Role of N-Linked Glycosylation in Rat Renal Na/P\textsubscript{i}-Cotransport*

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Our laboratory recently identified a sodium-dependent transport system for phosphate from rat kidney cortex (NaP\textsubscript{2}-2; Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., Biber, J., and Murer, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5979-5983). In the present study we have investigated whether or not this cotransporter is glycosylated and the role of N-glycosylation in determining its function. Glycosidase digestion of the NaP\textsubscript{2}-2 protein from rat brush border membranes, in vitro translation studies, or oocyte expression of the NaP\textsubscript{2}-2 cRNA indicate that the mature protein is glycosylated. Glycosidase treatment reduces the size of the protein from ~70-110 kDa to ~60-65 kDa. We therefore used site-directed mutagenesis to identify which of the putative consensus sites for N-linked glycosylation are utilized in the mature NaP\textsubscript{2}-2 protein. Altering the nucleotide sequences encoding both of the Asn-288 and Asn-328 residues to Gln produced mutants that are completely devoid of glycosylation, whereas mutants in which each of these sites were mutated separately are glycosylated when expressed in oocytes. These results suggest that both of these sites are modified by N-linked glycosylation in the mature protein. Surface expression of glycosylated and unglycosylated NaP\textsubscript{2}-related proteins was documented by biotinylation experiments. In contrast to the wild-type (fully glycosylated) transporter, immunocytochemistry provides evidence for a partial intracellular localization of mutant unglycosylated cotransporters. Na/P\textsubscript{i} cotransport was studied in oocytes expressing wild-type or mutagenized NaP\textsubscript{2}-related proteins using tracer or electrophysiological techniques. Although the transport rates are lower (by a factor of 2-3) after expression of the unglycosylated NaP\textsubscript{2} protein, the P\textsubscript{i} transport characteristics (pH dependence, apparent affinity for P\textsubscript{i}, or Na\textsuperscript{+}) are similar in oocytes expressing either wild-type or glycosylation-deficient proteins.

Inorganic phosphate (P\textsubscript{i}) homeostasis in the body is achieved largely by the regulated reabsorption of P\textsubscript{i} in the proximal convoluted tubule of the kidney. Proximal tubular epithelial cells utilize specific apical and basolateral membrane transport proteins for the vectorial reabsorption of P\textsubscript{i} (2, 3). Physiological regulation of transcellular proximal tubular P\textsubscript{i} flux involves alterations in the rate of apical Na/P\textsubscript{i} cotransport (2).

Our laboratory has recently cloned cDNAs encoding three closely related NaP\textsubscript{i} cotransport systems from human and rat kidney cortex and from the opossum kidney (OK) cell line which, upon expression in Xenopus laevis oocytes, all display sodium-dependent P\textsubscript{i} transport with substrate specificities and kinetic properties resembling those of proximal tubular brush border membrane transport proteins (1, 4). The deduced protein sequences of these cotransporters are highly homologous but are different to the previously cloned sodium-dependent P\textsubscript{i} transporter from the rabbit (NaP\textsubscript{1}; Ref. 5). Northern blot and/or reverse transcriptase-polymerase chain reaction analysis have identified the mRNA encoding the rabbit (NaP\textsubscript{1}) and rat (NaP\textsubscript{2}) cotransporters within the proximal tubule of the kidney cortex (6, 7). In addition, immunoblot and immunohistochemical analyses, using antibodies directed against the NaP\textsubscript{1} and NaP\textsubscript{2} proteins, have confirmed this distribution and further localized these proteins to apical brush border membranes within the proximal convoluted tubule (6, 8). The distribution of the NaP\textsubscript{1} and NaP\textsubscript{2} proteins suggests that both types of Na/P\textsubscript{i} cotransport systems play a role in proximal tubular apical Na/P\textsubscript{i} cotransport. It therefore seems likely that differences in the function of the NaP\textsubscript{1}, -2, -3, and -4 cotransporters contribute to the apparent heterogeneity in NaP\textsubscript{i} cotransport observed in isolated brush border membrane vesicles (2, 9).

The deduced primary structure of the NaP\textsubscript{2} cotransporter predicts a protein of 637 amino acids with a calculated mass of approximately 68 kDa. Notably, the deduced amino acid sequence of the NaP\textsubscript{2} protein includes several putative consensus sites to signal N-linked glycosylation, two of which are located within a putative extracellular domain (Refs. 1 and 10 and Fig. 3A). Immunoblot analysis of isolated renal brush-border membranes suggests that the mature NaP\textsubscript{2} protein is subject to post-translational modification (glycosylation) as its molecular mass is ~80-90 kDa, that is ~10-20 kDa larger than that predicted for the primary amino acid sequence (7). Therefore, in the present study we have investigated whether or not the NaP\textsubscript{2} cotransporter is glycosylated and the role of N-glycosylation in determining the function of this protein. We have used in vitro mutagenesis to alter two consensus sequences for N-linked glycosylation, present within a putative extracellular domain of NaP\textsubscript{2}, and analyzed the glycosylation state and the transport function of the mutagenized and "wild-type" NaP\textsubscript{2} cotransporters.

MATERIALS AND METHODS

X. laevis Oocytes

All methods and reagents used for the preparation and manipulation of oocytes have been described in detail elsewhere (11). Plasmids encoding NaP\textsubscript{2} and mutant cDNAs were linearized using XbaI and used for the in vitro synthesis of cRNA, including capping, using T7 RNA polymerase (12). 50 nl of water or water containing cRNA (native or...
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mutagenized NaP-2 was injected into oocytes using a semi-automatic injector (Inject-Matic system, J. A. Gabay, Geneva). Tracer studies, measuring the uptake of 32PO$_4$-$_2$ were performed 1–3 days after injection; electrophysiological measurements of P, inward current were performed 3–8 days after injection, and immunocytochemistry was performed 4 days after injection.

Membrane Purification

Rat kidney brush-border membranes were isolated according to a magnesium-precipitation technique as described (13). Yolk-free homogenates were prepared from oocytes 1–3 days after injection with water or cRNA (5 ng). 20–25 oocytes were washed twice in Barth’s solution (mM 86 NaCl, 1 KCl, 0.82 mM MgSO$_4$, 0.41 mM CaCl$_2$, 0.33 mM Ca(NO)$_3$, 2.4 mM NaHCO$_3$, 10 mM Hepes-Tris-HCl, pH 7.4, and 2 mg/ml gentamicin) and once in oocyte homogenization buffer (mM 250 sucrose, 0.5 mM EDTA, 5 mM Tris-HCl, pH 6.9, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/mL leupeptin) before homogenization in 4 µl of homogenization buffer/oocyte by 10 passages through a 25-gauge needle. Homogenates were centrifuged twice at 100,000 x g for 10 min. (4°C) in order to sediment the yolk proteins. The supernatant was used directly for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or immunoprecipitation or stored at -70°C. Protein determinations were obtained using the Bradford assay (14).

Gel Electrophoresis and ImmunobLOTS

Membrane proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes (15). Protein size standards from Bio-Rad were used to estimate the relative molecular mass of SDS-PAGE separated proteins. Polyclonal antibodies, raised against synthetic peptides corresponding to the deduced amino-terminal amino acid sequence of NaP-2 protein, were used to detect NaP-2 related immunoreactivity (7). Similar results are obtained using a polyclonal antibody raised against the carboxy-terminal amino acid sequence of NaP-2 (data not included). The specificity of these antibodies was previously established by the fact that inclusion of antigenic peptides in the immunogen sequence of the NaP-2 protein, were used to detect NaP-2 related proteins from 20 µg of NaP-2 cRNA solubilized in 1% SDS by boiling for 2 min. Samples were then diluted at a ratio of 1:10 in 20 mM sodium phosphate, pH 7.2, 10 mM sodium azide, 50 mM EDTA, 0.5% (w/v) Nonidet P-40 and boiled again for 2 min. After cooling, peptide/N-glycosidase F (Boehringer Mannheim) was added to a concentration of 2 units/µl and the samples were incubated at 37°C for 24 h. NaP-2 related proteins were immunoprecipitated from the denatured homogenates prior to SDS-PAGE and autoradiography.

In Vitro Mutagenesis

Oligonucleotides N290Q (5‘-GAGTCCCGAGGAGCAAGGTCCT-3‘) and N328S (5‘-GGCCAGCTGCGACAAGGGGGCAGA-3‘) (Microsynth, Windisch, Switzerland) were used for site-directed mutagenesis of the nucleotide sequences encoding the NaP-2 Asn-298 and Asn-328 residues to Glu. The NaP-2 cRNA was subcloned into the pAl vector and the Altered Sites in vitro Mutagenesis System (Promega) was used to perform the mutagenesis according to the manufacturer’s instructions. Mutants were identified by nucleotide sequence analysis (18).

Cell Surface Biotinylation

The cell surface biotinylation procedure was adapted from a published protocol (19) and was performed 3 days after injection of oocytes with water or cRNA (10 ng). Oocytes were washed five times for 5 min in Barth’s solution containing 10 mM triethylenamine, pH 9.0, at 4°C, then incubated in the Barth’s triethylenamine solution supplemented with 2.2 mM (1,200 dilution from frozen aliquots in dimethyl sulfoxide) of the biotinylation reagent sulfohydroxymethylboiin long chain (NHS-LC-biotin, Pierce) at 4°C. After 30 min the solution was replaced and the oocytes incubated a further 30 min at 4°C in the presence of the biotinylation reagent. The labeling solution was then replaced with a Barth’s triethylenamine solution containing 5 mM glycine for 10 min, and the oocytes were finally washed twice for 5 min in Barth’s triethylenamine solution. Yolk-free homogenates were prepared as described (see “Membrane Purification”). Biotinylated NaP-2-related proteins were detected after immunoprecipitation of NaP-2-related proteins and streptavidin blotting. Immunoprecipitation and blotting were performed as described (see “Glycosidase Digestion and Immunoprecipitation”).

Immunocytochemistry

Oocytes were fixed by immersion in a solution of 3% paraformaldehyde in PBS for 20 min and stored in 15% sucrose, PBS at 4°C for 4 days. Single eggs were then frozen onto thin cork slices using liquid nitrogen cooled liquid propane. Cryosections (5 µm) were cut and processed as described previously for immunohistochemistry (7). Control oocytes were incubated with preimmune rabbit serum or with antisera preabsorbed for 30 min with 100 µg/ml antigenic peptide. Immunofluorescence was revealed by laser scanning microscopy (Zeiss LSM 310, Zeiss Oberkochen, Germany).

Transport Studies

Measurement of Tracer Uptake—Uptake experiments were performed using 0.5 µM 32PO$_4$-$_2$ (20 µCi/ml) in the presence of 100 µM Na$^+$ as described previously (11). Total P, uptake is reported; when 1 µg of

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Endo-H, endoglycosidase H.
Fig. 1. Immunoblot analysis of rat brush-border membrane proteins (A), yolk-free homogenates from water- and NaPi-2 cRNA-injected oocytes (B). Samples were mixed with SDS-PAGE gel loading buffer (2% SDS, 20% glycerol in 120 mM Tris-HCl, pH 6.8 (final concentration) in the presence (B) or absence (A) of 100 mM dithiothreitol, heated to 95 °C for 2 min, and separated on a 9% polyacrylamide gel prior to the electrotransfer of proteins to nitrocellulose (15). Electrobotted proteins were incubated with polyclonal antibodies raised against a synthetic peptide corresponding to the NH2 terminus of NaPi-2 (7). The higher molecular mass bands seen in immunoblots from brush-border membranes (A) are related to NaPi-2 and could represent some undefined aggregates (7). C, autoradiograph of an SDS gel showing NaPi-2-related immunoprecipitates from metabolically labeled water- and NaPi-2 cRNA-injected oocytes. Immunoprecipitation was performed using the NH2-terminal anti-(NaPi-2) antiserum as described under "Materials and Methods." The approximate molecular mass of proteins is indicated (in kilodaltons) beside all figures showing SDS-PAGE in this paper.

NaPi-2 cRNA is injected into oocytes, the sodium-independent uptake of Pi is approximately 5% of total Pi uptake (1). Uptake by water-injected oocytes was used as a control.

Electrophysiological Measurements—Two-electrode current- and voltage-clamp recordings were performed in an external solution (sucrose) containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (titrated with NaOH or HCl to the pH indicated) at room temperature as described (20). Phosphate was added to the superfusate at a concentration of 1 mM or as indicated (Fig. 7B). In all experiments phosphate-induced current (Ip) was measured at a holding potential of +50 mV. The size of the Ip varied 2–3-fold depending on the time period after cRNA injection and on the different batches of oocytes (different animals). Therefore experimental data obtained on a single day from multiple oocytes derived from one batch is presented.

RESULTS

Immunodetection of the NaPi-2 Protein—Antibodies directed against the deduced amino-terminal amino acid sequences of the cloned NaPi-2 cotransporter were used to identify the NaPi-2-related proteins in immunoblots from rat brush-border membranes and NaPi-2 cRNA-injected X. laevis oocytes. The native cotransporter from rat brush-border membranes exhibits an apparent molecular mass of ~80–90 kDa (Fig. 1A and Ref. 7), whereas in immunoblots prepared from cRNA-injected oocytes, immunoreactive ~80–110-kDa proteins, as well as a smaller, distinct NaPi-2-related protein with an apparent molecular mass of ~70 kDa were observed (Fig. 1B). It seems likely that the larger molecular weight bands apparent in Fig. 1A are aggregates of NaPi-2-related proteins, as they are not observed in the presence of antigenic peptide or when the brush-border membrane samples are treated with 10–100 mM dithiothreitol prior to SDS-PAGE (data not shown). Metabolic labeling, using [35S]Met, and immunoprecipitation were also used to identify NaPi-2-related proteins expressed in cRNA-injected oocytes; labeled proteins ranging in size from ~70 to 110 kDa, similar to those detected by immunoblot analysis, were identified (Fig. 1C). These proteins are not detected in water-injected oocytes (Fig. 1, B and C) and were not detected by preimmune sera (results not shown). Together, the results presented in Fig. 1 suggest glycosylation of NaPi-2-related proteins, since the apparent molecular mass of these proteins is ~10–40 kDa larger than that predicted from the deduced primary amino acid sequence of the NaPi-2 protein (1).

Endo-H Digestion of in Vitro Translated NaPi-2 Proteins—NaPi-2, related in vitro translation products were synthesized from the NaPi-2 cRNA in the presence of [35S]Met, separated by SDS-PAGE, and visualized by autoradiography. As we have shown previously, the major NaPi-2 translation products synthesized in the presence of microsomes are larger than the ~66-kDa products synthesized in the absence of microsomes (Ref. 1 and Fig. 2A). The microsome-dependent shift in the size of the NaPi-2 translation products was completely reversed by Endo-H treatment, indicating that the decreased mobility observed after translation in the presence of microsomes is due to glycosylation (Fig. 2A).

Peptide-N-Glycosidase F Digestion of Oocyte-expressed NaPi-2 Proteins—Peptide-N-glycosidase F cleaves the bond between asparagine and N-acetyl-D-glucosamine (GlcNAc) residues that joins the carbohydrate moiety to glycosylated proteins, thereby liberating all N-linked oligosaccharides from glycoproteins (unless located at amino or carboxyl termini (21)). This enzyme was therefore used to examine whether or not the mature NaPi-2 cotransporter from cRNA-injected oocytes is glycosylated. Homogenates from [35S]Met-labeled NaPi-2 cRNA-injected oocytes were incubated in the presence or absence of peptide-N-glycosidase F. NaPi-2-related proteins were then immunoprecipitated, separated by SDS-PAGE, and autoradiographed in order to visualize NaPi-2-related proteins. The results in Fig. 2B indicate that the mature protein is deglycosylated by this enzyme to reduce the apparent size of the (radio)labeled cotransporter to ~60–65 kDa. In contrast to the broad band of the untreated NaPi-2 sample, the deglycosylated products migrate as a single sharp band under SDS-PAGE. This indicates that the apparent higher molecular mass of the mature cotransporter is due to the addition of asparagine-linked core oligosaccharide to the primary structure of the NaPi-2 protein. The migration of the glycosylated cotransporter suggests that mature NaPi-2 proteins range in size from ~80 to 110 kDa. This is likely to be due to heterogeneity in the extent of glycosylation of the mature cotransporter. Similar data were obtained by applying the same procedure to isolated rat renal brush-border membranes (data not shown).

Mutagenesis of Putative Glycosylation Sites within the NaPi-2 Cotransporter—The primary structure of the NaPi-2 protein includes several putative consensus sites for N-linked glycosylation (Fig. 2B). The microsome-dependent shift in the size of the NaPi-2 translation products was completely reversed by Endo-H treatment, indicating that the decreased mobility observed after translation in the presence of microsomes is due to glycosylation (Fig. 2A).

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A schematic representation of the structure of the sodium-dependent phosphate cotransporter NaP,-2 is shown. Asn residues, within the NaP,-2 structure, are shown for details, see Refs. 1 and 10. (A) A, a schematic representation of the putative secondary structure for the rat proximal tubular sodium-dependent phosphate cotransporter NaP,-2. The putative location of four potential glycosylation sites, Asn residues, within the NaP,-2 structure is shown (for details, see Refs. 1 and 10). B, autoradiograph of an SDS gel showing the [35S]Met-labeled immunoprecipitates from wild-type (NaP,-2) and mutant (N298Q, N328Q) or the double mutant (N298Q+N328Q) NaP,-2 proteins expressed in cRNA-injected oocytes. NaP,-2-related proteins are not detected in control water-injected oocytes.

The relative size of the labeled translation products was analyzed by SDS-PAGE and autoradiography (Fig. 3B; see also Figs. 1C and 2B). Expression of the double glycosylation mutant (N298Q+N328Q) results in the detection of a single sharp protein band with an apparent size of ~60–65 kDa (Fig. 3B). Notably, this size is in close agreement with that of the enzymatically deglycosylated cotransporter from cRNA-injected oocytes (Fig. 2B). In contrast, injection of cRNAs encoding each of the single mutants (N298Q or N328Q) results in the expression of larger, glycosylated, forms of the cotransporter (Fig. 3B). These results indicate that both of the Asn-298 and Asn-328 residues are glycosylated and that these residues are the major sites of glycosylation in the mature NaP,-2 cotransporter. Similar results were obtained from immunoblot analysis of oocyte-expressed mutants (data not shown).

Surface Location of Oocyte-expressed NaP,-2-related Proteins (Wild-type and "Unglycosylated")—In order to decide whether or not the glycosylated NaP,-2-related protein (N298Q,N328Q; double glycosylation mutant) is exposed at the oocyte surface, we have performed surface biotinylation experiments followed by immunoprecipitation with an anti-NaP,-2 antibody and streptavidin blotting (Fig. 4B). These experiments show that both forms of NaP,-2-related proteins (wild-type and "double glycosylation mutant") are efficiently transported to the oocyte surface. Reciprocal experiments, in which surface biotinylation was followed by streptavidin-agarose precipitation and immunoblotting with a NaP,-2-specific antibody, provided qualitatively similar results (data not shown).

Immunocytochemical detection, using an antibody that specifically recognizes NaP,-2-related proteins, shows intense staining at the oolemma in oocytes expressing either wild-type NaP,-2-related proteins or the double glycosylation mutant (Fig. 5). Interestingly, we have repeatedly observed intracellular staining in oocytes expressing the double glycosylation mutant, but not in oocytes expressing the wild-type transporter (Fig. 5). This observation suggests a greater intracellular location of the double mutant (see "Discussion").

P, Transport by Glycosylation Mutants—The functional role of glycosylation was investigated by studying the expression of the cotransporter in X. laevis oocytes. NaP,-2-related P, transport can be measured by tracer fluxes (1) or by electrophysiological techniques (20). Fig. 6A shows that oocytes injected with either the wild-type or the glycosylation-deficient double mutant cRNA exhibit a significant enhancement in their ability to transport radiolabeled P, compared with water-injected oocytes. Furthermore, in electrophysiological studies, the addition of P, (1 mM) to the extracellular fluid induced a net inward current (I) across the cell membrane of oocytes injected with cRNA encoding either the wild-type or the double-mutant NaP,-2 cotransporters (Fig. 6B). Therefore, it appears that glycosylation of the cotransporter is not required for the transport of P, by the NaP,-2 cotransport system, since the double mutant retains transport function. However, the amount of transport mediated by expression of the double mutant is always less than that mediated by the wild-type transporter (see "Discussion").

The sodium concentration dependence (Fig. 7A), the P, concentration dependence (Fig. 7B) and the pH dependence (Fig. 8) of P, transport were all found to be similar between the wild-type NaP,-2 protein, the two single mutants, and the double mutant. Therefore, the data presented in Figs. 7, A and B, and 8 suggest that glycosylation does not, on a qualitative level, influence the transport function of the NaP,-2 protein. It should be mentioned that Na/P, cotransport most likely operates at a 3:1 (Na/P,) stoichiometry which is supported by the simple Michaelis-Menten behavior of the P, concentration dependence and the sigmoidal dependence on sodium concentration (see also, Refs. 1 and 20)). The pH dependence of NaP, cotransport is not fully understood; recent studies suggest a decreased Na+ affinity at low pH as the major mechanism for the pH dependence of I, but other mechanisms such as preferential transport of divalent P, may also take place (1, 20).
A protein includes four consensus sites for N-linked glycosylation, two of which (Asn-298 and Asn-328) are located in the extracellular domain within the current secondary structure model for NaP,-2 (Refs. 1 and 10 and Fig. 3A). The potential use of one or several of these consensus sites is suggested by the observation that the apparent molecular mass of NaP,-2-related proteins from rat kidney brush border membranes and NaP,-2 cRNA-injected X. laevis oocytes is ~10-40 kDa larger than that predicted by the primary amino acid sequence of this protein (Fig. 1 and Ref. 7). Furthermore, treatment of homogenates from NaP,-2 cRNA-injected oocytes (and rat kidney brush border membranes; data not shown) with peptid-N-glycosidase F resulted in a reduction in the apparent molecular mass of the protein from ~70-110 kDa to ~60-65 kDa. Finally, the microsome-dependent increase in the apparent molecular mass of the NaP,-2 products obtained by in vitro translation is completely reversed by treatment with endoglycosidase H (Fig. 2A, Endo H and Ref. 1). In total, these results indicate that the mature NaP,-2 protein is a glycoprotein.

In the present study we have used site-directed mutagenesis to further document the glycosylation of the NaP,-2 protein and to analyze the functional role of glycosylation. As secondary structure predictions place two of the consensus sites for glycosylation, Asn-298 and Asn-328, extracellularly (see above) we have focussed on the replacement of these two sites. Three mutants were created such that either and/or both of these residues were replaced with Gln. The double mutant (N298Q,N328Q) does not appear to be glycosylated, since metabolic labeling and immunoprecipitation of NaP,-2-related proteins from oocytes expressing this mutant reveal a single protein species with a lower apparent molecular mass than any of the glycosylated forms of NaP,-2. Notably, the size of the ~60-65-kDa translation product from this mutant is consistent with that of the enzymatically deglycosylated (peptid-N-glycosidase F-treated) NaP,-2 protein (Figs. 2B and 3B). The oocyte-expressed translation products from each of the single site NaP,-2 mutants include proteins that are larger than the primary translation product. These results indicate that both of these residues (Asn-298 and Asn-328) are glycosylated in the mature NaP,-2 cotransporter (Fig. 3B). Evidence for glycosylation of Asn-298 and Asn-328 substantiates the positioning of these residues within an extracellular domain of the protein. Furthermore, the lack of glycosylation in the double mutant suggests that the other consensus sites for glycosylation are most likely not used.

The functional role of glycosylation was investigated by studying the expression of the wild-type and glycosylation-deficient mutant NaP,-2 proteins in oocytes. Oocytes expressing the fully glycosylated wild-type and the unglycosylated double mutant NaP,-2 proteins are both capable of P, transport with unaltered qualitative properties, indicating that glycosylation is not essential for the functional expression of this protein (Figs. 6-8). However, in both tracer and electrophysiological experiments the amount of transport mediated by the glycosylation-deficient mutants appears to be reduced relative to that of the wild-type cotransporter.

Oocytes expressing the unglycosylated transporter exhibit a reduced (by a factor of 2-3) transport rate of NaP,-2 cotransport in comparison with the wild-type transporter (see above). This difference in transport rate might be a consequence of different amounts of NaP,-2-related protein being present at the oocyte surface or due to an alteration in transfer rate per transporter molecule. Unfortunately, the differences in transport rates (maximally a factor of 2-3) and oocyte variability preclude a reliable quantification (within a factor of 2-3) of surface exposed NaP,-2-related protein and do not allow a conclusion as to whether or not the transport rate per transporter is altered (by a factor of 2-3) by protein glycosylation. There are several theoretical and experimental reasons for not being able to perform such quantification with sufficient accuracy (required for the observed difference in transport). (i) The efficiency of interaction between the antibody used for immunocytochemistry as well as with reagents used for surface labeling (e.g. biotinylation) might be significantly influenced by the presence or absence of glycosylation; (ii) glycosylated and unglycosylated NaP,-2-related proteins show a completely different behavior in electrophoresis, making a comparative quantification by densitometry difficult (broad versus sharp band); (iii) there is significant variability in the capacity of individual oocytes to express proteins. Therefore, we cannot offer a firm statement that surface expression of NaP,-2-related protein is equal or different for the wild-type and unglycosylated NaP,-2 cotransporters. Nevertheless we have consistently observed that the transport rate of the unglycosylated NaP,-2 protein is reduced relative to...
that of the wild-type protein and, using immunocytochemistry, that only oocytes expressing the unglycosylated protein exhibit intracellular NaPi-2-related staining. Therefore, we suggest that the transport rate of glycosylated and unglycosylated NaPi-2 proteins at a holding potential of ~50 mV, at 96 mM Na+ and a pH of 7.4. Ip was induced by superfusion of 1 mM Pi, for 30 s. The Ip in the NaPi-2, N298Q, N328Q, and N298Q/N328Q cRNA-injected oocytes was 129.7 ± 36.4, 23.9 ± 3.3, 36.6 ± 3.4, and 31.7 ± 5.6 nA, respectively, where data are represented as the mean ± S.E. (n = 6–10 oocytes from a representative experiment for all electrophysiological measurements in this paper).

The affinity of the cotransporter for P_1 and Na^+ and the characteristic pH dependence of sodium-dependent P_1 transport appears to be independent of the glycosylation state of the protein (Figs. 7 and 8). These results are consistent with the finding that baculovirus-mediated transfection of insect (Sf9) cells with the cDNA encoding NaPi-2 leads to the expression of a fully functional unglycosylated NaPi-2 protein with P_1, Na^+, and pH dependence that is indistinguishable from that of the native cotransporter (22). Finally, these results are also consistent with the findings of Douas and co-workers (23) who found no significant difference in the rate of sodium-dependent P_1 uptake by renal brush-border membrane vesicles in response to in vitro enzymatic deglycosylation by Endo-H or endoglycosidase F. In addition, the in vivo inhibition of α-mannosidase, a key enzyme in the processing of glycoproteins in the Golgi apparatus, by alkaloid swainsonine did not affect the rate of NaPi-2 symport across renal brush-border membrane vesicles (23).

In conclusion, we have shown that the mature NaPi-2 cotransport protein from rat brush-border membranes and cRNA-injected oocytes is a glycoprotein. Furthermore, site-directed mutagenesis identified two sites for the N-linked glycosylation of the mature NaPi-2 protein at Asn-298 and Asn-328. The apparent molecular mass of a double mutant, in which
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Fig. 8. pH dependence of Ip. The experimental conditions correspond to those given in the legend to Fig. 5B. The Ip at pH 6.3 for the wild-type (WT, NaPi-2), N298Q, N328Q, and double (N298Q/N328Q) mutants, given as a percentage of Ip at 7.3 (arbitrarily defined as 100%), were 42 ± 4, 33 ± 4, 33 ± 2, and 36 ± 3 (n = 6 oocytes for each cRNA), respectively.

both of these sites were altered to Gln, is consistent with that of the enzymatically deglycosylated cotransporter protein. However, it appears that glycosylation is not required for NaPi-2 mediated sodium-dependent Pi transport, since the unglycosylated mutant is also able to transport Pi in a sodium-dependent manner.

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REFERENCES

1. Magagnin, S., Werner, A., Markovich, D., Sombas, V., Stange, G., Biber, J., and Murer, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 89, 5979-5983
2. Murer, H., Werner, A., Rehkin, R., Waarlin, F., and Biber, J. (1991) Am. J. Physiol. 260, C885-C890
3. Rehkin, S. J., Forgo, J., and Murer, H. (1990) Pflugers Arch. 418, 554-560
4. Sombas, V., Markovich, D., Hayes, G., Stange, G., Forgo, J., Biber, J., and Murer, H. (1994) J. Biol. Chem. 269, 6615-6621
5. Werner, A., Moore, M. L., Biber, J., Semenza, G., and Murer, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9608-9612
6. Custer, M., Meier, F., Schlatter, R., Gregor, R., Garcia-Perez, A., Biber, J., and Murer, H. (1993) Pflugers Arch. 424, 203-209
7. Custer, M., Lotacher, M., Biber, J., Murer, H., and Kaisling, B. (1994) Am. J. Physiol. 266, F767-F774
8. Biber, J., Custer, M., Werner, A., Kaisling, B., and Murer, H. (1993) Pflugers Arch. 424, 210-215
9. Murer, H. (1992) J. Am. Soc. Nephrol. 2, 1649-1685
10. Biber, J., and Murer, H. (1994) Cell Physiol. Biochem. 4, 185-197
11. Werner, A., Biber, J., Forgo, J., Palacin, M., and Murer, H. (1990) J. Biol. Chem. 265, 12531-12536
12. Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988) Nucleic Acids Res. 16, 7583-7600
13. Biber, J., Stueger, B., Haase, B., and Murer, H. (1981) Biochim. Biophys. Acta 647, 169-176
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
15. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
16. Baron, J., and Verrey, P. (1994) Am. J. Physiol. 266, C1278-C1290
17. Markovich, D., Stange, G., Bertran, J., Palacin, M., Werner, A., Biber, J., and Murer, H. (1993) J. Biol. Chem. 268, 1362-1367
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
19. Sargiacomo, M., Lisanti, M. P., Graeve, L., Le Bivic, A., and Rodriguez-Boulin, E. (1988) J. Membr. Biol. 107, 277-286
20. Busch, A. E., Waldegger, S., Herrera, T., Biber, J., Markovich, D., Hayes, G., Murer, H., and Lang, F. (1994) Proc. Natl. Acad. Sci. U. S. A., in press
21. Maley, F., Trimboli, R. B., Tarentino, A. L., and Plummer, T. H. (1989) Anal. Biochem. 180, 195-204
22. Fucentese, M., Murer, H., and Biber, J. (1994) Experientia (Basel) 50, S15-S27
23. Yiasouf, A. K., Szczepanska-Konkel, M., and Dousa, T. P. (1988) J. Biol. Chem. 263, 13689-13691