Characterization of the placental brush border membrane Na⁺/H⁺ exchanger: identification of thiol-dependent transitions in apparent molecular size

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We examined the protein and mRNA encoding the amiloride-sensitive Na⁺/H⁺ exchanger from human placenta. Reverse transcriptase PCR of human placental RNA and a human chorio-carcinoma cell line showed that the message for the amiloride-sensitive Na⁺/H⁺ exchanger is present in the placenta and its derived cell line. Northern blot analysis showed only one species of Na⁺/H⁺ exchanger mRNA, of about 5 kb in size. To examine the Na⁺/H⁺ exchanger protein two different affinity-purified antibodies were produced against the C-terminal cytoplasmic region of the Na⁺/H⁺ exchanger. The antibodies both identified a

105 kDa protein in human placental brush border membrane vesicles. Under non-reducing conditions the amount of 105 kDa protein was greatly decreased, while a 205 kDa protein became apparent. This is probably a dimer of the 105 kDa protein. The monomer-to-dimer transition was dependent on the concentration of β -mercaptoethanol. The results show that the amiloride-sensitive Na⁺/H⁺ exchanger is relatively abundant in human placenta and that it can exist as a larger 205 kDa protein linked by disulphide bonds.

INTRODUCTION

The Na⁺/H⁺ exchanger is a widely distributed protein which regulates intracellular pH in many mammalian cells by extruding one H⁺ in exchange for Na⁺ when decreases in intracellular pH occur. It is responsible for recovery of intracellular pH in many cell types following acidification [1–3]. Pharmacologically distinguishable types of antiporters exist on the apical and basolateral surfaces of cultured porcine kidney cells [4], and it has been shown that there may be more than one form of the Na⁺/H⁺ exchanger in a number of tissues [4–7].

Despite its key role in the regulation of intracellular pH, the Na⁺/H⁺ exchanger appears to be present in small quantities in the plasma membranes of various cell types. Its activity has been demonstrated in many cell types, but only some preliminary work has been accomplished on the purification and characterization of the protein itself. Studies have recently isolated and cloned several isoforms of the cDNA of the Na⁺/H⁺ exchanger (NHE-1–NHE-4); however, there has been little progress in characterizing the proteins involved [8]. Attempts have been made to identify the particular protein(s) responsible for Na⁺/H⁺ exchange in various cell lines using antibodies or the Na⁺/H⁺ exchange inhibitor amiloride and its analogues. Some results have suggested that a 100–110 kDa protein or a 65 kDa protein is the exchanger [9–11]. Sardet et al. [12] made an antibody against a fusion protein of the amiloride-sensitive human Na⁺/H⁺ exchanger which immunoprecipitated a 110 kDa protein in fibroblasts [13]. However, using an antibody raised against a synthetic peptide antibody other smaller proteins have also been identified as the putative exchanger, often in the 65–75 kDa range [14]. The smaller proteins have been suggested to correspond to the low amiloride affinity form of the exchanger [14], or alternatively could be fragments of the larger protein. We have previously shown the presence in the rabbit myocardium of

a Na⁺/H⁺ exchanger that has marked sequence identity to the amiloride-sensitive (NHE-1) form described earlier by Sardet et al. [12,13,15]. We found that the deduced amino acid sequence of this clone was over 96% identical to that of the amiloride-sensitive human form described earlier by Sardet et al. [13]. Similarly, Hildebrandt et al. [16] have recently isolated and sequenced a rabbit renal Na⁺/H⁺ exchanger which was 95% identical to the human exchanger. These results suggest that the Na⁺/H⁺ exchanger is highly conserved between various tissues and species.

The purpose of this study was several-fold. We have recently shown [15] that in rabbit skeletal muscle a second, smaller, related form of the Na⁺/H⁺ exchanger mRNA exists. In the present study we examined mRNA from human placenta to see if this other isoform was present. We also studied the relative abundance of the exchanger message in several tissues. According to hydrophobicity plots, the C-terminal region of the amiloride-sensitive Na⁺/H⁺ exchanger should be an antigenic region of the protein. We wished to produce antibodies against this region of the protein, as this would give us a tool with which to examine the abundance and distribution of the protein. We wanted to immunologically identify the amiloride-sensitive Na⁺/H⁺ exchanger in the placenta and study its characteristics. We also characterized the effects of thiol reduction on the native subunit structure of the protein.

We found that the placental brush-border membrane is enriched in 105 kDa exchanger antigen and conclude that the placenta may be a good tissue with which to begin studies on purification and characterization of the protein. The protein appears to be present as a dimer, and under non-reducing conditions had an apparent molecular weight of 205 kDa. The exchanger message is of one type only, of 5 kb, and was present both in the placenta and in BeWo cells, which are derived from human placenta.

Abbreviation used: IPTG, isopropyl β -D-thiogalactoside.

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EXPERIMENTAL

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Bethesda Research Laboratories. Taq polymerase was from Bio/Can Scientific (Mississauga, Ont., Canada). The plasmids pEX-1 and pGEX-3X were from Boehringer Mannheim Canada (Laval, Que., Canada) and Pharmacia LKB Biotechnology Inc. respectively. [α - 32 P]ATP (20–40 Ci/mmol) was purchased from Amersham (Oakville, Ont., Canada). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH, U.S.A.) or Bio-Rad (Richmond, CA, U.S.A.), and nylon membranes were from Amersham. SDS/PAGE reagents and protein standards were purchased from Bio-Rad, and horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio/Can. Acridine Orange and *p*-aminophenyl- β -D-thiogalactopyranoside-agarose were obtained from Sigma (St. Louis, MO, U.S.A.). The synthetic peptide was obtained from the Alberta Peptide Institute (Edmonton). Phenylarsine oxide was a gift from Dr. Charles Holmes of the Department of Biochemistry, University of Alberta. General laboratory reagents used were of analytical grade or better.

Preparation of purified membranes

Human placental syncytiotrophoblast brush-border membrane vesicles were prepared as described by Balkovetz et al. [17] from normal term human placentas. Some placental brush-border membranes used in this study were a gift from Dr. V. Ganapathy, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA, U.S.A. Protein was determined by the method of Bradford [18]. In some experiments human placental syncytiotrophoblast brush-border membrane vesicles were also prepared in the presence of 1 mM iodoacetamide to prevent thiol reduction and possible alteration of the Na⁺/H⁺ exchanger during the isolation procedure. In these experiments, 1 mM iodoacetamide was included in the buffers used to wash and lyse the cells and during isolation of the brush-border membrane fraction. The inclusion of iodoacetamide to prevent structural alterations during isolation is similar to the procedure described earlier for the rat insulin receptor [19].

Polyacrylamide gels and immunostaining

SDS/PAGE was on 9% polyacrylamide gels (or on 7% gels as indicated) as described earlier [20], essentially by the method of Laemmli [21]. After separation, proteins were transferred electrophoretically on to nitrocellulose membranes [22]. Immunostaining of nitrocellulose membranes was carried out in the presence of 1% skim milk powder using affinity-purified antibodies essentially as described earlier [23], or using the Amersham Enhanced Chemiluminescence Western Blotting and Detection System as described by the manufacturer.

Measurement of Na⁺/H⁺ exchange in placental vesicles

Placental brush-border membrane vesicles were loaded with 25 mM Mes, 5 mM Tris and 200 mM mannitol, pH 5.5, as previously described [17]. Changes in pH gradient across the vesicular membrane were monitored by following the fluorescence quenching of Acridine Orange, using a Shimadzu RF-5000 spectrofluorophotometer (excitation 492 nm; emission 528 nm; slit-widths 3 nm). The temperature was maintained at 15 °C with a thermoregulated cuvette holder. Placental brush border membrane vesicles were added to a final concentration of

70 μ g of protein/ml. A calibration curve of % quench against Δ pH was prepared using the above buffer at pH values ranging from 6.5 to 8.0, giving a Δ pH of 1–2.5 pH units. For functional assays, the external pH was 7.5. Proton extrusion was initiated by the addition of 10 mM NaCl. Each experiment was done in duplicate or triplicate using different placental membrane preparations.

Expression of Na⁺/H⁺ exchanger protein and antibody production

A portion of the rabbit Na⁺/H⁺ exchanger cDNA clone corresponding to the C-terminal 178 amino acids was expressed in *Escherichia coli* using the pEX-1 vector, which expresses proteins as a fusion protein with β -galactosidase as described earlier [24]. The plasmid was transformed into host cells and the protein induced and purified as described earlier [24] with a *p*-aminophenyl- β -D-thiogalactopyranoside-agarose column. For immunization studies, partially purified protein was separated by SDS/PAGE as described above and briefly stained with 0.05% Coomassie Brilliant Blue R-250 in water (without methanol or acetic acid). The gel was destained briefly with water and the 135 kDa Na⁺/H⁺-exchanger- β -galactosidase fusion protein [24] was excised and used for immunization of rabbits in Freund's complete adjuvant, followed by three booster injections in Freund's incomplete adjuvant at 2-week intervals. For affinity purification of these antibodies a portion of the rabbit Na⁺/H⁺ exchanger cDNA clone corresponding to the C-terminal 344 amino acids of the rabbit cardiac Na⁺/H⁺ exchanger was cloned into the *Sma*I-*Eco*R1 sites of expression plasmid pGEX-3X using PCR and primers of sequence 5' AAGTAACTTC-CCCATGTG(CT)GACCTGTTCTCAC 3' and 5' AAGAA-TTCTACTGCCC(C/T)TTGGGGA(A/T)GAA, designed from bp 4–41 and 1027–1047 respectively of the rabbit cardiac Na⁺/H⁺ exchanger clone described earlier [15]. (The redundancies are present in the second primer for use with the human Na⁺/H⁺ exchanger message as described in Sardet et al. [13].) PCR was carried out as described below by the method of Saiki et al. [25]. After subcloning of the fragment, production of the Na⁺/H⁺-exchanger-pGEX-3X fusion protein was in DH5- α cells, which were induced with 0.2 mM isopropyl β -D-thiogalactoside (IPTG). Cells were lysed with a French press and the pellet fraction (10000 g, 10 min) containing the Na⁺/H⁺-exchanger-pGEX-3X fusion protein was separated by size by SDS/PAGE as described above. The separated proteins were transferred to nitrocellulose and the fusion protein was identified using the synthetic peptide antibody (described below). The appropriate nitrocellulose strip was used to affinity-purify the antibody against the Na⁺/H⁺-exchanger- β -galactosidase fusion protein as described earlier [20].

For production of another antibody against the cytoplasmic domain of the Na⁺/H⁺ exchanger, a synthetic peptide was synthesized encoding the C-terminal 13 amino acids of the rabbit cardiac Na⁺/H⁺ exchanger ([14], EPGEPEFPKGGQ). This was coupled to keyhole-limpet haemocyanin and used to immunize rabbits as described above. To affinity-purify this antibody, synthetic peptide coupled to BSA was linked to CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. The resulting matrix was used to purify the antibody against the Na⁺/H⁺ exchanger similar to as described above.

RNA isolation and analysis

Poly(A)⁺ RNA was isolated from human placenta and Bewo cells with a modified procedure similar to that described earlier

[15]. The tissue was powdered in a mortar and pestle cooled to the temperature of liquid nitrogen and was homogenized in 12 ml/g RNAase free buffer containing 4.5 M guanidinium thiocyanate, 50 mM EDTA, 25 mM sodium citrate, pH 7.0, 0.1 M β -mercaptoethanol and 2% lauroyl sarcosine. The tissue was homogenized with an Omni 2000 homogenizer for 30 s at 15000 rev./min. The tissue was then extracted with phenol, the aqueous phase centrifuged at 10000 *g* for 20 min and the supernatant precipitated two times with ethanol. The resulting RNA pellet was purified with a PolyAtract mRNA isolation kit as described by the manufacturer (Promega). Samples (10 μ g) of poly(A)⁺ RNA were separated by electrophoresis in a denaturing formaldehyde 1.5% agarose gel stained with ethidium bromide and transferred to Immobilon-N filters as described earlier [15]. The Northern blot was probed with ³²P-labelled random-primed cDNA. The fragment used for labelling was bp 1–688 of the rabbit cardiac cDNA clone [15], corresponding to a portion of the cytoplasmic C-terminus of the protein. Hybridization and washes were as described earlier; routinely, the final wash of blots was at low-to-medium stringency with 2 $\times$ SSC at 60 °C [15] (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate).

Reverse transcriptase PCR

Total RNA was isolated as described above and 5 μ g was reverse-transcribed with the Invitrogen cDNA cycle kit using the procedures described by the manufacturer. Samples (5 μ l) of the cDNA were used for PCR [25] with synthetic primers of the sequence 5' TCTGTGGCCACTACGG 3' and 5' AAGAA-TTCTACTGCC(C/T)TTGGGGA(A/T)GAA 3'. The primers were based on the sequences of the known rabbit (nucleotides 206–221 and 1027–1048 [15]) and human (nucleotides 1610–1625 and 2428–2448 [12]) Na⁺/H⁺ exchanger cDNA clones. Samples were added to a reaction mixture (50 μ l) containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each deoxynucleotide and 1 unit of Taq polymerase. Amplification of cDNA was repeated 32 times under the conditions of 95 °C for 40 s, 58 °C for 40 s and 72 °C for 3 min on a Coy TempCycler. A 5 μ l aliquot of these reactions was examined on 5% polyacrylamide gels and on a 1% agarose gel, and samples were analysed for hybridization to cDNA probes by Southern blotting as described above for Northern blotting.

Cell culture

BeWo cells were generously provided by Dr. C. Cass, Department of Biochemistry, University of Alberta.

RESULTS

To confirm the presence of the Na⁺/H⁺ exchanger mRNA in intact tissue and in a placentally derived cell line we used reverse transcriptase PCR. Total RNA was reverse-transcribed and subjected to 32 cycles of amplification with the primers described in the Experimental section. The results are shown in Figure 1. The expected band of 846 bp was evident most notably in the sample derived from human placenta mRNA. The reverse-transcribed BeWo cell line mRNA also gave a PCR product, although it was somewhat less evident, possibly reflecting a difference in amount of the Na⁺/H⁺ exchanger message. The identity of the PCR product was confirmed by Southern blotting and hybridization with the appropriate fragment of the Na⁺/H⁺ exchanger cDNA clone (results not shown).

We have earlier shown the presence of a smaller isoform of the Na⁺/H⁺ exchanger mRNA in rabbit fast-twitch skeletal muscle which hybridizes under reduced stringency [15]. Figure 2 shows Northern blot analysis of the human placental mRNA probed with a fragment of the rabbit cardiac cDNA as described in the Experimental section. Only one band was apparent upon exposure of the Northern blot after hybridization with the cDNA probe, even with exposure after washing at reduced stringency. The size of this band was slightly over 5 kb, as calculated when compared with the positions of the 28 S and 18 S ribosomal subunits, which were run on the same gel (not shown).

To study the Na⁺/H⁺ exchanger protein we produced antibodies against the C-terminus of the exchanger. We used the expressed terminal 178 amino acids of the Na⁺/H⁺ exchanger protein fused to β -galactosidase (described in [24]). The purified fusion protein produced antibodies of high titre, although this included antibodies against β -galactosidase as well as against the Na⁺/H⁺ exchanger. To affinity-purify the antibodies, the terminal 344 amino acids of the rabbit cardiac Na⁺/H⁺ exchange protein

Figure 1 Reverse transcription PCR of total RNA samples of human placenta and BeWo cells

Samples were prepared as described in the Experimental section, separated by electrophoresis on 5% polyacrylamide gels and stained with ethidium bromide. mRNA samples are: lane 1, control (no mRNA); lane 2, BeWo cell line; lane 3, human placenta. Lane M contains markers, a mixture of λ DNA *Eco*RI- and *Hind*III-cut plus *Hae*III-digested pBR322. The sizes of the distinct bands shown are, from top to bottom, 20–3.5 kb, 2 kb, 1.9 kb, 1.6 kb, 1.3 kb, 983 bp, 831 bp, 587 bp, 540 bp, 504 bp, 458 bp, 434 bp, 267 bp, 234 bp, 213 bp, 192 bp, 184 bp.

Figure 2 RNA blot analysis of poly(A)⁺ RNA from intact human placenta

Residues 511–1265 of the rabbit cardiac cDNA clone [14] were used to probe 10 μ g of poly(A)⁺ RNA as described in the Experimental section. The relative positions of the 28 S and 18 S ribosomal subunits are indicated, and were determined from samples of total RNA run simultaneously on the same gel.

Figure 3 Immunological detection of expression of Na^+/H^+ -exchanger-glutathione transferase fusion protein in *E. coli*

A portion of the rabbit cardiac Na^+/H^+ exchanger corresponding to the terminal 344 amino acids was cloned into the expression vector pGEX-3X. Control and fusion protein plasmids were transfected into *E. coli* and cells were induced with 0.2 mM IPTG. The cells were pelleted, and protein was separated by SDS/PAGE and transferred to nitrocellulose. Lane 1, Bio-Rad prestained markers; lane 2, cell extract of control plasmid with no insert; lanes 3 and 4, extracts of plasmids with the Na^+/H^+ exchanger insert. The nitrocellulose was reacted with antibody against a C-terminal synthetic peptide of the C-terminal 13 amino acids of the rabbit Na^+/H^+ exchanger.

[15] were expressed using the expression vector pGEX-3X. To confirm that we had correctly subcloned the appropriate fragment in the correct reading frame we used an independently made antibody against a synthetic peptide of the C-terminal 13 amino acids of the rabbit cardiac Na^+/H^+ exchanger protein. Figure 3 shows a Western blot of the expressed C-terminal 344 amino acids of the exchanger protein fused to glutathione transferase and reacted with the antibody against the synthetic peptide. Samples were whole cell lysates of *E. coli*. Lane 2 shows the control (pGEX-3X with no Na^+/H^+ exchanger insert), while lanes 3 and 4 show the strong immunoreactivity of the antibody with the expressed protein made from two independent clones in *E. coli*. Some evidence of degradation of the fusion protein was noted (Figure 3). The fusion protein was found to be enriched in a low-speed pellet fraction, possibly in inclusion bodies. This fraction was separated by SDS/PAGE and the proteins transferred to nitrocellulose. The larger immunoreactive protein on the nitrocellulose, denoted by the arrow in Figure 3, was used to affinity-purify the antibody as described earlier [20]. In addition to the antibodies produced against the fusion protein, we also affinity-purified antibodies against the C-terminal synthetic peptide.

The affinity-purified antibody against the C-terminal 178 amino acids of the Na^+/H^+ exchanger was used to identify the Na^+/H^+ exchange protein in syncytiotrophoblast brush-border membranes prepared as described in the Experimental section [17]. Figure 4(a) (lane 1) shows the pattern of Coomassie Blue staining of the membranes. Only very small amounts of stained protein were visible at the size of the 106 kDa Bio-Rad prestained marker. Lane 2 shows the pattern of immunostaining with the affinity-purified antibody against the C-terminal 178 amino acids of the protein. Very strong immunoreactivity was shown at 105 kDa. This is in agreement with the size of the Na^+/H^+ exchanger predicted from the human cDNA clone isolated earlier [12,13]. The protein was enriched in the brush-border membrane fractions and was not present in sufficient quantities to be detected in crude microsome fractions of whole placenta (results not shown). In addition, we noted the presence of a minor

Figure 4 Analysis of protein composition and immunoreactivity of human placental syncytiotrophoblast brush border membranes

(a) Lane 1 contains Coomassie Blue-stained proteins of SDS/PAGE (9%) of human placental syncytiotrophoblast brush-border membranes prepared as described in the Experimental section. Lane 2 is a Western blot showing the immunoreactivity of human placental syncytiotrophoblast brush-border membranes with affinity-purified antibody against the C-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger. Lane 3 is a Western blot showing the immunoreactivity of human placental syncytiotrophoblast brush-border membranes with affinity-purified antibodies against the C-terminal 13 amino acids of the rabbit cardiac Na^+/H^+ exchanger. The blots were developed using the Amersham Enhanced Chemiluminescence Western Blotting and Detection System. The position and sizes of the prestained standards are indicated. (b) and (c) Immunoreactivity of human placental syncytiotrophoblast brush-border membranes with affinity-purified antibodies under reducing and non-reducing conditions. Membrane proteins were run on SDS/7% polyacrylamide gels as described in the Experimental section. Samples are in the presence (R; reducing) and absence (NR; non-reducing) of 3% β -mercaptoethanol. The positions and sizes of the Bio-Rad prestained standards myosin (205 kDa) and β -galactosidase (116.5 kDa) are indicated. (b) Immunoreactivity of human placental syncytiotrophoblast brush-border membranes with affinity-purified antibody against the C-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger. (c) Immunoreactivity with antibody against the C-terminal 13-amino-acid synthetic peptide of the Na^+/H^+ exchanger.

immunoreactive band in the brush-border membranes, of approx. 170 kDa. The identity of this protein is not yet known. Pre-immune sera showed no reactivity with any membrane proteins (results not shown). To confirm the identity of the 105 kDa protein we used affinity-purified antibody against the synthetic peptide encoding for the terminal 13 amino acids of the Na^+/H^+ exchanger. Figure 4(a) (lane 3) shows that the same 105 kDa

Figure 5 Concentration-dependence of the mobility shift of the Na⁺/H⁺ exchanger

Placental syncytiotrophoblast proteins were run on 7% SDS/PAGE as described in Figure 4 and solubilized with sample buffer in the absence of β -mercaptoethanol. β -Mercaptoethanol was then added to the final concentrations of (%) = 1 (A), 0.1 (B), 0.01 (C), 0.001 (D), 0.0001 (E), 0.00001 (F) and 0 (G)%. The immunoblot was developed with antibody against the C-terminal 13 amino acid synthetic peptide of the Na⁺/H⁺ exchanger.

protein was recognized by this antibody. In addition, the presence of an 82 kDa antigen was noted. Control (preimmune) sera of rabbits showed no reactivity with any of the above-described antigens.

To examine the effects of reduction of the disulphide bonds on the mobility of the Na⁺/H⁺ exchanger we immunoblotted the human placental syncytiotrophoblast brush-border membranes separated on SDS/7%-polyacrylamide gels with the affinity-purified antibodies in the standard SDS protein sample buffer, but in the presence and absence of 3% β -mercaptoethanol (Figures 4b and 4c). When samples were prepared in the absence of β -mercaptoethanol there was a dramatic decrease in the intensity of the 105 kDa antigen and the appearance of a 205 kDa antigen which is not present in the reduced sample. Both the antibody against the synthetic peptide (Figure 4c) and the antibody against the fusion protein (Figure 4b) gave the same results. We examined the concentration-dependence of this effect in order to show more carefully the mobility shift of the exchanger from the 205 to the 105 kDa form. Membranes were solubilized with sample buffer in the absence of β -mercaptoethanol, which was added to give the final concentration indicated (Figure 5). The results showed that the shift in mobility from dimer to monomer occurred between 0.001% and 0.1% β -mercaptoethanol (final concentration). The amount of 205 kDa antigen in the absence of β -mercaptoethanol was usually somewhat less than the 105 kDa antigen in the presence of β -mercaptoethanol. It appears as though the efficiency of transfer of the 205 kDa protein from protein gels to nitrocellulose is not as high as with the 105 kDa protein.

To investigate whether β -mercaptoethanol has any effect on Na⁺/H⁺ exchanger activity, placental brush-border membrane vesicles (7 mg protein/ml) were incubated in the presence or absence of β -mercaptoethanol for 30 min at room temperature. The rate at which 10 mM NaCl collapsed a gradient of 2.0 pH units (acid inside) was determined by following the quenching of Acridine Orange, as described in the Experimental section. In the absence of β -mercaptoethanol, a rate of 5.77 ± 0.66 ($n = 4$) pH units/min per mg of protein was observed. Preincubation with 1% β -mercaptoethanol had no significant effect on exchanger activity [6.33 ± 0.52 (3) pH units/min per mg]. As a control, identical experiments using the vicinal dithiol reagent phenylarsine oxide gave 50% inhibition of exchanger activity at 0.5 mM, similar to results described earlier by Ganapathy and co-workers [26].

To determine whether the 105 kDa antigen is normally a dimer in the cell or may be the product of rearrangement during the

Figure 6 Comparison of placental brush-border membranes isolated in the presence or absence of iodoacetamide

Placental brush-border membranes were prepared as described in the Experimental section in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1 mM iodoacetamide. Samples were separated on SDS/7%-PAGE as described in Figure 5 and solubilized with sample buffer in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of β -mercaptoethanol (3%). The positions and sizes of the Bio-Rad prestained standards myosin (205 kDa) and β -galactosidase (116.5 kDa) are indicated.

isolation procedure, we isolated placental brush border membranes in the presence of 1 mM iodoacetamide. The inclusion of iodoacetamide to prevent structural alterations during isolation is similar to the procedure described earlier for the rat insulin receptor [19]. A placenta was divided in half and brush-border membranes were isolated in either the presence or the absence of 1 mM iodoacetamide (Figure 6). Control (lanes 1 and 3) and iodoacetamide-treated (lanes 2 and 4) membranes were separated on SDS/PAGE in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of β -mercaptoethanol. In the presence of β -mercaptoethanol there was a noticeable increase in the amount of 105 kDa antigen. In the absence of β -mercaptoethanol there was an increase in both the 205 and 105 kDa antigens.

DISCUSSION

The purpose of this study was to further characterize both the protein and mRNA for the amiloride-sensitive Na⁺/H⁺ exchanger of human placenta. We examined the human placenta since, in addition to being an easily available source of tissue, earlier studies have suggested that it is enriched in Na⁺/H⁺ exchanger activity [17]. Reverse transcriptase PCR was used to confirm the presence of exchanger message in human placenta. We obtained a strong signal from whole placenta and from the BeWo cell line, which is derived from a malignant human choriocarcinoma [27,28]. Since the primers are based on the amiloride-sensitive Na⁺/H⁺ exchanger, the results (Figure 1) strongly support the presence of this form of the exchanger in this tissue. The function of the Na⁺/H⁺ exchanger in the placental brush-border membrane may be in the transfer of Na⁺ from the mother to the foetus. It may be the main mechanism of transport of Na⁺ across this membrane, perhaps accounting for its presence and relative abundance there [17].

To further examine the Na⁺/H⁺ exchanger mRNA we used Northern blot analysis. We have earlier demonstrated the presence of a smaller (3.8 kb) isoform of the Na⁺/H⁺ exchanger message in rabbit skeletal muscle which cross-hybridizes at low stringency [15]. We found (Figure 2) that only the 5 kb message for this protein is found in the placenta. This is the same size as found earlier for the amiloride-sensitive Na⁺/H⁺ exchanger [12,13,15], again confirming the presence of this isoform in this tissue. The smaller message, which we have also found in the rabbit myocardium (L. Fliegel and J. R. B. Dyck, unpublished work), may represent the amiloride-insensitive exchanger, which is suggested to be of a smaller size than the amiloride-sensitive form [14]. The conclusion that the Na⁺/H⁺ exchanger of the placenta brush-border membrane is the amiloride-sensitive form is in agreement with the observations of Balkovetz et al. [17],

who characterized the sensitivity of the exchanger to inhibition by amiloride analogues.

In order to examine the Na^+/H^+ exchanger protein we produced antibodies against it. To achieve this end we expressed the C-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger [24] as a fusion protein in *E. coli* and used it to produce antibodies against this region of the protein. We affinity-purified the resulting polyclonal antibody with a second, independently produced, Na^+/H^+ exchanger fusion protein. To confirm that we had correctly expressed the protein in both cases we used an antibody against a synthetic peptide encoding the C-terminal 13 amino acids of the rabbit cardiac Na^+/H^+ exchanger (Figure 3).

The resulting affinity-purified antibodies were used to examine the Na^+/H^+ exchanger protein in syncytiotrophoblast brush-border membrane vesicles from human placenta. Both affinity-purified antibodies immunostained a 105 kDa protein (Figure 4a). This is in agreement with the predicted size of the protein derived from its cDNA sequence [12] and from studies which immunoprecipitated the protein from other tissues [13]. To our knowledge this is the first immunological identification of the protein in this tissue. Since both the antibodies were based on the amiloride-sensitive form of the Na^+/H^+ exchanger, the results confirm the presence of this Na^+/H^+ exchanger isoform in placenta brush-border membranes. It is of note that Coomassie Blue staining of the placental membranes showed only small amounts of protein present at 105 kDa on the gel (Figure 4a, lane 1), suggesting that the Na^+/H^+ exchanger makes up only a small component of the membrane fraction, despite its relative enrichment in this compared with other tissues.

We found that the Na^+/H^+ exchanger can exist in its native form as a dimer. When samples of placental syncytiotrophoblast brush-border membrane vesicles were examined by immunoblotting under non-reducing conditions, the amount of 105 kDa protein was dramatically decreased, while a 205 kDa antigen appeared (Figures 4b and 4c). This mobility shift was noted with both affinity-purified antibodies and was dependent on the concentration of β -mercaptoethanol (Figures 4b, 4c and 5). These results are not totally surprising. Kinetic analysis has suggested that the Na^+/H^+ exchanger does exist as either an oligomer or a complex in the membrane [29,30]. The present study provides the first direct evidence for dimerization in the membrane. We cannot yet determine whether the protein is a homodimer or a heterodimer with another protein of the same size. Because the mobility shift corresponds almost exactly to a doubling of the exchanger mass, it seems likely that it is a homodimer. Future experiments may resolve this question.

It has been noted that vicinal dithiol groups are essential for activity of the Na^+/H^+ exchanger [26]. We further examined the effects of β -mercaptoethanol on the Na^+/H^+ exchange activity of placental brush-border membrane vesicles. We found that pre-incubation of the placental brush-border membrane vesicles with 1% β -mercaptoethanol had no effect on Na^+/H^+ exchanger activity. However β -mercaptoethanol at 1% did result in formation of a monomer in the presence of SDS (Figure 5). This suggests that the reduction of the particular disulphide bond involved in dimerization is not essential to the activity of the exchanger. We cannot yet determine whether the subunits have in fact dissociated from each other in the intact vesicles incubated with β -mercaptoethanol.

One possibility is that the 205 kDa Na^+/H^+ exchanger antigen could be a product of rearrangement of thiols during the isolation procedure. To investigate this possibility we isolated human placental brush-border membranes in the presence or absence of iodoacetamide. Iodoacetamide (1 mM) has been used previously

to prevent thiol reduction and possible structural alterations during isolation procedures [19]. The total amount of 105 kDa immunoreactive antigen in the presence of β -mercaptoethanol was greatly increased when the isolation was done in the presence of iodoacetamide (Figure 6, lanes 1 and 2). The reason for the increased quantity of immunoreactive antigen is unknown, although iodoacetamide has been used to prevent degradation of this protein in other studies [12]. We have noted that the Na^+/H^+ exchanger is very sensitive to treatment with proteinases (L. Fliegel and R. S. Haworth, unpublished work).

When placental brush-border membranes were isolated in the absence of iodoacetamide there was little 105 kDa antigen present in samples run without β -mercaptoethanol (Figure 6, lane 3). β -Mercaptoethanol converted all of the 205 kDa antigen to 105 kDa antigen in these membranes (Figure 6, lane 1). Although the amounts of 105 kDa antigen appear to be larger than the quantity of 205 kDa protein, this is attributable to difficulties in transfer of the larger proteins [31,32]. In contrast to control membranes, membranes isolated in the presence of iodoacetamide contained both the 205 kDa and the 105 kDa antigens when prepared for SDS/PAGE without β -mercaptoethanol (Figure 6, lane 4). Both antigens are also increased in quantity compared with control membranes (lane 3). There was an especially marked increase in the 105 kDa antigen relative to control membranes. β -Mercaptoethanol also converted all of the 205 kDa antigen to 105 kDa in iodoacetamide-treated membranes (Figure 6, lane 2). The reason for the large increase in the apparent amount of 105 kDa antigen is not yet certain. Since the 205 kDa antigen was still present and increased in quantity in iodoacetamide-treated membranes, it seems unlikely that all of the 205 kDa protein is the product of rearrangement of the 105 kDa antigen during isolation. The results, however, suggest that a large portion of the protein exists as a monomer in the cell in addition to the dimer. Possibly iodoacetamide protects the monomer against degradation, accounting for its increased levels despite increased levels of 205 kDa antigen. Future experiments will explore this possibility.

We also note that in addition to the Na^+/H^+ exchanger the affinity-purified antibodies also reacted with a limited number of other proteins. One protein of approx. 170 kDa (based on comparison with Bio-Rad prestained standards) reacted with antibodies against the C-terminal 178 amino acids, and another of 82 kDa reacted with antibody against the synthetic peptide. The identity of either protein is not yet known. Recently we have affinity-purified antibodies against the C-terminal 178 amino acids by another procedure, using antigen coupled to a Sepharose column. These antibodies do not cross-react with the 170 kDa protein (results not shown). We note also that the 82 kDa antigen which cross-reacts with the synthetic peptide antibody is the same size as an intensely staining band in Coomassie Blue-stained SDS/polyacrylamide gels of the placental membranes (Figure 4). This suggests that the cross-reactivity may be rather weak, and may not be based on strong identity between the proteins. Alternatively, the cross-reactivity could reflect the presence of a different minor co-migrating protein. Future experiments may resolve the identity of the cross-reacting protein.

Overall, our study has identified the presence and confirmed the identity of the amiloride-sensitive Na^+/H^+ exchanger protein in the placental syncytiotrophoblast brush-border membrane. We show that two different types of antibodies against the Na^+/H^+ exchanger protein identify a protein of 105 kDa in these vesicles. In membranes prepared under non-reducing conditions the protein appears as a dimer, and it appears as though a significant portion of the protein may exist in its native state as a dimer. We note also that the placenta and the brush-border membranes are a relatively easily available enriched source of the

exchanger, although the protein is still present only in small amounts. Future studies may involve solubilization, purification and characterization of the protein from this tissue.

This research was supported by research grants from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada to L.F. J.R.B.D. is supported by a studentship from the Heart and Stroke Foundation of Canada. L.F. is a Scholar of the Heart and Stroke Foundation of Canada and the Alberta Heritage Foundation for Medical Research. We thank Dr. C. Cass (Department of Biochemistry, University of Alberta) for her gifts of BeWo cells. We thank Dr. Charles Holmes of the Department of Biochemistry, University of Alberta, for his gift of phenylarsine oxide. We thank A. Barr for technical assistance. We are most grateful to Dr. V. Ganapathy for his gift of highly purified placental brush-border membranes with which we began this study.

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