Multiple carbohydrate moieties on the Na^+/H^+ exchanger

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Affinity-purified antibodies against the C-terminal region of the Na^+/H^+ exchanger (NHE-1) were used to analyse the carbohydrate moiety of the protein. The Na^+/H^+ exchanger in human placental brush-border membranes has an apparent molecular mass of 105 kDa. Incubation of intact or detergent-solubilized membranes with glycopeptidase F removed the carbohydrate moiety and increased the apparent mobility of the exchanger. Digestion with endoglycosidase-F caused a similar change in mobility, but endoglycosidase-H had no effect, suggesting that the placental Na^+/H^+ exchanger is a glycoprotein of the bi-antennary complex type. Removal of the carbohydrate moiety with glycopeptidase F had no effect on the ability of the protein to promote the exchange of Na^+ for H^+, and had no detectable effect on the sensitivity of the exchanger to trypsin. Limited digestion with glycopeptidase F and neuraminidase indicated the presence of two intermediate forms between the fully glycosylated and the deglycosylated protein. This suggests the presence of at least two, and possibly three, N-linked carbohydrate moieties.

INTRODUCTION

The mammalian Na^+/H^+ exchanger has an essential role in the maintenance of cell volume and the regulation of intracellular pH and cell division [1]. Na^+/H^+ exchange activity is present in most tissues. There are several tissue-specific isoforms of the exchanger, which vary in molecular mass [2–4] and in their sensitivity to the inhibitor amiloride [2]. Recently, several isoforms of the Na^+/H^+ exchanger (NHE-1–NHE-4) have been cloned [5]. The amiloride-sensitive isoform (NHE-1) is inhibited by nanomolar concentrations of amiloride analogues such as ethylisopropylamiloride. An amiloride-resistant form requires micromolar concentrations of amiloride analogues for inhibition [2–6]. The amino acid sequence of NHE-1 has been deduced from the nucleotide sequence of a cDNA clone [7]. The protein is predicted to have a molecular mass of 91 kDa [7,8], with three potential sites for N-linked glycosylation [7]. A previous study noted that the amiloride-sensitive exchanger from epidermal cells was in fact a glycoprotein [8]. The predicted topology of the protein, based on the Engelman algorithm, suggests that only two of the three sites are on the external side of the membrane [7]. The function of the carbohydrate moiety on the Na^+/H^+ exchanger is not yet clear. Others have shown that N-asparagine-linked oligosaccharides have an important role in the biosynthesis [9], processing [10], translocation [11] and membrane insertion [9] of proteins to which they are attached. Carbohydrates may also be important in conferring resistance to proteolysis [12], and in some cases may have an important functional role [13].

In this study, we have taken advantage of a specific antibody raised against the C-terminal region of the amiloride-sensitive exchanger to investigate the structure and function of the carbohydrate moiety of the NHE-1 isoform of the Na^+/H^+ exchanger [14]. We examined the native protein from human placental brush-border membranes (BBM), since *in situ* it will be in its native conformation. This preparation is also a relatively rich source of the amiloride-sensitive (NHE-1) isoform of the Na^+/H^+ exchanger [14,15].

EXPERIMENTAL

Materials

Acridine Orange, glycopeptidase F, neuraminidase, α-mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase and trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All reagents used were of the highest grade available.

BBM vesicle preparation

BBMs were isolated from normal term human placenta essentially as described previously [14,15]. The final preparation of BBM vesicles was washed twice with loading buffer (25 mM Mes/5 mM Tris/200 mM mannitol, pH 5.5), and resuspended to 7 mg of protein/ml in loading buffer by passage through a 23-gauge needle.

Protein analysis

SDS/PAGE was on 7% polyacrylamide gels as described earlier [16]. Some experiments used large high-resolution 16 cm × 16 cm polyacrylamide gels. Proteins were transferred electrophoretically on to nitrocellulose membranes [16]. Immunostaining of the nitrocellulose membranes was done with the Amersham Enhanced Chemiluminescence Western Blotting and Detection System. Sizes of proteins were based on comparison with Bio-Rad prestained molecular-mass markers.

Carbohydrate analysis

(a) Intact vesicles. Intact BBM vesicles were washed twice with 5 ml of glycolytic buffer (50 mM KCl/20 mM NaH_2PO_4, pH 7.2), and resuspended to 1 mg of protein/ml in the same buffer containing 50 mM EDTA and a cocktail of proteinase inhibitors [16]. Samples (50 μl) were incubated in the presence of glycopeptidase F (8 units/ml), endoglycosidase F (Endo-F) (0.1 unit/ml) or endoglycosidase H (Endo-H) (0.1 unit/ml) for 14 h at 37 °C.

Abbreviations used: BBM, brush-border membrane; Endo-F, endoglycosidase-F; Endo-H, endoglycosidase-H.

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Partial deglycosylation of intact BBM vesicles was performed by incubating vesicles (350 μg) with glycopeptidase F (8 units/ml) for 0, 1 and 2 h. Fully deglycosylated exchanger was prepared by overnight incubation with glycopeptidase F (16 units/ml) and neuraminidase (5 units/ml).

(b) Solubilized vesicles. BBM vesicles (1 mg of protein) were pelleted as above, and washed twice with glycolytic buffer before digestion. The final pellet was resuspended in 200 μl of buffer containing proteinase inhibitors [16]. SDS (10%) and β-mercaptoethanol (10%) were added to final concentrations of 0.5% and 0.1%, respectively, and samples were boiled for 5 min. Buffer (750 μl) was added, plus n-octyl glucoside (10%) to a final concentration of 0.5%. Enzyme or buffer (4 μl) was added to samples of the solubilized membranes (50 μl). The final enzyme activities were: glycopeptidase F, 8 units/ml; α-mannosidase, 3.52 units/ml; neuraminidase, 2.56 units/ml; β-galactosidase, 8 units/ml; β-*N*-acetylglucosaminidase, 0.096 unit/ml. EDTA was added to a final concentration of 50 mM to all samples except that containing α-mannosidase. The samples were incubated overnight at 37 °C, and processed as described above.

Exchanger activity

Intact BBM vesicles were digested with glycopeptidase F (8 units/ml) for 6 h as described above. The samples of control or deglycosylated vesicles were washed twice with loading buffer (5 ml), and resuspended to 5 mg of protien/ml. Na^+/H^+ exchange was assayed by using the fluorescence quenching of Acridine Orange as described previously [14]. Internal and external pH were 5.5 and 7.5 respectively. Without added NaCl, the pH gradient of BBM vesicles had a half-life of 10–20 min. NaCl (0–50 mM final concn.) was added 1 min after the vesicles. Where used, amiloride (stock 10 mM solution in dimethyl sulphoxide; 250 μM final concn.) was added 2 min before the vesicles. K_m and $V_{max.}$ values were determined from Lineweaver–Burk plots.

Trypsin sensitivity

Control (20 μg of protein) or glycopeptidase F-treated (25 μg of protein) BBM vesicles were incubated with 0.0036–0.36 unit of trypsin for 15 min at 37 °C. Trypsin inhibitor (10 μg in 1 μl) was added to stop the digestion. Samples were analysed by immunoblotting as described above. Trypsin was made in a solution of 1 mM HCl (360 units/ml) and stored at −20 °C.

Antibodies

Antibodies against the Na^+/H^+ exchanger raised in rabbits were directed against a fusion protein that contained the C-terminal 178 amino acids of the NHE-1 isoform of the Na^+/H^+ exchanger [17]. Only affinity-purified antibodies were used, prepared as described previously [14,17].

Statistics

The SDS/PAGE gels and immunoblots presented are typical of three or more experiments performed on different preparations of vesicles. Other data are means ± S.E.M. Statistical differences between groups were evaluated by Student's *t* test.

RESULTS AND DISCUSSION

Using an antibody directed against the C-terminal cytoplasmic region of the amiloride-sensitive Na^+/H^+ exchanger (NHE-1), we detected a protein of molecular mass approx. 105 kDa in BBM vesicles from human placenta (Figure 1, lane 3). Another independently prepared antibody against the C-terminal 13 amino acids of the Na^+/H^+ exchanger [17] recognized a protein of the same molecular mass (result not shown), confirming that we had correctly identified the protein.

To determine the extent of the glycosylation of the Na^+/H^+ exchanger, human placental BBM vesicles were incubated with glycopeptidase F. This digestion caused a large change in the apparent molecular mass of the protein, from 105 to 98 kDa (Figure 1, lane 2). Thus the exchanger is a glycoprotein with a molecular-mass shift of 7 kDa upon treatment with glycopeptidase F. Such treatment does not seem to result in a completely deglycosylated exchanger, as incubation with glycopeptidase F in the presence of neuraminidase yields a lower-molecular-mass band (Figure 3, lane 4). This suggests that access to at least one carbohydrate chain may be sterically hindered when sialic acid residues are present. Digestions with O-glycanase in the presence of neuraminidase caused no further increase in the mobility of the immunoreactive antigen, in comparison with neuraminidase alone. This suggests that there are no O-linked carbohydrate groups attached to the Na^+/H^+ exchanger (results not shown).

To determine which type(s) of carbohydrate moiety are present on the exchanger, we treated the native protein with Endo-F and Endo-H. Figure 1 shows the results of these digestions. Endo-F (lane 4) had a similar effect to glycopeptidase F (lane 2), decreasing the apparent molecular mass of the exchanger by 7 kDa. In contrast, Endo-H (lane 1) had no effect on mobility. Endo-F removes the core and terminal moieties of biantennary-complex, hybrid and high-mannose-type N-oligosaccharide groups, whereas Endo-H only affects hybrid and high-mannose-type groups. Since Endo-F decreased the apparent molecular mass of the Na^+/H^+ exchanger, whereas Endo-H had no effect, the results suggest that the human Na^+/H^+ exchanger carries one or more N-linked biantennary-complex-type oligosaccharide groups.

To characterize the nature of the carbohydrate moiety further, we used other glycolytic digestions of solubilized BBM vesicles. The results of these digestions are shown in Figure 2. As with intact BBM vesicles, glycopeptidase F (lane 2) treatment produced a large change in apparent molecular mass. Neuraminidase (lane 4) also caused a change in mobility, although not to such an extent. None of the other enzymes tested altered the mobility of the Na^+/H^+ exchanger. α-Mannosidase, which cleaves terminal mannose residues, had no significant effect on the mobility of the exchanger (lane 5), indicating that the carbohydrate group is not

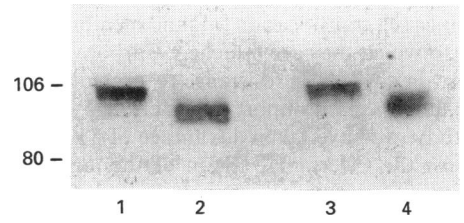

Figure 1 Western-blot analysis of human placental BBM with polyclonal antibody against the amiloride-sensitive Na^+/H^+ exchanger after digestions with glycopeptidases

Intact BBM vesicles were prepared as described previously [14]. Samples containing 50 μg of protein were incubated for 14 h at 37 °C in the presence of Endo-H (0.1 unit/ml), Endo-F (0.1 unit/ml), glycopeptidase F (8 units/ml) or buffer (1 μl). Samples were analysed by SDS/PAGE, as described in the Experimental section; 20 μg of protein per lane was loaded. Lanes 1–4 are Endo-H, glycopeptidase F, control and Endo-F respectively. The relative positions of Bio-Rad prestained markers phosphorylase *b* (106 kDa) and BSA (80 kDa) are indicated. Sizes of bands: lanes 1 and 3, 105 kDa; lanes 2 and 4, 98 kDa.

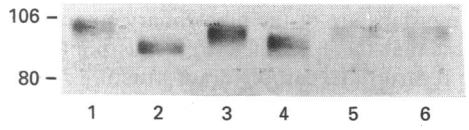

Figure 2 Western-blot analysis of solubilized human placental BBM with polyclonal antibody against the amiloride-sensitive Na^+/H^+ exchanger after digestions with glycopeptidases

BBM vesicles were prepared as described in [14], and solubilized with SDS and n-octyl glucoside as described in the Experimental section. Samples containing 50 μg of protein were incubated for 14 h at 37 °C in the presence of buffer (1 μl), glycopeptidase F (8 units/ml), α-mannosidase (3.52 units/ml), neuraminidase (2.56 units/ml), β-galactosidase (8 units/ml) or β-*N*-acetylglucosaminidase (0.096 unit/ml). Samples were analysed by SDS/PAGE, as described in the Experimental section, loading 20 μg of protein per lane. Lanes 1–6 are control, glycopeptidase F, β-galactosidase, neuraminidase, α-mannosidase and β-*N*-acetylglucosaminidase respectively.

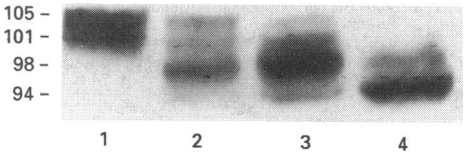

Figure 3 Effect of limited digestion with glycopeptidase F on the mobility of the exchanger

Intact BBM vesicles were prepared as described previously [14]. Samples containing 50 μg of protein were incubated at 37 °C. Lane 1 is control BBM vesicles incubated overnight in the absence of enzyme; lane 2 is a 1 h incubation with glycopeptidase F (8 units/ml); lane 3 is a 2 h incubation under the same conditions; and lane 4 is an overnight incubation with glycopeptidase F (16 units/ml) and neuraminidase (5 units/ml).

Table 1 Effect of deglycosylation on kinetic parameters of the Na^+/H^+ exchanger

Data were obtained as described in the Experimental section. Values shown are means ± S.E.M., and are from six separate experiments using three different placental BBM vesicle preparations. There were no significant differences between control and treated vesicles when compared by a paired Student's *t* test.

	$V_{max.}$ (d(ΔpH)/min per mg of protein)	K_m (mM)	Amiloride-sensitive exchange (%)
Control	82.6 ± 15.0	30.9 ± 8.7	88.0 ± 0.8
Glycosidase F	75.5 ± 21.2	25.1 ± 8.3	86.0 ± 1.4

of the high-mannose or hybrid type. This, together with the observation that neuraminidase had an intermediate but significant effect on mobility, supports the conclusion that the carbohydrate moiety is of the complex type. Treatment of solubilized BBM with both α-mannosidase and β-*N*-acetylglucosaminidase apparently affected the quantity of exchanger present. This may be due to proteolysis, although no lower-molecular-mass bands were identified. It is doubtful whether α-mannosidase activity affects the ability of the antibody to react with intact antigen, because removal of carbohydrates does not affect the ability of the antibody to recognize the exchanger (Figure 1, lane 2; Figure 2, lane 2). Also, the mobility of the remaining immunoreactive antigen was not altered.

To establish the number of carbohydrate moieties attached to the exchanger, we used partial digestions with glycopeptidase F. The products were analysed on large high-resolution polyacrylamide gels, followed by immunostaining with C-terminal antibody. The results are shown in Figure 3. Lane 1 shows control BBM vesicles incubated overnight without glycopeptidase F. Two bands were apparent, at 105 and 101 kDa. We were routinely able to identify the presence of two bands in this higher-resolution SDS/PAGE system, but were generally unable to distinguish the bands with smaller SDS/PAGE gels. The second band may be due to differential glycosylation of the exchanger. Alternatively, it may represent partial proteolysis of the exchanger during the initial preparation of the vesicles. It should be noted, however, that both bands were present even in preparations made in the presence of a cocktail of 10 different protease inhibitors [16]. Lanes 2 and 3 (Figure 3) are incubations with glycopeptidase F for 1 and 2 h respectively. Lane 4 is an overnight digestion with glycopeptidase F and neuraminidase. A total of four bands can be distinguished in the sum of all the lanes, two starting products (105 and 101 kDa), another intermediate (98 kDa), and a final smaller product (94 kDa). The smallest band represents the completely deglycosylated exchanger, since overnight incubation with glycopeptidase F and neuraminidase together results in the major band of this size. The observation that overnight incubation with glycopeptidase F gives one major band argues against the 101 kDa band in the control (lane 1) being a proteolytic product, and suggests that the intact protein exists in differentially glycosylated forms.

There are two possible explanations for the results of the partial-deglycosylation experiments. The simplest interpretation is that the Na^+/H^+ exchanger exists in two forms with either two or three carbohydrate moieties. Partial deglycosylation of these two forms results in the observed intermediates; complete deglycosylation results in the observed single product. An alternative explanation is that there are only two carbohydrate groups, which are significantly different in size in the intact protein. This could also result in the pattern of four immunoreactive bands and an eventual single immunoreactive deglycosylated protein. Both explanations suggest there are at least two sites of glycosylation of the protein, and possibly a third. The Na^+/H^+ exchanger contains three consensus sequences for asparagine-linked oligosaccharides, at amino acids 75, 370 and 410 [7]. However, a model based on hydrophobicity plots [7] suggests that only amino acids 75 and 370 are extracellular. The present series of experiments provides evidence that at least two of these proposed sites, and possibly the third, are extracytoplasmic and glycosylated. It should be noted that a recent report has shown the presence of cytoplasmically orientated N-glycosylation [18]. It is unlikely that the protein observed in the present series of experiments contains cytoplasmically orientated sugars. Incubation of right-side-out vesicles [15] with glycosidases (Figure 2) had the same effects as treatment of the solubilized protein (Figure 1).

To investigate the effect of removal of the carbohydrate group on exchanger activity, we digested intact BBM vesicles with glycopeptidase F. To determine the appropriate digestion time for this experiment, the effect of incubation time on cleavage was assessed. Removal of the carbohydrate group from the exchanger was essentially complete after 6 h under the conditions described in the Experimental section (results not shown). We used this incubation time for functional assays. The kinetic parameters for the exchanger in vesicles incubated with either buffer (control) or glycopeptidase F were not significantly different (Table 1), indicating that the carbohydrate moiety is not essential for Na^+/H^+-exchanger function. Pretreatment with glycopeptidase F also did not decrease the degree of inhibition of the BBM exchanger by 250 μM amiloride (Table 1), with almost 90% of Na^+-dependent proton movement being sensitive to amiloride in both treated and untreated vesicles. These results contrast with those reported by Yusufi et al. [19]. They examined the rat renal BBM Na^+/H^+ exchanger and found that the carbohydrate moiety

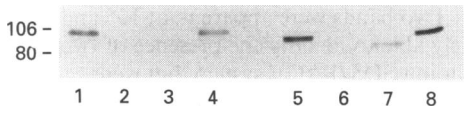

Figure 4 Effects of glycopeptidase F treatment on sensitivity to digestion by trypsin

Samples were analysed by Western-blot analysis of treated membranes. Intact BBM vesicles were prepared as described previously [14]. Vesicles were incubated with buffer or glycopeptidase F (8 units/ml) for 6 h, and subsequently treated with trypsin for 15 min at 37 °C, as described in the Experimental section. Lanes 1–4 are control BBM vesicles (20 μg of protein/lane) incubated with; 1 μl of 1 mM HCl (1) or 0.36 (2), 0.036 (3) and 0.0036 (4) unit of trypsin in 1 mM HCl. Lanes 5–8 are glycopeptidase F-treated BBM vesicles (25 μg of protein/lane) similarly treated with vehicle or trypsin.

is essential to the activity of the protein. Removal of the carbohydrate moiety with Endo-F resulted in decreased rates of Na^+/H^+ antiport. However, the Na^+/H^+ exchanger in this membrane is the amiloride-resistant (NHE-2) isoform [3–5], and may therefore possess different characteristics compared with the amiloride-sensitive (NHE-1) type. To summarize, it appears as though in the NHE-1 isoform glycosylation is not essential for activity, and in the NHE-2 isoform glycosylation is essential.

In several cases glycosylation of proteins has been shown to confer resistance to proteolysis [12]. We examined the sensitivity of the Na^+/H^+ exchanger to trypsin after treatment with glycopeptidase F (Figure 4). Control and glycopeptidase F-treated vesicles were equally sensitive to trypsin over the range investigated (0.0036–0.36 unit/digestion), suggesting that the carbohydrate groups do not confer resistance to proteolysis. In some experiments we were able to detect a small amount of immunoreactive 98 kDa protein (Figure 4). The exact identity of this species is unknown. One possibility is that it is an intermediate of the glycosylation process, from incompletely processed membrane proteins contaminating our preparation. The size of this immunoreactive band is the same as the major intermediate shown in Figure 3 (lane 3), suggesting that this is a possible explanation. It is unlikely that the 101 kDa immunoreactive antigen (Figure 3, lane 1) also originates from incompletely processed membranes, since it is present in similar abundance to the 105 kDa protein.

In conclusion, our studies indicate that the human amiloride-sensitive Na^+/H^+ exchanger (NHE-1 isoform) carries two or three N-linked carbohydrate moieties. Evidence suggests that the carbohydrate groups are of the biantennary complex type. The results suggest that there is a heterogeneous population of the protein with regard to its carbohydrate structure. There may be some protein with three carbohydrate groups and some with only two groups. Alternatively, there may be two populations of protein present with heterogeneity in the degree of glycosylation at only two sites. It may be that the function of the carbohydrate moiety lies in supporting proper folding, membrane insertion and orientation of the protein, or in directing the protein to the plasma membrane. Future studies will explore these possibilities.

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