Effect of cross-linkers on the structure and function of pig-renal sodium–glucose cotransporters after papain treatment

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Kidney brush-border membranes contain two sodium-dependent glucose transporters, one with low and one with high affinity for phlorizin, the specific inhibitor of these transporters. Using Scatchard analysis of phlorizin binding and Western blotting with specific antibodies against these transporters, we demonstrate in this study that although both transporters were proteolysed by papain treatment, only the high-affinity phlorizin-binding sites were decreased. Papain treatment followed by cross-linking with homobifunctional disuccinimidyl tartarate restored only the structure of the low-affinity phlorizin-binding protein (approx. molecular mass 70 kDa) without modifying the phlorizin-binding sites. When disuccinimidyl tartarate was replaced with dithiobis(succinimidyl acetate), another homobifunctional cross-linker with a higher spacer arm, the low- and high-affinity sites were both restored, with reappearance of two phlorizin-binding proteins with approx. molecular masses of 70 and 120 kDa. We conclude that high-affinity phlorizin-binding sites depend on the presence of the heterodimeric 120 kDa protein.

INTRODUCTION

All studies carried out to date on small-intestinal and kidney brush-border membranes (BBMs) are consistent with the existence of at least two structurally different sodium–glucose cotransporters. Based on kinetic measurements of D-glucose uptake and phlorizin binding to native BBM [1–3], this observation has been strengthened by identification of human genetic defects characterized by impaired, preferentially intestinal glucose transport [4]. Molecular cloning and expression studies in *Xenopus* oocytes have recently proved that a clone termed SGLT1, isolated from small intestine and kidney, represents the high-glucose/low-phlorizin-affinity transporter [5]. Using an identical approach, several investigators have assigned low-glucose/high-phlorizin-affinity transport to another independent SGLT2 [6] or SAAT-pSGLT2 clone [7]; others have attributed it to a modulation of the SGLT1 protein unit by a regulatory RS1 subunit [8]. Utilization of a different protocol, using polyclonal antibodies against the 70 kDa phlorizin-binding component of sodium–glucose transporter from pig-kidney BBM, allowed us to identify in the same BBM a heterodimeric 120 kDa structure containing the 70 kDa presumed to be SGLT1 and another related 60 kDa subunit [9].

Based on papain treatment of pig small-intestinal and renal BBM followed by cross-linking experiments, this immunological and kinetic study demonstrates a correlation between the proteolysis of the 120 kDa protein and the decrease in high-affinity phlorizin-binding sites. The change in affinity for phlorizin, and consequently probably for D-glucose, thus appears to require the association of two protein subunits.

EXPERIMENTAL

BBM vesicles from small intestine and renal cortex of pig were prepared by the magnesium-precipitation method of Kessler et al. [10] and Vannier et al. [11], respectively. BBM vesicles at 20 mg/ml were then frozen in liquid nitrogen until use.

Papain digestion

BBM vesicles were washed in 50 mM potassium phosphate, pH 6.2, then pelleted at 40000 ***g*** for 30 min at 4 °C. Pellets were suspended in the same buffer at a final protein concentration of 9 mg/ml and incubated with or without activated papain (1:50 [w/w] ratio of papain to membrane protein) for 15 min at 37 °C. Papain was activated in potassium phosphate buffer, pH 6.2, containing 0.03 mM dithiothreitol and 1 mM cysteine. After papain digestion, membranes were immediately used for cross-linking experiments or washed twice in 300 mM mannitol/10 mM Hepes-Tris, pH 7.4, and centrifuged at 40000 ***g*** for 30 min for phlorizin-binding measurements.

Dithiobis(succinimidyl acetate) (DTSA) synthesis

DTSA was prepared according to Bodansky [12]. Dithiodiglycolic acid in solution (10 mmol) was added to 10 mmol of dicyclohexyl carbodiimide dissolved in distilled dimethylformamide. After 10 min at 0 °C, 20 mmol of *N*-hydroxysuccinimide were added to the mixture and kept at 0 °C overnight. Dicyclohexylurea was removed by precipitation with ethyl acetate and filtration, and the excess *N*-hydroxysuccinimide was removed by washing with a mixture of citric acid/$NaHCO_3$, 10% (v/v). DTSA was twice recrystallized from isopropanol and its purity was checked by TLC on silica gel (using dichloromethane and ethyl acetate) and NMR.

Cross-linking experiments

Intestinal and renal BBMs in sodium phosphate buffer, pH 6.2, (1 mg of membrane protein) adjusted to pH 7.5 by addition of alkaline potassium phosphate buffer, pH 8.5, were incubated for 30 min at room temperature in the presence of disuccinimidyl tartarate (DST) or DTSA dissolved at 5 mM final concentration in dry DMSO. Membranes were then washed twice in 300 mM

Abbreviations used: BBM, brush-border membrane; DST, disuccinimidyl tartarate; DTSA, dithiobis(succinimidyl acetate).

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mannitol/10 mM Hepes-Tris buffer, pH 7.4, and centrifuged at 40000 ***g*** for 30 min for phlorizin-binding measurements.

Phlorizin-binding measurements

Phlorizin binding was carried out by adding [^{3}H]phlorizin at concentrations ranging from 0.2 to 8 μM to membrane vesicles (100–200 μg of protein) in 100 mM NaSCN, 10 mM Hepes-Tris, pH 7.4, in the absence (total binding) or presence (non-specific binding) of 1 mM unlabelled phlorizin. Renal BBM was incubated for 15 min at 25 °C in a total volume of 90 μl. Each sample was filtered on a 0.65 μm Sartorius filter, rapidly washed and counted for radioactivity. Specific sodium-dependent phlorizin binding was calculated by subtracting binding in the presence of 1 mM phlorizin from binding in the absence of unlabelled phlorizin.

Western immunoblots

Protein samples (30 μg) solubilized in 4 % SDS (v/v) and boiled for 3 min were separated on a 4–15 % acrylamide (w/v) linear gradient using the Mini-Protean II Cell (Bio-Rad). Western blots were obtained with the IgG fraction derived from antisera raised in rabbit against the 70 kDa antigen or a synthetic octadecapeptide (amino acid residues 402–419) made from the major extracytoplasmic loop of SGLT1 using a Mini Trans Blot apparatus (Bio-Rad). Electrophoresis and Western-blot buffers and procedures have been described previously [9,13].

RESULTS AND DISCUSSION

The treatment of membranes by the proteinases papain and trypsin has been used extensively to study the effects of these proteinases on transporters and to achieve negative purification of the carriers, provided the proteinases do not affect the function of the transporters. This is the case for the sodium–glucose cotransporter from small-intestine BBM, whose phlorizin-binding capacity is not modified [14]. In contrast, conflicting results have been reported concerning phlorizin binding on kidney BBM after proteinase treatment; this is probably due to measurement of the binding to proteinase-treated BBM at a single phlorizin concentration [15,16]. As both high- and low-affinity binding sites have been detected in rabbit kidney [17], we measured the binding of phlorizin to renal BBM treated or not treated by papain at several phlorizin concentrations.

Pig-kidney BBM were incubated in the presence of activated papain (see Experimental section) for 15 min at 37 °C, washed twice and assayed for phlorizin binding at concentrations ranging from 0.2 to 8 μM in the presence of Na^+. Results clearly revealed the presence of two sets of binding sites in native kidney BBM, with the set of high-affinity binding sites decreasing drastically in papain-treated BBM (Figure 1A). The same experiment with pig small-intestinal BBM was impaired by the presence of phlorizin hydrolase; after 5 s of incubation, phlorizin binding was not modified by papain treatment of small-intestinal BBM vesicles (not shown), as previously reported [14].

Western blotting of kidney BBM allowed identification of two proteins of 120 and 70 kDa, which reacted immunologically with polyclonal antibodies against the 70 kDa glucose-inhibited phlorizin-binding fraction (Figure 2B, lane 1). Only the 70 kDa protein was detected in small intestinal BBM from the same animal species (Figure 2A, lane 1). Identical results were obtained using sequence-specific (Ser^{402}–Lys^{420}) antibodies directed against a synthetic peptide corresponding to the extracytoplasmic loop of SGLT1 (Figure 3, lane 1). Similar experiments were carried out using intestinal and renal BBMs treated by papain, as indicated above. As shown in Figures 2A, 2B and 3 (lane 2), the 120 and 70 kDa immunoreactive proteins were both proteolysed by papain. Two possible explanations exist for preservation of the low-affinity phlorizin-binding sites after papain digestion: either proteolytic cleavage occurs away from the molecular sites of the phlorizin binding (although phlorizin binding probably involves

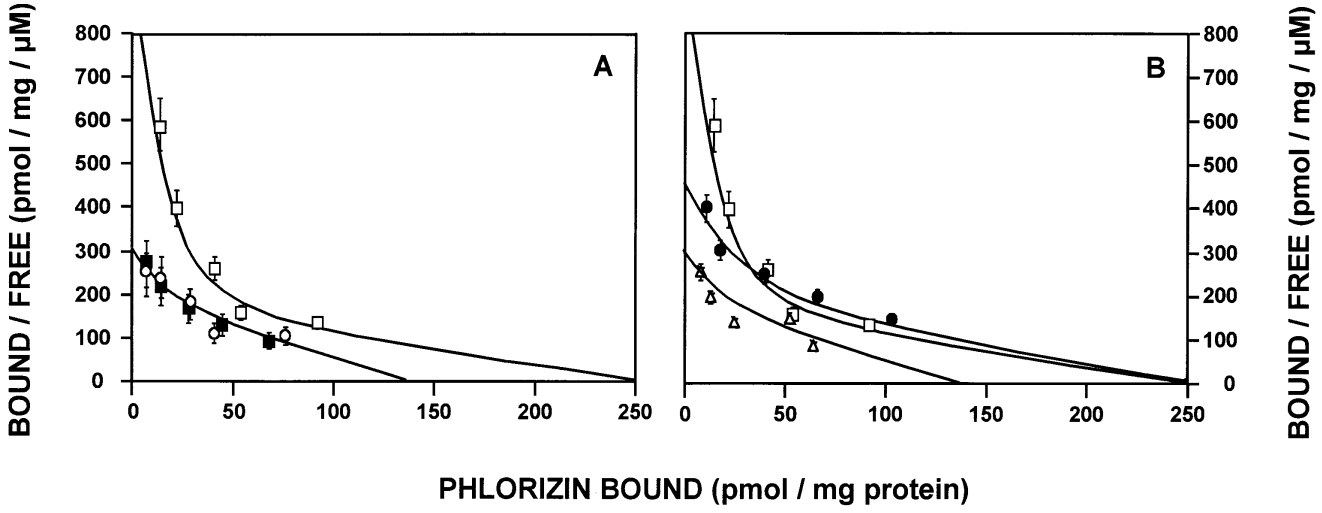

Figure 1 Scatchard analysis of sodium-dependent phlorizin binding to pig-kidney BBM vesicles treated by papain and cross-linkers

Vesicles (7–8 mg/ml) were incubated for 15 min at 37 °C in the absence (□) or presence (○) of activated papain in 50 mM potassium phosphate buffer, pH 6.2, as described in Experimental. Papain-treated vesicles were then incubated with (A) 5 mM DST (■) or (B) 5 mM DTSA (●) in 50 mM potassium phosphate buffer, pH 7.0, for 30 min at 25 °C, and washed twice by centrifugation at 105000 ***g*** for 30 min at 4 °C in 300 mM mannitol/10 mM Hepes-Tris buffer, pH 7.4. Vesicles treated successively with papain and DTSA were incubated in the presence of 50 mM dithiothreitol in 50 mM sodium acetate buffer, pH 4.5, for 30 min at 25 °C, then washed in mannitol-Hepes buffer (△). Aliquots (100–200 μg) of vesicles were then incubated in 100 mM NaSCN/10 mM Hepes-Tris buffer, pH 7.4, with different phlorizin concentrations for 15 min at 25 °C. The measurements were corrected for non-specific binding (see Experimental). Scatchard plots represent mean values of two experiments in triplicate samples with standard deviations. The curves were fitted assuming two inhibition sites using a curve-fitter program.

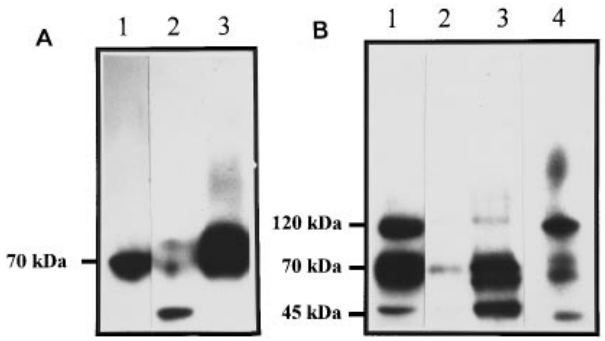

Figure 2 Immunoblot analysis of proteins from BBM vesicles treated by papain and cross-linkers

Pig small intestinal (A) and kidney (B) BBM vesicles were incubated in the absence (lane 1) or presence of papain (lane 2) followed by cross-linking with DST (lane 3) or DTSA (lane 4), as described in Figure 1. Aliquots (30 μg) were applied on a 4–15% SDS-polyacrylamide linear gradient mini gel after addition of 4% SDS and boiling for 3 min. The slab gel was run at 200 V and 4 °C for 1 h. Immunoblotting was performed with antibodies directed against the 70 kDa protein (see Experimental). The protein markers used were thyroglobulin subunit (330 kDa), ferritin (220 kDa), phosphorylase b (94 kDa), BSA (67 kDa), catalase subunit (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa).

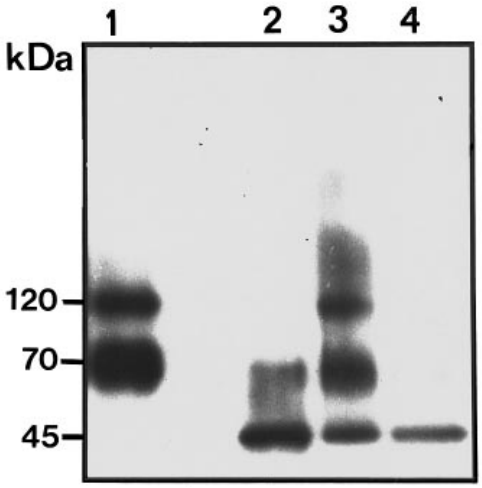

Figure 3 Immunoblot analysis of proteins from kidney BBM using SGLT1 sequence-specific antibodies

Pig-kidney BBM vesicles (lane 1) treated by papain (lane 2) were cross-linked with DTSA (lane 3), then incubated in the presence of 50 mM dithiothreitol (lane 4). All conditions of incubations were identical to that described in Figure 1, except that papain was inactivated by 0.04 M NaOH after 15 min of incubation for BBM treated only by papain (lane 2). Electrophoresis and immunoblotting were performed as reported in Figure 2 using sequence-specific antibodies (Ser^{402}–Lys^{420}) directed against a synthetic peptide corresponding to the extracytoplasmic loop of SGLT1.

several parts of the transporter [18]), or the three-dimensional structure of the 70 kDa protein, buried in the membrane, is conserved despite proteolysis. In this last case protein fragmentation would occur during detergent denaturation before electrophoresis.

To test this hypothesis, cross-linking experiments were conducted on small-intestinal and kidney BBMs treated or not treated by papain. DST was selected as the bifunctional cross-linker because it has the shortest spacer arm. Intestinal and renal BBMs treated by papain were incubated in the presence of DST for 30 min at room temperature. Western blots (Figures 2A and 2B, lane 3) revealed that DST cross-linked the proteolysed fragments of the 70 kDa protein from intestinal and renal BBM. No reconstitution of the initial structure was seen for the 120 kDa protein identified in kidney BBM. In addition, similar values were found for phlorizin binding by kidney BBM treated by papain, followed or not by cross-linking with DST (Figure 1A). The DST cross-linker thus failed to restore the high-affinity phlorizin binding. The presence of the 70 kDa protein following DST cross-linking cannot be due to the papain inactivation by the cross-linker, since papain digestion was almost achieved after 15 min of incubation at 37 °C, as proved by 80 and 50% solubilization, respectively, of aminopeptidase and alkaline phosphatase from renal BBM. Moreover, only 30–40% of sodium–glucose transport activity was found in residual renal BBM, which corresponded with the high-affinity glucose transport.

We previously reported that the 120 kDa protein is a heterodimeric structure containing the 70 kDa, low-affinity glucose-inhibited phlorizin-binding protein associated with a 60 kDa protein [9]. In our experiments papain treatment also led to cleavage of the 60 kDa protein in smaller fragments linked by DST (see Figure 2B, lane 3), but the dimeric structure was not reconstituted. The decrease in high-affinity phlorizin binding in kidney BBM after papain treatment thus appears correlated with the disappearance of the 120 kDa protein.

The 6 Å spacer arm of DST may be too short in this case to link the two subunits of the 120 kDa protein; this is why we next synthesized DTSA, a thiol-cleavable homobifunctional cross-linker with a 9 Å spacer arm.

When kidney BBM, treated by papain and incubated in the presence of DTSA for 30 min at room temperature, were assayed for phlorizin binding, the high-affinity phlorizin-binding sites were partially restored (Figure 1B), and the 120 kDa protein fraction immediately reappeared in the Western blot (Figure 2B, lane 4 and Figure 3, lane 3). The same experiment carried out on small-intestinal BBM displayed only the 70 kDa protein (not shown).

When kidney BBM treated successively by papain and DTSA was incubated for 30 min at room temperature in the presence of the reducing agent, dithiothreitol, at 50 mM final concentration, then washed twice in mannitol/Hepes buffer, pH 7.4, the Scatchard curve of phlorizin binding became similar to that for BBM treated only with papain (Figure 1B). In this case, the 70 and 120 kDa proteins were no longer seen in the Western blots (Figure 3, lane 4).

These studies are consistent with the previous observation that a phlorizin-binding component of 110 kDa with a high affinity for phlorizin (K_d approx. 0.5 μM) is yielded in radiation-inactivation experiments on renal BBM [19].

Our data clearly demonstrate that there are two renal sodium–phlorizin-binding entities that account for the different affinities for phlorizin binding. While the monomeric 70 kDa SGLT1 is indisputably the high-glucose-affinity transporter with low phlorizin affinity, the structure of the low-glucose-affinity transporter with high phlorizin affinity remains controversial. The recent isolation from kidney cDNA libraries of clones encoding a low-glucose-affinity protein closely related with SGLT1, named SGLT2 [6,7], supports the hypothesis of coexpression of distinct SGLT1 and SGLT2 proteins with 76% identity at the amino acid level [20]. However, it is apparently inconsistent with the presence of the heterodimeric structure seen in our experiments. Nevertheless, it cannot be excluded that SGLT2 synthesized from cRNA injected into oocytes may join with endogenous SGLT1 present at low levels in oocytes [21]. On the other hand,

structural identity exists between the two monomeric 70 and 60 kDa proteins which make up the heterodimeric 120 kDa [9]. If this hypothesis is true, it would explain the presence of two substrate sites on a single functional transporter [22].

In conclusion, the high-affinity glucose transporter appears to be the immunoreactive 70 kDa protein found in small-intestinal as well as kidney BBM, whereas the oligomeric 120 kDa protein is apparently the low-glucose-affinity transporter specific to renal BBM.

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