Biochimica et Biophysica Acta, 1103 (1992) 37–44

BBAMEM 75475

Glycosylation of the rabbit intestinal brush border Na^+/glucose cotransporter

Bruce A. Hirayama and Ernest M. Wright

Department of Physiology, UCLA School of Medicine, Los Angeles, CA (USA)

(Received 18 June 1991)

Key words: Glycoprotein; Membrane protein; Glycosylation structure; Sodium ion/glucose cotransporter

The glycosylation of the mature form of the rabbit intestinal Na^+/glucose cotransporter was investigated by using both glycosidases and chemical treatment. The protein was identified on Western blots using polyclonal antibodies directed against peptide sequences from the cloned transporter as a M_r 68000 polypeptide. The effect of these treatments on the size of the transporter is consistent with the major post-translational processing being a single N-linked glycosylation of either the tri- or tetra-antennary complex type. Either method of deglycosylation reduced the SDS-PAGE size by 11000 to M_r 57000. These results also suggest that O-linked glycosylation, if present, contributes little to the apparent size of the transporter. The relative size of the deglycosylated mature protein appears to be greater than that of the *in vitro* primary transcript (M_r 45000), suggesting either a difference in a stable conformational state insensitive to reduction and denaturation by SDS or an additional post-translational modification. In addition, deglycosylation of the native transporter does not affect transport activity in brush border membrane vesicles. The transporter, an integral membrane protein having several membrane-spanning regions, has an anomalous mobility in SDS-PAGE as shown by Ferguson analysis. We estimate that the actual size of the mature Na^+/glucose cotransporter is 86000, and that N-linked glycosylation contributes about 15000 to the mass.

Introduction

Our group has cloned a rabbit intestinal brush border Na^+/glucose cotransporter [1], having the transport properties of the classical Na^+/glucose cotransporter [2]. The coding region predicts a protein of 662 amino acids having a calculated mass of 73080. *In vitro* translation experiments suggest a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) size of 52000, and provide evidence for N-glycosylation at a single site [3]. The initial high-mannose glycosylation, observed for translation in the presence of pancreatic microsomes, contributes ≈ 6000 in apparent mass. The mature form of the transporter is a broad band centered at M_r 70000 [4]. This size agrees with the consensus size for the mature protein in brush borders from rabbit intestine determined using biochemical methods (M_r 68000–77000 [5–8]).

In this study we characterize the carbohydrate decoration of the mature form of the intestinal brush border Na^+/glucose cotransporter as a step in the definition of its structure, and attempt to account for the difference in apparent size between the primary amino acid transcript and the mature protein.

Methods

Removal of glycosylation from the brush border cotransporter was monitored by Western blotting with anti-peptide antibodies specific for the transporter [4]. Both chemical and enzymatic methods were used to determine the extent and general types of oligosaccharide structures present on the mature protein. The sizes of the mature and deglycosylated transporter proteins were compared to the size of the primary transcript of the cloned rabbit intestinal Na^+/glucose cotransporter translated in reticulocyte lysate.

Correspondence: B. Hirayama, Department of Physiology, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90024-1751, USA.

Reagents

Trifluoromethanesulfonic acid (TFMS), invertase, fetuin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), leupeptin, aprotinin and pepstatin A were from Sigma (St. Louis, MO). Endo-β-acetylglucosaminidase-H from *Streptomyces plicatus* (Endo-H), endo-β-acetylglucosaminidase-F from *Diplococcus pneumoniae* (Endo-F), peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase-F from *Flavobacterium meningiosepticum* (PNGase-F) and α-*N*-acetylneuraminosylglycohydrolase from *Vibrio cholerae* and *Arthrobacter ureafasciens* (neuraminidase) were purchased from Boehringer-Mannheim (Indianapolis, IN) or Genzyme (Boston, MA). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase was from CalBiochem (La Jolla, CA). Streptavidin conjugated to horseradish peroxidase, the protein assay kit, and all electrophoresis reagents were from Bio-Rad (Richmond, CA). Dialysis tubing was Spectrapor 7, molecular weight cutoff 25000 (Spectrum, Los Angeles, CA). Rabbit reticulocyte lysate and RNAsin were from Promega (Madison, WI) and [^{35}S]methionine was purchased from ICN (Irvine, CA). Radioactive methylated protein standards were from Amersham (Arlington Heights, IL). D-[^{3}H]Glucose was purchased from New England Nuclear (Boston, MA). All other reagents were of the highest grade commercially available.

Na^+/glucose cotransporter from brush border membranes

Brush border membranes used in this study were isolated from the small intestine of New Zealand White rabbits using a Ca^{2+} precipitation method [9] and were stored in liquid nitrogen until used. Rates of transport for 50 μM D-glucose were measured in the presence or absence of a 100 mM NaCl gradient as described in Kaunitz and Wright [10]. Typical values for initial rates of Na^+-dependent transport were 40–100 pmol/mg per s.

In some experiments, the Na^+/glucose cotransporter was partially purified by size-fractionation using preparative SDS-PAGE. A standard gel (Protean, Bio-Rad) 1.5 mm thick, was run with 4 mg of brush border protein in a preparative well. After completion of the run, 0.5–1.0 cm wide horizontal slices in the M_r 50000–100000 range were taken and the proteins electroeluted (Geluter, EC Apparatus, St. Petersburg, FL). The eluted fractions were analyzed on Western blots for the presence of the Na^+/glucose cotransporter. Positive fractions were then lyophilized, deglycosylated and analyzed as described for whole brush borders.

In vitro translation product

The primary transcript of the rabbit intestinal Na^+/glucose cotransporter clone was produced from cRNA using rabbit reticulocyte lysate, with or without canine pancreatic microsomes, and labeled with [^{35}S]methionine [3]. For a 25 μl translation volume, 0.5 μg of cRNA was used.

Deglycosylation

Several enzymes were chosen, based on their specificities, to examine the N-linked glycosylation of the mature cotransporter. Since there may be glycosylation at sites which are not efficiently removed by enzymes, either because of their structure or by steric hindrances from the polypeptide even after denaturation, we also employed a non-specific chemical deglycosylation protocol.

Enzymes

The protocols follow the recommendations of Boehringer-Mannheim.

Endo-H

Brush borders were solubilized in SDS at a protein:SDS ratio of 1:1.2 in 50 mM Na acetate, 10 mM EDTA, 10 mM β-mercaptoethanol (pH 5.5) at a protein concentration of 0.5 mg/ml by boiling for 3 min. The potential inhibitory effect of SDS on the activity of the enzyme was neutralized by sequestering free SDS in a 6-fold excess of Triton X-100, added from a 10% (v/v) stock solution. Endo-H was added to the mixture at a concentration of 100–200 mU/ml. Proteinase inhibitors (PMSF, 0.5 mM, leupeptin, 25.5 μg/ml, pepstatin A, 6.25 μg/ml and aprotinin 250 units/ml) were added and then the mixture was incubated at 37°C for approx. 16 h.

Endo-F

The brush border proteins were prepared as above except that the buffer was 250 mM Na acetate, 10 mM EDTA, 10 mM β-mercaptoethanol (pH 6.5). Enzyme was added to a concentration of 0.25–16 units/ml.

PNGase F

Solubilization was as for endo-H except that the buffer was 25 mM Na phosphate, 10 mM EDTA, 10 mM β-mercaptoethanol (pH 7.5). Enzyme was added to a concentration of 40 units/ml.

The activity of the endoglycosidases and PNGase-F was monitored using the test substrate invertase (containing only N-linked glycosylation) and monitoring its change in mass by SDS-PAGE with Coomassie blue staining.

Neuraminidase

Two types of neuraminidase were used in the analysis; *Vibrio cholerae* and *Arthrobacter ureafaciens*, 125 mU/ml. Activity for both enzymes was verified using fetuin by SDS-PAGE and in a fluorometric assay [11].

At the pH used in the experiments, pH 7.5 in PNGase F buffer or pH 5.5 in endo-H buffer, the *A. ureafaciens* enzyme retains at least 70% of its maximal activity [11].

Trifluoromethanesulfonic acid

Chemical deglycosylation was performed using TFMS as described in Refs. 12 and 13. Brush border membranes (1 mg) were lyophilized and placed into a pre-cooled 5 ml glass vial (Kimble 49200 with teflon-lined cap) in an ice bath. The reaction was started by addition of 100 μl of anhydrous anisole and 200 μl of trifluoromethanesulfonic acid, both cooled to 0°C. The mixture was immediately gassed for 30 s with dry nitrogen, vortexed to solubilize the sample, and incubated on ice for 3 h.

The reaction was quenched with 800 μl of ice-cold 60% (v/v) aqueous pyridine, added dropwise to minimize heating of the mixture, and 25 μl of 1 M NaOH. The mixture was then dialyzed overnight at 4°C against two changes of 4 liters of 10 mM NH_4HCO_3, 0.05% (w/v) SDS. Before electrophoresis the proteins were concentrated by either lyophilization or trichloroacetic acid precipitation. Control glycoproteins were fetuin (having both N- and O-linked carbohydrate) and invertase.

Antibodies

Antibodies used in this study were produced against peptides synthesized from the sequence of proposed major intracellular and extracellular hydrophilic loops of the rabbit intestinal Na^+/glucose cotransporter [4]. Antibody Ab-E is directed against a nonadecapeptide (Ser-402–Lys-420) and Ab-C is directed against a dodecapeptide (Arg-602–Asp-613). The antibodies were IgG fractions obtained via Protein-A affinity chromatography. All blots shown were probed with Ab-C; identical results were obtained with Ab-E.

Detection of the Na^+/glucose cotransporter

Na^+/glucose cotransporter was detected on Western blots [4]. Control brush borders, or deglycosylated samples, containing from 5 to 20 μg protein were solubilized in sample buffer and the proteins were separated by SDS-PAGE [14] on 8% minigels. Relative molecular weights (M_r) were estimated by linear regression of a semilog transformation of biotinylated molecular weight standards (Bio-Rad).

Identity of the transporter was verified by comparing the blot probed with peptide-absorbed antibodies to the blot probed in the absence of peptide [4]. The difference in banding pattern between '+ peptide' and '− peptide' revealed the transporter protein.

The blots and autoradiograms were scanned with a Hoefer GS300 densitometer (San Francisco, CA) and analyzed with companion data acquisition and analysis software. The limits of immunoreactivity were characterized by estimation of the width of the band at half of the maximal peak height.

Glycoprotein detection

The efficiency of degiycosylation was monitored by assaying for the presence of glycoproteins with a carbohydrate derivatization/antibody binding method using the Glycan Detection Kit (Boehringer-Mannheim). For this purpose a duplicate blot of the deglycosylated samples was run and compared to the starting material for the presence of residual glycosylation.

Ferguson analysis

We applied Ferguson analysis to determine if the transporter behaves anomalously in SDS-PAGE, following Matsushita et al. [16], in which a series of autoradiograms and Western blots were taken from SDS-PAGE gels over a range of total acrylamide concentration (6–10% T) with constant ratio of crosslinker (2.7% C). The rate of migration is determined for each gel, and the result is plotted according to Ferguson [17]:

$$\log R_f = \log Y_o + K_r T \quad (1)$$

with $\log R_f$ as the abscissa and %T as the ordinate. The slope of the regression line, K_r (retardation coefficient), reflects the effect of the size of the protein on its migration through the gel matrix and Y_o (free-solution mobility) is the migration rate without matrix sieving effects.

Proteins were transferred to nitrocellulose, immunoprobed and scanned for R_f values as previously described. Values for the retardation coefficient (K_r) were derived from linear regression analysis as the slope of %T vs. $\log R_f$ [17]. Molecular size was estimated from linear regression analysis of $\log M_r$ vs. $\log K_r$, using biotinylated standard proteins in all determinations.

Deglycosylation of functional transporter

The Na^+/glucose cotransporter was treated with PNGase-F *in situ* in brush border membrane vesicles. A 100 μg sample of brush borders, suspended in 5 μl of 300 mM mannitol, 2% bovine serum albumin, 10 mM Hepes-Tris (pH 7.4), was treated with 1.25 units of PNGase-F in a total volume of 10 μl. Control membranes were treated with aliquots of the buffer supplied with the enzyme stock (50% glycerol, 150 mM NaCl, 1 mM EDTA, buffered with 80 mM phosphate (pH 7.5)). Incubation was 16–20 h at 22°C. Bovine serum albumin was found to enhance stability of transporter activity during this prolonged incubation (Hirayama, B.A. and Wright, E.M., unpublished observations).

The membranes were separated by centrifuging twice in 2 ml of (in mM) 150 KCl, 0.1 $CaCl_2$, 0.1 $MgSO_4$, 10

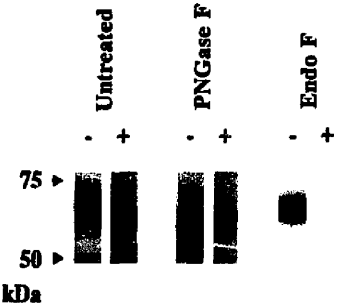

Fig. 1. Effect of PNGase F and Endo-F on the size of the Na^+/glucose cotransporter. Brush border membranes were treated with PNGase-F, to remove N-linked glycosylation, and analyzed by Western blotting, the relevant portions of the blots are shown. The paired strips show the total (−) and peptide-blocked (+) immunoreactive patterns. The broad band which was eliminated by preincubation of the antibody with the relevant peptide is the Na^+/glucose cotransporter. In control brush borders the Na^+/glucose cotransporter had M_r 71000 (half-peak width 14000). Treatment with PNGase-F reduced the band to M_r 60000. Endo-F and Endo-H (not shown) had no effect on the size or breadth of the band. A summary is presented in Table I.

TABLE I

Enzymatic deglycosylation of the Na^+/glucose cotransporter

Whole brush borders were deglycosylated as described in Methods. The treated proteins were then analyzed by Western blotting, after SDS-PAGE on 8% gels, with Ab-C. The *in vitro* product was detected by autoradiography of the [^{35}S]methionine-labeled product. PNGase-F and TFMS were the only reagents that reduced the size of the transporter. Blots were scanned by densitometer to determine R_f values, which were compared to standard proteins by linear regression analysis of log molecular weight of the standards.

Treatment	Peak mol. wt. ($M_r \times 10^{-3}$)	Peak width half-maximum ($M_r \times 10^{-3}$)	*n*	Change peak mol. wt.	Change width
Control	67±2	16	27	0	0
PNGase-F	56±2	11	7	−11	−5
TFMS[a]	57±1	9	15	−11	−4
Endo-H	68±1	11	3	+1	−5
Endo-F	67±1	14	5	0	−2
Neuraminidase	66+1	15	9	−1	−1
Neuraminidase + Endo-H	66±1	17	2	−1	+1
PNGase-F + neuraminidase	61	11	1	−6	−5
In vitro	45±0	13	2	NA	NA

[a] In this case purified transporter was used. Results from control and enzyme treated purified samples were identical to those reported in the table: ($M_r \times 10^{-3}$) control = 68±2 (n = 17); Endo-F = 66±1 (n = 2); PNGase-F = 57±1 (n = 2).

Hepes-Tris (pH 7.4), in a Type 50.3Ti rotor at 49000 rpm for 30 min.

Results

Fig. 1 shows an example Western blot comparison of the mature cotransporter before and after enzymatic deglycosylation. The untreated transporter had M_r 71000 (half-maximal peak width 14000). PNGase-F reduced the apparent size of the specific immunoreactive band by 11000 to 60000 and the band sharpened slightly (half-maximal width 9000). There was no observable change in apparent mass from the control sample after treatment with either Endo-F or Endo-H.

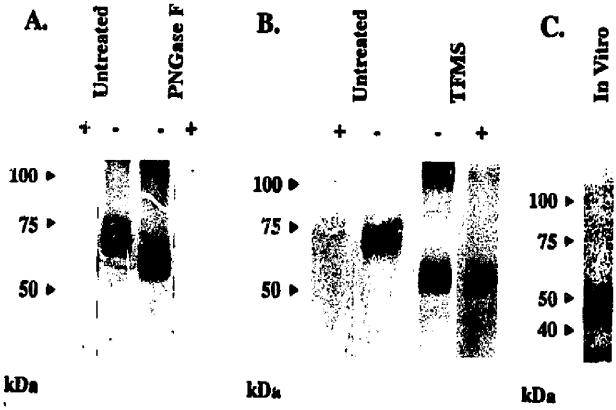

Fig. 2. Effect of chemical deglycosylation on the size of the Na^+/glucose cotransporter. Partially purified Na^+/glucose cotransporter was treated with trifluoromethanesulfonic acid or PNGase-F and analyzed by Western blotting. The strips show the total immunoreactive pattern (−) compared to that observed with peptide-blocked (+) antibody. Mature Na^+/glucose cotransporter was M_r 68000. Treatment with either TFMS and PNGase-F reduced the size to M_r 57000. An autoradiogram from an *in vitro* translation of the Na^+/glucose cotransporter clone (M_r 45000), which was analyzed on the same gel, is shown for comparison. Note that bands of smaller size were observed in some TFMS experiments using purified transporter, however, since the 57000 band was already deglycosylated (Fig. 3), we interpret the presence of smaller bands as incidental damage to the polypeptide in spite of our precautions. In addition, bands corresponding in size to dimeric forms of the transporter were occasionally observed. No other specific bands were observed.

nor was a change observed with either form of neuraminidase (Table 1). Sequential treatment with neuraminidase, either before treatment with Endo-H or after initial digestion with PNGase F, also made no alteration of apparent mass.

Chemical deglycosylation

Fig. 2 compares the effects of enzymatic and chemical deglycosylation on partially purified mature Na^+/glucose cotransporter with the *in vitro* primary transcript. Panels A and B show that the final size of the transporter after each treatment was a band centered at about M_r 57000. The size of the transporter decreased by about 11000 upon enzymatic deglycosylation and the band remained broad (9000 half-maximal width). After TFMS treatment the samples were reduced to the same size (M_r 57000), also in a band spanning 9000. The overall result of this treatment was identical for the Na^+/glucose cotransporter in whole brush border samples. For comparison, Fig. 2 shows that the *in vitro* translation product in rabbit reticulocyte lysate had M_r 45000. The results of this series of experiments are summarized in Table I.

Residual glycosylation

Fig. 3 shows the result of probing the transporter-enriched M_r 70000 protein fraction for carbohydrate

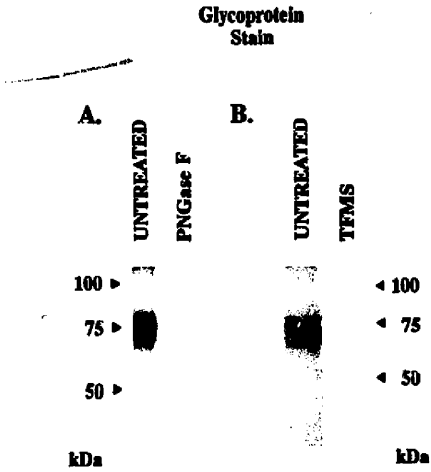

Fig. 3. Assay for residual glycosylation after TFMS treatment. The same fractions used in Fig. 2 were probed for the presence of carbohydrate. The samples were analyzed by SDS-PAGE and transferred to nitrocellulose. The left lane, Untreated, is the starting material, showing a strong reaction indicative of the presence of carbohydrate. In Panel A, after PNGase-F treatment, faint bands are still detected in the original and lower mw, indicative of a small amount of residual glycosylation remaining on proteins at these sizes. In Panel B, after TFMS treatment, there is a very faint band corresponding to the upper portion of the starting broad band at about M_r 70000. There is no staining below this band.

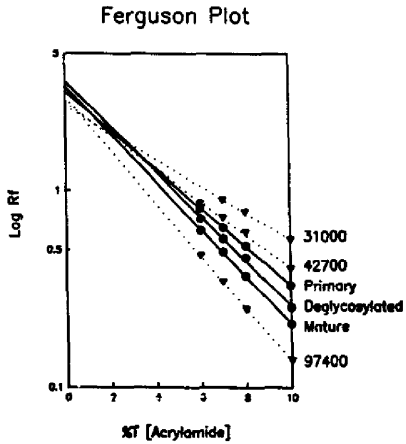

Fig. 4. Ferguson plot of mature, deglycosylated and primary transcript forms of the Na^+/glucose cotransporter. SDS-PAGE gels of 6, 7, 8 and 10% T were run with identical protein samples and the results were analyzed in Ferguson plots as described in Methods. The *in vitro* translation of the clone was analyzed on the same gels. Lanes containing the radioactive samples were removed, fixed, treated with Enhance (New England Nuclear), dried and exposed to film (XAR, Kodak, Rochester, NY) at −80°C. The remainder of the gel was treated as described for Western blotting. Standard proteins have a common free-solution mobility (Y_0), as indicated by their common *Y*-intercept. The different forms of the Na^+/glucose cotransporter intercept the *Y*-axis at a common point of slightly higher value. This indicates that the most appropriate method for estimation of size is a comparison of the retardation coefficients (K_r). By this analysis the size of the mature transporter was M_r 81000; degiycosylated mature transporter, M_r 75000; primary amino acid transcript from the cloned Na^+/glucose cotransporter, M_r 59000. Standards are phosphorylase B, 97400; bovine serum albumin, 66200; ovalbumin, 42700; carbonic anhydrase, 31000. Lines are for linear regression. Size estimates are from linear regression of K_r vs. molecular weight. The data from the 66000 standard were not plotted in this figure for clarity.

after enzymatic or chemical treatment. There was a strong staining in the untreated sample, indicating the presence of glycosylation on the proteins. After PNGase-F there was a marked reduction in staining, although three regions of light staining remained. This was not unexpected since any O-linked sugars would remain after PNGase-F treatment. After TFMS treatment there was a very light residual staining at about M_r 70000 but there was no evidence of glycosylation at lower molecular weights. This shows that all of the proteins, including the Na^+/glucose cotransporter, were deglycosylated by both enzymatic or chemical treatments.

Ferguson analysis

Fig. 4 is a representative Ferguson plot of mature and deglycosylated transporter and primary transcript of the cloned transporter. The plot shows that the

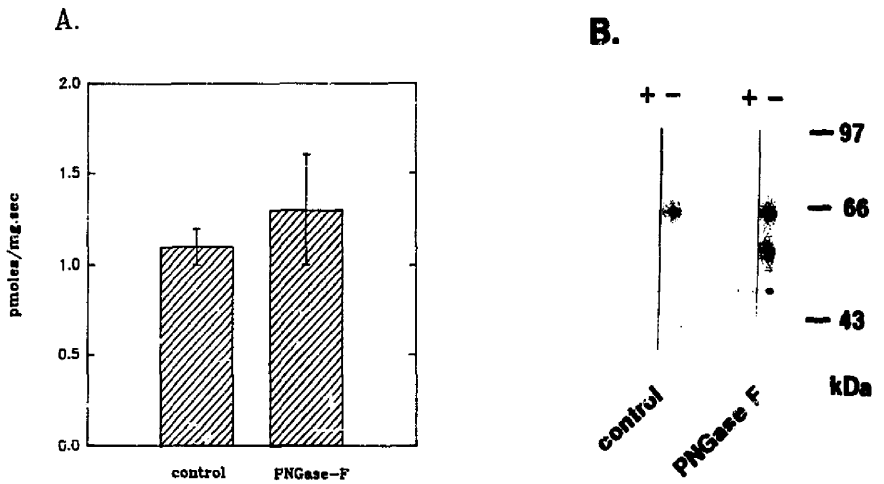

Fig. 5. Effect of deglycosylation on the native transporter. Whole rabbit brush border membrane vesicles were treated with PNGase-F as described in Methods. Panel A shows Na^+-dependent glucose uptake in control (1.1 pmol/mg per s) and enzymatically deglycosylated transporter (1.3 pmol/mg per s). Panel B shows Western blots of the control and PNGase-F treated brush border vesicles, probed with Ab-C with (+) and without (−) peptide-absorption. A single transporter band was detected in control membranes. After PNGase-F treatment the transporter was split into an upper band, the normal glycosylated form, and a lower band which corresponds to the deglycosylated transporter. The transport data are for triplicate samples (±S.D.), each strip of the Western blot contained 3 μg protein.

standard proteins had a common y-intercept (Y_0) between 2.6 and 3.0. In all forms of the Na^+/glucose cotransporter this value was consistently higher, from 3.1 to 3.4. This anomalous mobility of the transporter precludes a reliable estimate of the cotransporter size through comparison to the standard proteins using a single %T gel (since the estimate of size for the transporter varies as a function of the gel concentration). An estimate of the size based on the relative mobility, as K_r vs. molecular mass, is more reliable than that estimated from single concentration gels [18]. In this analysis the mature Na^+/glucose cotransporter was M_r 81 000, falling to 75 000 after PNGase-F treatment. The primary amino acid transcript was M_r 59 000 and, in a single experiment, the presence of pancreatic microsomes resulted in an increase of 6000 compared to the primary transcript.

Effect of deglycosylation on function

Fig. 5 shows the effects of enzymatic deglycosylation on Na^+-dependent transport. In Panel A, Na^+-dependent transport of PNGase-F-treated membranes (1.3 pmol/mg per s) was slightly higher than in the control membranes (1.1 pmol/mg per s). Note that the 16–20 h incubation at 22°C resulted in a loss of activity from the starting Na^+-dependent transport rate (30 pmol/mg per s). Panel B shows that PNGase-F was able to deglycosylate approx. 50% of the transporter as shown by the appearance of a band corresponding to the size of the deglycosylated transporter. Treatment with larger amounts of PNGase-F (up to 4-fold) gave similar results.

Discussion

As a first step in understanding the functional significance of post-translational influences on the expression of the Na^+/glucose cotransporter we have investigated the glycosylation of the mature form of the rabbit small intestinal Na^+/glucose cotransporter. The primary amino acid sequence of the transporter has two consensus N-linked glycosylation sites, Asn-306 and Asn-248. *In vitro*, only Asn-248 is glycosylated [3] increasing the M_r by 6000 over the size of the primary amino acid transcript.

Glycosylation structure

We exploited the specificities of several glycosidases to investigate the mature glycosylation structure of the brush border protein, as summarized in Table I. The initial high mannose core is evidently modified in the mature transporter. The lack of effect of Endo-H on the mature transporter indicates that the glycosylation structure is no longer of the high-mannose form. Endo-H prefers high-mannose oligosaccharides, but high concentrations will cleave hybrid oligosaccharides, so the lack of effect of high concentrations of Endo-H suggests that the structure is not hybrid [19]. The ability of PNGase-F, which cleaves all types of N-lin-

TABLE II

Ferguson plot estimation of the mass of the Na^+/glucose cotransporter

The mature Na^+/glucose cotransporter from rabbit intestinal brush borders, PNGase-F treated and control, was compared to the *in vitro* translation product from cRNA from the cloned Na^+/glucose cotransporter using Ferguson plots. The size of the polypeptides was estimated by comparison of K_r (retardation factor) to the known masses of standard proteins. The results from SDS-PAGE gels of 6% to 10% T are summarized. The mature transporter was detected by Western blotting; the *in vitro* product was radioactively labeled with [^{35}S]methionine.

Treatment	Mol. wt. ($M_r \times 10^{-3}$)	n
Control	86±4	7
PNGase-F	71±5	4
In vitro	61±5	5

ked oligosaccharides (except if located at amino or carboxy termini [19]) to reduce the mass by about 11000, suggests that the glycosylation structure has increased in complexity, since the size difference of the transporter *in vitro* is only 6000 with microsomes. Endo-F, which can cleave biantennary complex structures but it is ineffective against tri- and tetra-antennary glycosylation [20], had no effect on the size of the transporter. These results suggest that all of the mature glycosylation is a tri- or tetra-antennary complex structure.

The observation that both chemical deglycosylation and enzymatic removal of N-linked oligosaccharides reduces the apparent molecular mass to the same value (Table I) indicates that the major carbohydrate modification is restricted to N-linked types. Our observation that the mass is reduced in a single step supports the prediction that there is only one N-linked glycosylation site used in the mature protein [3].

There was no observable effect of neuraminidase, either before or after N-deglycosylation, suggesting that there are no significant amounts of sialic acid present on either N- or O-linked oligosaccharides. In addition, sequential treatment with neuraminidase and Endo-H showed no alteration in apparent mass, indicating that the activity of Endo-H is probably not inhibited by terminal sialic acid on a high mannose structure.

Chemical digestion of peripheral glycosylation structures by TFMS removes all glycosylation except the terminal N-linked and O-linked residues, which are removed more slowly [12]. The removal of all glycosylation was verified after TFMS treatment of purified transporter. The apparent size of the transporter falls by 11000, to M_r 57000, which is identical to the size of the enzymatically deglycosylated protein. TFMS reportedly does not affect the integrity of the polypeptide chain under the conditions used in these experiments [12,13].

Deglycosylated and primary transcript size as determined by SDS-PAGE: Ferguson plots

The observed size of the deglycosylated mature form of the Na^+/glucose cotransporter and the primary amino acid transcript appear to be different (Table I). Hydrophobic integral membrane proteins frequently exhibit anomalous migration in SDS-PAGE. We used Ferguson's analysis [17] to compare mobility of the different forms of the transporter. All of the forms of the Na^+/glucose cotransporter consistently have a slightly higher mobility than the water-soluble standard proteins. This finding makes estimation of size based on a single %T gel unreliable since the size determined will depend on the acrylamide concentration of the gel. In this case the most appropriate analysis, with reservations, is made by using a plot of K_r vs. molecular weight [18]. Fig. 4 shows the results of this plot, summarized in Table II. The data suggest that the actual size of the mature transporter is about 86000, with glycosylation contributing 15000 to the apparent size. There is, however, a difference between the size of the primary transcript and the deglycosylated form of the mature transporter. The close values for Y_0 between the primary transcript and deglycosylated mature transporter indicate that this comparison is valid, suggesting an actual difference in size between these two forms.

The anomalous migration of the transporter in SDS-PAGE indicates that either the binding ratio of SDS:protein and/or the relationship of molecular weight and hydrodynamic size is not the same as for the standards. Even if the Y_0 values are identical it must be acknowledged that differences in K_r, such as those due to different conformational states, may result in apparent size differences.

Effect of deglycosylation on transport

Post-translational modifications of proteins, such as glycosylation, can have various influences on the expression of function (see Ref. 21). In membrane proteins the effect of such a change can be manifested either directly on function or indirectly, as in targeting to the plasma membrane [22]. The purpose of N-glycosylation in the expression of Na^+-dependent glucose transport has yet to be elucidated, however it does not appear to be required for the protein to transport its substrates.

Previous studies on the Na^+/glucose cotransporter suggested that N-linked glycosylation is not required for transport function since mutation of the N-glycosylation site did not eliminate the functional expression of the cotransporter in oocytes [3]. In the present study we were able to remove glycosylation from 40–50% of

the transporters using PNGase-F while retaining transport activity which was not different from the control (Fig. 5). These results support the contention that N-linked glycosylation is not required for transporter activity. There is also evidence that functional expression of Na^+-dependent glucose transport in renal brush border membrane vesicles does not require a specific N-linked glycosylation structure. Yusufi et al. [23] showed that while treatment of rats with swainosine, or normal renal brush border membranes treated with Endo-F, reduced the V_{max} for Na^+/H^+ exchange in renal brush border membrane vesicles, there as no effect on Na^+-dependent transport of glucose, proline or phosphate.

In summary, these studies indicate that the mature form of the cloned rabbit intestinal Na^+/glucose cotransporter is glycosylated at a single N-linked site. The initial high mannose glycosylation is uniformly modified to a tri- or tetra-antennary complex structure, without terminal sialic acid. O-linked glycosylation is either small or absent. As estimated from Ferguson plots this carbohydrate structure contributes about 15000 to the size of the transporter. N-linked glycosylation appears to be not required for transport activity.

Acknowledgments

We thank Dr. A.M. Pajor for assistance with the *in vitro* translation experiments and Drs. C.D. Smith and E. Turk for helpful discussions and critical comments on the manuscript. This work was supported by NIH grant DK19567.

References

1 Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 330, 379–381.
2 Ikeda, T.S., Hwang, E-S., Coady, M.J., Hirayama, B.A., Hediger, M.A. and Wright, E.M. (1989) J. Membr. Biol. 110, 87–95.
3 Hediger, M.A., Mendlein, J., Lee, H.-S. and Wright, E.M. (1991) Biochim. Biophys. Acta 1064, 360–364.
4 Hirayama, B.A., Wong, H.C., Smith, C.D., Hagenbuch, B.A., Hediger, M.A. and Wright, E.M. (1991) Am. J. Physiol. 261, C296–C304.
5 Hosang, M., Vasella, A. and Semenza, G. (1981) Biochemistry 20, 5844–5854.
6 Schmidt, U.M., Eddy, B., Fraser, C.M., Venter, J.C. and Semenza, G. (1983) FEBS Lett. 161, 279–283.
7 Peerce, B.E. and E.M. Wright. (1984) Proc. Natl. Acad Sci. USA 81, 2223–2226.
8 Peerce, B.E. and Wright, E.M. (1984) J. Biol. Chem. 259, 14105–14112.
9 Stevens, B.R., Ross, H.J. and Wright, E.M. (1982) J. Membr. Biol. 66, 213–225.
10 Kaunitz, J.D. and Wright, E.M. (1984) J. Membr. Biol. 79, 41–51.
11 Meyers, R.W., Lee, R.T., Lee, Y.C., Thomas, G.H., Reynolds, L.W. and Ucida, Y. (1980) Anal. Biochem. 101, 166–174.
12 Edge, A.S.B., Faltynek, C.R., Hof, L., Reichert, L.E., Jr. and Weber, P. (1981) Anal. Biochem. 118, 131–137.
13 Sojar, H.T. and Bahl, O.M. (1987) Methods Enzymol. 138, 341–350.
14 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
15 Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
16 Matsushita K., Patel, L. and Kaback, H.R. (1984) Biochemistry 23, 4703–4714.
17 Ferguson, K.A. (1964) Metabolism 13, 985–1002.
18 Frank, R.N. and Rodbard, D. (1975) Arch. Biochem. Biophys. 171, 1–13.
19 Maley, F., Trimble, R.B., Tarentino, A.L. and Plummer, T.H., Jr. (1989) Anal. Biochem. 180, 195–204.
20 Tarentino, A.L. and Plummer, T.H., Jr. (1987) Methods Enzymol. 138, 770–778.
21 Paulson, J.C. (1989) Trends Biochem. Sci. 14, 272–275.
22 Sumikawa, K. and Miledi, R. (1989) Mol. Brain Res. 5, 183–192.
23 Yusufi, A.N.K., Szczepanska-Konkel, M. and Dousa, T.P. (1988) J. Biol. Chem. 263, 13683–13691.