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Biosynthesis of the cloned intestinal Na^+/glucose cotransporter

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The initial stages in the biosynthesis of the cloned Na^+/glucose cotransporter were examined by *in vitro* expression of the protein in the absence and presence of pancreatic microsomes. Glycosylation was detected by endoglycosidase-H shifts in the apparent size of the proteins on SDS-PAGE. In the presence of microsomes, M_r increased from 52000 to 58000, and this was reversed by endo-H. This demonstrates that the protein is glycosylated and that there is no large cleavable signal sequence. Using partial transcripts and site-directed mutagenesis, we established that Asn-248 is glycosylated. Glycosylation was not required for the functional expression of the transporter in *Xenopus* oocytes. In terms of the topology of the protein, these results suggest that Asn-248 is on the external surface of the membrane.

Introduction

The primary sequence of the cloned intestinal brush border Na^+/glucose cotransporter contains two potential N-linked glycosylation sites [1]. Membrane transport proteins are normally glycosylated but the function of the carbohydrate is largely unknown. Recent experiments on the expression of ion channels in oocytes suggest that glycosylation is needed in some cases for efficient processing and insertion of functional channel proteins into the plasma membrane. For example, tunicamycin, an inhibitor of N-linked glycosylation, reduced the function expression of voltage-sensitive Na^+ channels but not voltage-sensitive K^+ and Ca^{2+} channels [2]. In this study we have examined the glycosylation of the transcribed protein in order to determine if either of these Asn residues are utilized, and to explore the role of glycosylation on the functional expression of the transporter. Our approach was to examine endoglycosidase-H sensitive shifts in the size of the protein expressed *in vitro* in the presence and absence of pancreatic microsomes, to use partial transcripts to determine which glycosylation site(s) are utilized, and then mutate the residues glycosylated and determine the effect on expression *in vitro* and *in vivo*. A preliminary account of some of these results has been presented [3].

Methods

The rabbit intestinal Na^+/glucose cotransporter cDNA (2.2 kb), which was inserted into Bluescript plasmid KS^+ [1], was used as a template for RNA synthesis. The template was linearized with *Not*I or with internal cleavage sites (*Sac*I, *Sma*I, *Stu*I) and RNA was synthesized with T3 RNA polymerase. The transcription and capping procedures were as described previously [1]. Restriction enzymes were obtained from Pharmacia LKB Biotechnology Inc., NJ.

In vitro translation. In general, we followed the method described by Mueckler and Lodish [4]. Canine pancreatic microsomes were obtained from Promega, WI, and we carried out the procedure recommended by the manufacturer. RNase inhibitor (RNasin) was from Promega, WI, and L-[^{35}S]methionine was from NEN, MA (1134 Ci/mmol). Deglycosylation was carried out by adding endoglycosidase-H (Boehringer Mannheim) and incubating at 30°C overnight. SDS sample buffer (containing 125 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 8M urea, 20% sucrose, 0.5 mg/ml Bromophenolblue) was added to each translation product, and the mixture was boiled for 5 min before electrophoresis on a 10% reducing gel [5]. After electrophoresis the gel was dried, treated with enhancer (Auto-

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fluor, National Diagnostics, NJ), and analyzed by autoradiography.

Examination of N-glycosylation sites. The two potential N-glycosylation sites of the Na^+/glucose cotransporter were investigated by *in vitro* translation of partial RNA transcripts which were obtained by enzymatic truncation of the Na^+/glucose cotransporter cDNA with the internal restriction enzymes *Sac*I, *Sma*I and *Stu*I (Fig. 2). *In vitro* translation was carried out (a) without microsomes, (b) with microsomes, and (c) with microsomes followed by treatment with endoglycosidase H, and the extent of the glycosylation of the partial translation products was tested by SDS gel electrophoresis.

Expression in Xenopus oocytes. RNA synthesized from the template was injected into *Xenopus* oocytes (1 μg/μl) and 3 days after injection the uptake of 50 μM ^{14}C-labelled methyl α-D-glucopyranoside (αMeGlc) was measured as described earlier [6].

Site-directed mutagenesis. To confirm that Asn-248 is the only N-linked glycosylation site in the protein, we used site-directed mutagenesis to replace Asn-248 with Gln. Oligonucleotide-directed *in vitro* mutagenesis was performed according to Kunkel [7,8], using the Bio-Rad (Richmond, CA) Muta-Gene M13 kit. The primer oligonucleotide (Genetic Designs, Inc., Houston, TX) was 5′-CTGTGGGATGGAGGTCTGTCCGTAGCTGATCTGGCTGGGAATG-3′. Mutagenesis with this sequence removed a *Bgl*II restriction site at position 751. *Bgl*II/*Eco*RI double digested plasmid DNA from mutants was analyzed by mini gel electrophoresis and a mutant was selected based on the restriction fragment pattern, i.e. absence of the 0.26 kb internal *Bgl*II fragment, increased molecular mass of the 3′-end *Bgl*II-*Eco*RI fragment from 1.47 to 1.73 kb, and no change in size of the 0.45 kb 5′-end *Eco*RI-*Bgl*II fragment. The mutagenesis was further confirmed by double stranded DNA sequencing using a T7 sequencing system (Pharmacia, New Jersey) and a synthetic oligonucleotide as a sequencing primer located upstream from the mutated region. RNA from the mutant Bluescript plasmid was either translated *in vitro* in the presence or absence of microsomes or was injected into oocytes as described above.

Results

Based on the amino acid sequence of the rabbit intestinal Na^+/glucose cotransporter there are two potential N-linked glycosylation sites (N-X-T/S) at positions 248 and 306 (Fig. 1). The approach we used to investigate the glycosylation of the two sites is outlined in Fig. 2. RNA transcripts were synthesized from the clone (Fig. 2, top left) using T3 RNA polymerase. Cleavage of the templates with the restriction enzymes *Sac*I, *Sma*I or *Stu*I was used to produce truncated transcripts. Using *Not*I the full-length Na^+/glucose cotransporter RNA transcript was obtained (Fig. 2, top right).

SDS-PAGE analysis of the *in vitro* translated products is shown in Fig. 3. It should be noted that the hydrophobic membrane protein tended to aggregate and so it was necessary to add high concentration of urea (8 M) and to boil the sample prior to electrophoresis. *In vitro* translation of the full length transcript in the absence of microsomes is shown in the first lane of both Figs. 3a and 3b. The full size protein appeared as a band at 52 kDa, but multiple additional bands were observed at lower molecular masses. These are probably due to premature termination of translation and/or proteolysis. The lower molecular mass bands were largely removed when translation was carried out in the

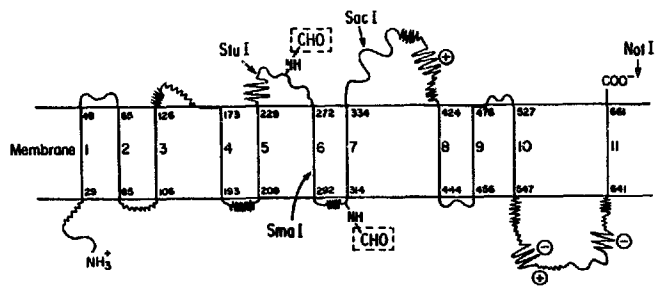

Fig. 1. Secondary structure model of the rabbit intestinal Na^+/glucose cotransporter. The two potential glycosylation sites Asn-248, and Asn-306 are shown (CHO). Also indicated are the restriction enzymes *Stu*I, *Sma*I, *Sac*I, and *Not*I which were used to produce truncated RNA coding for partial protein synthesis products.

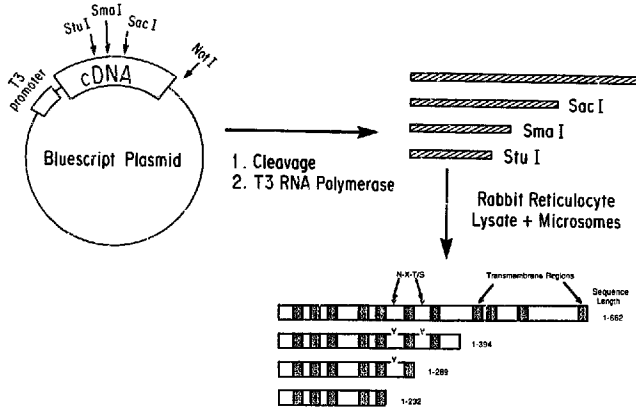

Fig. 2. Strategy used to study N-glycosylation of the Na^+/glucose cotransporter. The left side shows the Na^+/glucose cotransporter cDNA clone in Bluescript KS^+ plasmid. The clone was cleaved with the appropriate restriction enzyme (*Stu*I, *Sma*I, *Sac*I, or *Not*I). RNA was synthesized using T3 RNA polymerase, capped, and then used for *in vitro* translation with rabbit reticulocyte lysate together with dog pancreatic microsomes, in the presence of [^{35}S]methionine. The expected polypeptides are shown at the bottom of the figure. The transmembrane regions are indicated as shaded areas and the two potential N-glycosylation sites are marked (Y).

presence of microsomes, and when the microsomes were pelleted by centrifugation at the end of the reaction (lane 2, Figs. 3a and 3b). In the presence of pancreatic microsomes, the molecular mass of the full-length transcript increased to 58 kDa (lane 2, Fig. 3a), and this shift was completely reversed by endoglycosidase-H treatment. This indicates that the decreased mobility observed after translation in the presence of microsomes is entirely due to the addition of asparagine-linked core oligosaccharide [9]. In order to test for N-

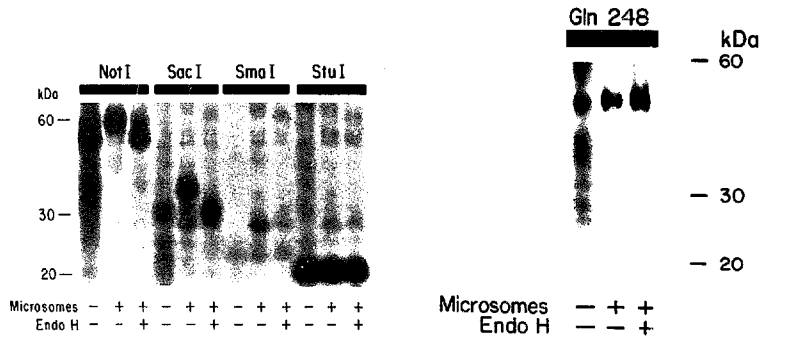

Fig. 3. Autoradiograph of an SDS gel showing the protein translation products (see Fig.2). (a) Truncated RNA. The restriction enzymes used to truncate the cDNA are indicated on top of the autoradiograph. There are three lanes for each restriction enzyme, one for translation without microsomes, one for translation with microsomes, and one for translation with microsomes followed by treatment with endoglycosidase H. (b) Mutant RNA. *In vitro* translation experiments were carried out on a mutant coding for Gln at position 248 instead of Asn.

TABLE I

Summary of the results obtained from the SDS gel shown in Fig. 3

The difference between the molecular masses in the absence and presence of microsomes (right side) indicates the extent of N-glycosylation. Similar results were obtained in two other separate experiments.

Template	Sequence	Apparent molecular mass (kDa)		
		without microsomes	with microsomes	Δ
*Not*I	1–662	52	58	6
*Sac*I	1–289	30	35	5
*Sma*I	1–289	22	27	5
*Stu*I	1–232	20	20	0

glycosylation of the two N-X-T/S sites, the restriction enzymes were chosen such that the corresponding translation products terminate before, after and between these sites. The SDS gel of the translation products is shown in Fig. 3 and the result is summarized in Table I. The left part of the table shows the enzymes used for truncation and the right part the relative molecular mass change due to glycosylation. The enzymes *Not*I, *Sac*I, and *Sma*I all resulted in translation products showing roughly the same 5–6 kDa molecular mass shift with microsomes. Since *Sma*I cleaves between the two potential N-glycosylation sites, this indicated that Asn-306 was not glycosylated. When using the restriction enzyme *Stu*I, which removes both glycosylation sites, no glycosylation was observed. This suggests that only Asn-248 is N-glycosylated.

To confirm this result, the glycosylation site at Asn-248 was replaced by Gln using site-directed mutagenesis. This mutation did not shift the apparent size of the primary *in vitro* translation product synthesized in the absence of microsomes (Fig. 3b). Furthermore, there was no increase in size when the *in vitro* translation reaction was carried out in the presence of microsomes and endoglycosidase H had no effect. We conclude that Asn-248 is glycosylated exclusively.

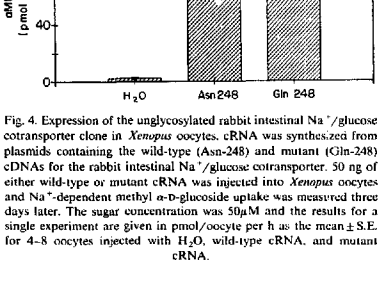

Fig. 4. Expression of the unglycosylated rabbit intestinal Na^+/glucose cotransporter clone in *Xenopus* oocytes. cRNA was synthesized from plasmids containing the wild-type (Asn-248) and mutant (Gln-248) cDNAs for the rabbit intestinal Na^+/glucose cotransporter. 50 ng of either wild-type or mutant cRNA was injected into *Xenopus* oocytes and Na^+-dependent methyl α-D-glucoside uptake was measured three days later. The sugar concentration was 50μM and the results for a single experiment are given in pmol/oocyte per h as the mean ± S.E. for 4–8 oocytes injected with H_2O, wild-type cRNA, and mutant cRNA.

The functional role of glycosylation at Asn-248 was investigated by studying the expression of the transporter in *Xenopus* oocytes. Fig. 4 shows the uptake of 50 μM αMeGlc into *Xenopus* oocytes injected with wild-type and mutant Na^+/glucose cotransporter cRNA. Replacing Asn with Gln at residue 248 only reduced sugar uptake about 25%. In three experiments with the mutant, the uptake was reduced 27 ± 3%. This suggests that glycosylation of Asn-248 is not essential for functional expression of this transport protein in oocytes.

Discussion

Glycosylation of the rabbit intestinal Na^+/glucose cotransporter asparagine residues 248 and 306 would be inconsistent with the secondary structure model (Fig 1) since complex-linked glycosylation is typically located only on the extracellular face of membrane proteins. Experiments to study the glycosylation of these sites involved *in vitro* translation of partial transcription products. There was no glycosylation for the partial peptide 1–232 (*Stu*I), whereas glycosylation of the partial peptides 1–289 (*Sma*I), and 1–394 (*Sac*I) was approximately the same as that of the intact transporter (1–662, *Not*I). This suggests that Asn-248 is glycosylated *in vitro*. However, truncations could have an effect on protein folding and membrane insertion that could influence glycosylation. This is unlikely in view of a recent detailed analysis of the secondary structure of a related transport protein, *lac* permease, using a powerful gene-fusion technique [10]. A set of *lac* permease-alkaline phosphatase gene fusions were expressed and the level of alkaline phosphatase activity was used to determine whether the fusion site normally faces the cytoplasm or periplasm. The results were in complete agreement with biochemical and immunological experiments on the topology of *lac* permease in the membrane. Since the C-terminal *lac* permease sequences were lost in the gene fusions, this clearly suggests that the transmembrane topology of the partial transcripts is independent of the C-terminal domains of the protein.

Nevertheless, we felt it was necessary to confirm our conclusion independently. Using site-directed mutagenesis, Asn-248 was replaced with Gln, and *in vitro* translation of this mutant gave no glycosylation in the presence of microsomes (Fig. 3b). We conclude that Asn-248 is the only site glycosylated *in vitro*.

Our experiments also suggest that the Na^+/glucose cotransporter does not contain a large (> 1 kDa) cleavable terminal signal sequence. As shown in Fig. 3, treatment of the glycosylated protein in pancreatic microsomes with endoglycosidase H did not reduce the size of the protein below that of the primary translation product. Similar results were obtained with another sugar transport protein, the human facilitated glucose transporter [4].

According to our secondary structure model of the Na^+/glucose cotransporter (Fig. 1), glycosylation of Asn-248 indicates that the hydrophilic loop between transmembrane segments 5 and 6 faces the extracellular side of the membrane. If this is correct, the N-terminus of the transporter should be found on the cytoplasmic face of the membrane. This orientation of the protein is probably also the case for the human intestinal Na^+/glucose cotransporter since the glycosylation site Asn-248 is conserved, and the amino acid sequence is virtually identical to that in rabbit [11]. Additional experiments of a different type are necessary to confirm the topology of the rabbit and human Na^+/glucose cotransport proteins in the brush border membrane.

We examined the role of N-linked glycosylation of the functional expression of the Na^+/glucose cotransporter in *Xenopus* oocytes. Na^+-dependent sugar uptake into oocytes (Fig. 4) was only reduced by 30% when oocytes were injected with cRNA transcribed from the Gln-248 mutant as opposed to wild-type cRNA. At this junction, we are unable to determine whether this small reduction is due to a defect in the transporter or to a decrease in the number of transporters in the membrane. Nevertheless, the result suggests that glycosylation of Asn-248 is not essential for the expression of transport activity in oocytes. The cloned Na^+/glucose cotransporter expressed in the mammalian cell line COS-7 [12] and in oocytes (unpublished data) was inhibited by 2 μg/ml tunicamycin (60%–80%). The discrepancy between these results with tunicamycin, an inhibitor of N-glycosylation and those with mutant Gln-248 (Fig. 4) suggests that tunicamycin at high concentrations may have a non-specific effect on protein synthesis and/or insertion.

Biochemical studies of the intestinal and renal brush border Na^+/glucose cotransporters have indicated that the molecular size of the mature protein is between 70 and 75 kDa [13,14]. Our *in vitro* translation experiments demonstrate that N-linked glycosylation accounts for part of the difference between the sizes of the primary translation product and the mature protein. In the presence of pancreatic microsomes, the relative molecular mass increased by 6 kDa, and this was reversed by endoglycosidase H. Additional complex N-linked glycosylations probably account for the increase in size of the protein to 70–75 kDa. Preliminary experiments in our group with intact brush border membranes confirm that the mature protein is glycosylated (Hirayama, B., personal communication). An increased molecular mass of this magnitude due to glycosylation is not uncommon for membrane proteins. For example, N-linked glycosylation accounts for the 18 kDa difference in size between the primary translation product and the mature facilitated glucose transporter [15,16].

Finally, this strategy may be used to determine if potential N-linked glycosylation sites in other cloned membrane proteins are actually glycosylated. The results provide important tools for examining the role of glycosylation in the function of the protein, and give clues about the topological arrangement of the cloned protein in the membrane.

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