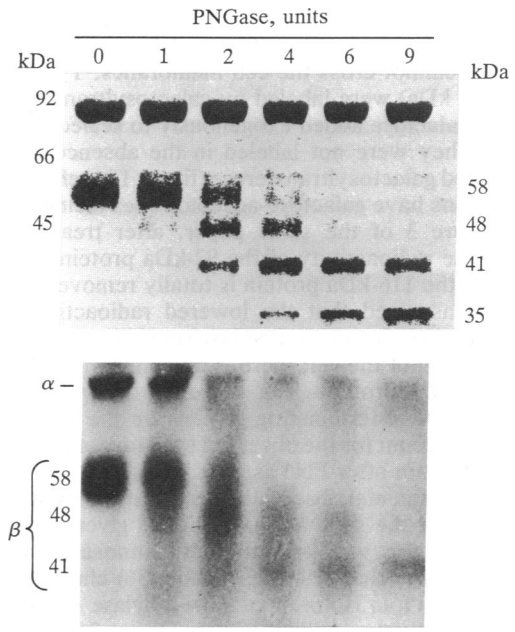

FIG. 3. (*Upper*) Effect of increasing concentrations of PNGase on the mobility of the Na^+-pump subunits. Purified Na^+-pump protein was treated with PNGase and the subunits were separated in a 12% gel. The molecular masses of standard proteins run in parallel are shown on the left. The apparent molecular masses calculated for the β subunit are shown on the right. (*Lower*) Autoradiogram of a gel similar to that shown in *Upper* with [^{14}C]galactose-labeled Na^+-pump protein. After electrophoresis the gel was dried and the autoradiography was done using Kodak X-Omat film with exposure for 10 days at −80°C. The positions of the α and β subunits are indicated.

The lack of change in the molecular weight of the α subunit after PNGase treatment (Fig. 3 *Upper*) suggests that the oligosaccharide chains bound to Na^+-pump α-subunit are probably smaller than those previously described for other N-linked carbohydrate chains (34). It should be noted that PNGase is able to hydrolyze from the protein N-linked oligosaccharides of various sizes, and it has recently been shown that the oligosaccharides need at least be a dimer of sugar residues N-linked to the protein for enzymatic cleavage to occur (35). Further studies are necessary to determine the size and composition of the oligosaccharides bound to the Na^+-pump α subunit.

Recent publications have described the existence of proteins that contain carbohydrates facing the cytosolic medium (reviewed in ref. 23). The bulk of these oligosaccharides are single GlcNAc residues which are O-linked to the proteins. However, a small proportion of these carbohydrates appear to be resistant to β-elimination and sensitive to PNGase. The significance of these observations has not been commented upon, but they suggest the existence of N-linked cytosolic oriented oligosaccharides on other proteins. For example, Holt *et al.* (20) labeled intact and permeabilized erythrocytes with galactosyltransferase and UDP-[^{3}H]galactose. They observed that permeabilization of the erythrocytes increased the galactosylation about 16 times; since 38% of the galactosylated oligosaccharides are resistant to β-elimination after permeabilization, as much as 32% of the intracellular radiolabeled oligosaccharides could be N-linked. In keeping with this, the cytosolic subcellular fraction separated by these authors appeared to contain a high percentage of galactosylated carbohydrates susceptible to PNGase degradation (see figure 2 in ref. 20). In a similar study, but using right-side-out sealed vesicles derived from the Golgi apparatus of rat liver, Capasso *et al.* (21) observed that many protein molecules were labeled by galactosyltransferase and UDP-[^{14}C]galac-

tose. Sealed Golgi vesicles accumulate UDP-galactose added exogenously; however, any exogenously added galactosyltransferase cannot cross the cell membranes. Two proteins (116 and 92 kDa) were labeled by galactosyltransferase and UDP-[^{14}C]galactose added exogenously to sealed Golgi vesicles, and they were not labeled in the absence of exogenously added galactosyltransferase (figure 1 in ref. 21). Thus, these proteins have galactose-acceptor sites facing the cytosol. In figure 3 of the same paper, after treatment with PNGase, the radioactivity of the 92-kDa protein is reduced and that of the 116-kDa protein is totally removed. Capasso *et al.* (21) assumed that the lowered radioactivity in the 92-kDa protein is due not to PNGase sensitivity but to a lower protein loading of the gel; there is no comment with respect to the 116-kDa protein. The presence of cytosolic-facing N-linked GlcNAc-terminating glycans on the 116-kDa protein would account for the observed total loss of radioactivity from this protein after PNGase treatment.

Other experimental observations suggest the existence of a mechanism for the synthesis of N-linked glycans which are smaller than the well-known high-mannose or complex N-linked oligosaccharides. Hase *et al.* (36) chromatographically separated four isoforms of Taka-amylase A, a glycoprotein produced by *Aspergillus oryzae*. Three of these isoforms have high-mannose oligosaccharides N-linked to the protein, but one has only a single N-linked GlcNAc. The authors speculate that the short N-linked glycan may have been produced from a more complex glycan by a glycosidase; it is also possible that this small glycan may be the product of a specialized mechanism of synthesis. In support of this alternative, Lehle and Tanner (37) have shown that the yeast enzyme, which transfers the oligosaccharide chain from the lipid intermediate DolPP-$Glc_3Man_9(GlcNAc)_2$ (DolPP = dolichyl diphosphate) to Asn residues of glycoproteins in the lumen of the endoplasmic reticulum (ER), is also capable of transferring short oligosaccharides from DolPP-$(GlcNAc)_2$-Man_1 and DolPP-$(GlcNAc)_2$ to endogenous glycoproteins. It is not known whether the transfer of the short oligosaccharides occurred in the cytosolic or lumenal side of the ER. Normally the transfer of oligosaccharides occurs after partial elaboration of the dolichol intermediate in the cytosol and following transmembrane flipping into the lumen of the ER. Sharma *et al.* (38) extended the previous observations by using a soluble glycosyltransferase and a synthetic peptide as substrate. Even though these are *in vitro* experiments, it is possible that the same or similar enzyme(s) may be responsible for the transfer *in vivo* of short N-linked glycans.

Small PNGase-sensitive glycans have not been reported in previous studies. The reason for this may be one of several possibilities. The small PNGase-sensitive glycans appear to account for only a small percentage of both protein and carbohydrate mass. Thus, following cleavage of the sugar–protein linkage the small oligosaccharides released could easily escape detection or be mistaken for small amounts of contaminating unincorporated radioactivity or degradation products. Of course, another possibility is that PNGase-sensitive GlcNac-terminating glycans may not be present on the previously well-studied glycoproteins.

The data presented in this communication demonstrate that the Na^+-pump α subunit is a glycoprotein with the carbohydrate chains facing the cytosolic medium. This unusual topographic arrangement of the carbohydrate has recently been reported in some integral membrane O-linked glycoproteins of ER (22) and Golgi apparatus (21) and other membrane-associated proteins (reviewed in ref. 23). In contrast to these proteins, the Na^+-pump α subunit is a glycoprotein that has N-linked carbohydrates facing the cytoplasmic medium. The existence of structural membrane glycoproteins with N-linked cytosolic-facing oligosaccharides challenges our current understanding of the mechanism of synthesis of N-linked oligosaccharides. Usually, N-linked oligosaccharides bind to Asn residues of the polypeptides that are in the sequence Asn-Xaa-Ser (or Thr), where Xaa may be any amino acid residue (39). The topographic orientation of the N-glycosylation described in this paper is consistent with the fact that the potential N-glycosylation sites of the α subunit are all located in what are assumed to be intracellular loops of the primary structure (40). However, the possibility that the cytosolic N-linked oligosaccharides bind to Asn residues which are not part of the consensus sequence described above cannot be ruled out.

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