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Purification of a putative Na^+/D-glucose cotransporter from pig kidney brush border membranes on a phlorizin affinity column

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Phlorizin, a potent inhibitor of the Na^+/D-glucose cotransporter, was derivatised to 3-aminophlorizin and subsequently coupled to Affi-Gel 15. Affinity chromatography of pig kidney brush border membranes solubilised in Triton X-100 allowed the purification of a 60 kDa protein on this resin. We consider this protein to be the Na^+/D-glucose cotransporter, or part of it, for the following reasons: (i) binding of this protein to Affi-Gel 15 specifically requires phlorizin covalently attached to the resin and is lowered when phlorizin is replaced by phloretin; (ii) binding of the 60 kDa protein to a phlorizin affinity column requires the presence of Na^+; (iii) polyclonal as well as monoclonal antibodies against the 60 kDa protein inhibit binding of phlorizin to brush border membranes from rabbit and pig kidney.

Affinity chromatography; Phlorizin; Na^+/D-glucose cotransporter; (Brush border, Pig kidney)

1. INTRODUCTION

Phlorizin, but not its aglycone phloretin, is a powerful reversible inhibitor of the Na^+/D-glucose cotransport system located in the brush border membranes of renal proximal tubule and small intestine ($K_i \sim 1\ \mu M$). Binding of phlorizin to the cotransporter has been shown to require sodium. The cotransporter represents less than 0.5% of the brush border membrane proteins. Identification of the cotransporter protein using radioactive phlorizin or glucose derivatives, which covalently bind to proteins, gave ambiguous results as to the molecular mass of the protein [1–4]. Because of the high affinity of phlorizin to the Na^+/D-glucose cotransporter and the low abundance of this protein, affinity chromatography using phlorizin covalently attached to agarose appeared to be a suitable tool to purify the cotransporter [4]. In this paper we describe the purification of a 60 kDa protein from pig kidney outer cortex brush border membranes which specifically binds to a phlorizin affinity column in the presence of sodium.

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Abbreviations: BBM, brush border membrane; DMS, dimethyl suberimidate; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; LLC-PK_1, pig kidney cells from American Type Culture Collection strain ATCC no. CL 101; M_r, relative molecular mass; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

BBMs from the pig kidney outer cortex were prepared as described [4]. The final membrane preparation contained 200 mM D-mannitol, 20 mM Hepes/Tris, pH 7.4, and 25 mg/ml protein, as assayed by the method of Lowry et al. [5]. It was enriched up to 10-fold in enzyme activities specific for BBMs such as leucine aminopeptidase and alkaline phosphatase [6], and about 0.1-fold in Na^+/K^+-ATPase activity present in the basal-lateral membrane [7]. The BBMs were subsequently solubilised in buffer containing 2% Triton X-100, 20 mM Hepes/Tris, pH 7.4, 0.2 mM DTT and 0.5 mM EDTA by gently stirring for 30 min at 4°C at a detergent/protein ratio of 4:1 [4]. After centrifugation for 1 h at 100000 × *g*, the supernatant was purified by affinity chromatography.

3-Aminophlorizin was synthesized from phlorizin (Fluka), as described [8]. 3-Aminophloretin was obtained by hydrolysing the intermediate 3-nitrophlorizin to 3-nitrophloretin according to a procedure described for the hydrolysis of phlorizin to its aglycon phloretin [9] and subsequent reduction of the nitro

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group. Phlorizin and phloretin affinity chromatography columns were prepared by coupling the respective amino derivatives (50 mM in isopropanol) to activated Affi-Gel 15 (BioRad). Both resins were tested for their ability to bind fluorescein labeled Con A (Vector). Binding of Con A was detected only on the phlorizin resin.

Prior to protein purification, the affinity column (2 ml bed volume) was equilibrated with 20 ml of the solubilisation buffer containing 0.1% instead of 2% Triton X-100. After loading the solubilisate onto the column, it was washed with 20 ml of the equilibration buffer to remove unbound protein. Bound proteins were eluted with 10 ml of the equilibration buffer containing in addition 5 mM phlorizin. Above buffers usually contained up to 100 mM NaCl which in some experiments was replaced by KCl or NH_4Cl. In one experiment 100 mM NaCl and 1 M D-glucose were present.

For further purification, HPLC on a C-1 reverse phase column (LKB, TSK TMS-250, 4.6 × 75 mm) was carried out. Eluent A was 0.1% TFA and eluent B was 0.05% TFA in 85% acetonitrile. A gradient from 30% B to 40% B was run for 30 min with a flow rate of 0.5 ml/min. UV absorbance was monitored at 230 nm.

In either case fractions of 1 ml were collected and aliquots run on an SDS-10% polyacrylamide slab gel prepared according to Laemmli [10]. Protein bands were visualized by staining with Coomassie brilliant blue R-250. Molecular mass standards were obtained from BioRad.

Rabbit polyclonal antibodies were raised against the 60 kDa protein purified by HPLC following standard immunization procedures [11]. Monoclonal antibodies against the 60 kDa protein were prepared after immunization of mice with total rabbit brush border membrane as described [12]. Monoclonal antibodies were dialyzed extensively against 10 mM Hepes/Tris, pH 7.2, to remove D-glucose, K^+, and Na^+ and polyclonal antibodies were purified on a protein A-Sepharose column (Pharmacia), as described [13].

For phlorizin binding studies 100 μg BBMs were first incubated with 80 μg antibody for 20 min and subsequently incubated with 0.2 μM [^{3}H]phlorizin (0.5 μCi) for 5 min at 21°C in a 100 μl total volume, containing 125 mM sucrose, 5 mM $MgCl_2$, 10 mM K^+/Hepes, pH 7.2, and either 100 mM NaCl or 100 mM KCl. The reaction was terminated with ice-cold non-radioactive buffer and the BBMs were subsequently washed by the rapid filtration method [14]. Radioactivity was measured in a liquid scintillation counter.

Protein samples of 50 μg, obtained after eluting the affinity column with phlorizin, were cross-linked with DMS (5 or 10 mg/ml) [15]. After 30 min reaction at 37°C, aliquots of 10 μg protein were separated by SDS-PAGE. Alternatively, BBMs were treated with DMS (10 or 25 mg/ml) for 30 min at 37°C. Subsequently, the protein was precipitated with $MeOH/CHCl_3$ to remove lipids [16]. After SDS-PAGE a Western blot analysis was carried out using polyclonal antibodies against the 60 kDa protein and protein A-HRP conjugate (BioRad) [17].

LLC-PK_1 cells [18] were grown in tissue culture using serum complemented minimal essential medium (Eagle's MEM). Apical membranes of these cells were prepared by $MgCl_2$-precipitation [19]. Membranes from rabbit renal medulla were prepared as described [20].

3. RESULTS

Solubilised renal brush border membrane proteins (250 mg) were applied to a phlorizin column in the presence of NaCl. Several proteins (~250 μg total) were bound to the column and eluted with phlorizin. The most abundant of these proteins has an apparent mass of 60 kDa on SDS-polyacrylamide gels. The minor proteins are of 40 and 55 kDa (fig.1B). This protein mixture was further separated by HPLC after loading 1 ml aliquots onto a C-1 reverse phase column. We obtained an almost homogeneous 60 kDa protein which was eluted at 30% acetonitrile (fig.2).

Binding of the three proteins to a phlorizin column was not markedly inhibited by 1 M D-glucose. In the absence of Na^+, significantly less of the 60 kDa protein was bound to the phlorizin column and eluted with phlorizin, compared to an experiment where 100 mM NaCl was present (fig.3). The amount of the 40 and 55 kDa proteins remained unchanged. When 100 mM Na^+ was replaced by K^+ or NH_4^+, a similar amount of the 60 kDa protein was bound to the phlorizin column. However, in the presence of 50 mM Na^+ significantly more of the 60 kDa protein was bound compared to an experiment where Na^+ was replaced by K^+. With 25 mM Na^+ or K^+ the

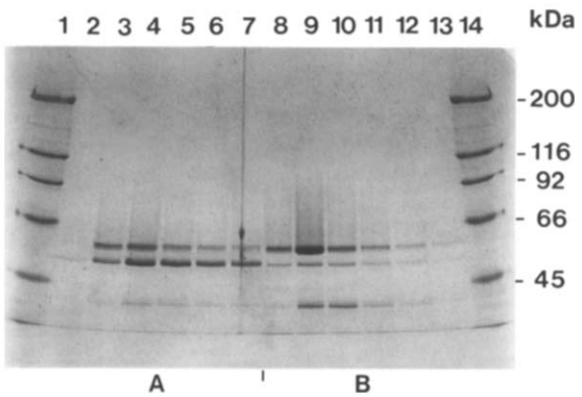

Fig.1. Affinity chromatography of solubilised pig kidney BBM proteins on a phloretin (A) and phlorizin column (B). Affinity chromatography was carried out as described in section 2. Aliquots of different fractions were separated by SDS-PAGE. Lanes 1 and 14, M_r markers; lanes 2–7, 100 μl of fractions 1–6 eluted with phlorizin from the phloretin affinity column; lanes 8–13, 100 μl of fractions 2–7 eluted with phlorizin from the phlorizin affinity column. Column load was 250 mg protein in each case.

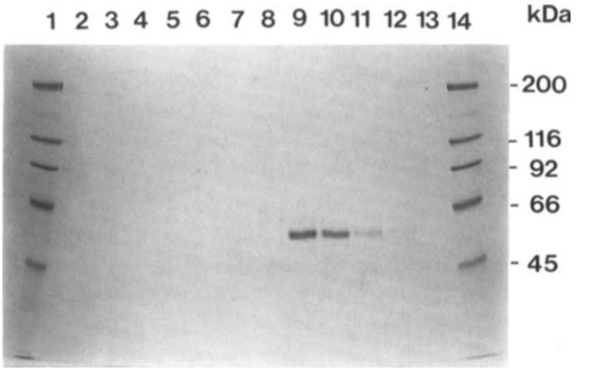

Fig.2. Reverse phase HPLC of proteins eluted with phlorizin from the phlorizin affinity column. 100 μl aliquots of fractions eluted between 25 and 35% acetonitrile were separated by an SDS-PAGE. Lane 9 corresponds to a fraction obtained by elution at 30% acetonitrile. For details see text.

amount of the 60 kDa protein bound was as low as in the absence of Na^+. Binding of the three proteins of 40, 55, and 60 kDa specifically required phlorizin coupled to the agarose matrix. When aminopiretanide (Hoechst, Frankfurt/M.), a reversible inhibitor of the Na^+,K^+,Cl^--cotransporter and not of the Na^+/D-glucose cotransporter, was coupled to the agarose column, none of the above proteins was bound to the column as judged by elution with phlorizin and subsequent SDS-PAGE. Carrying out an identical

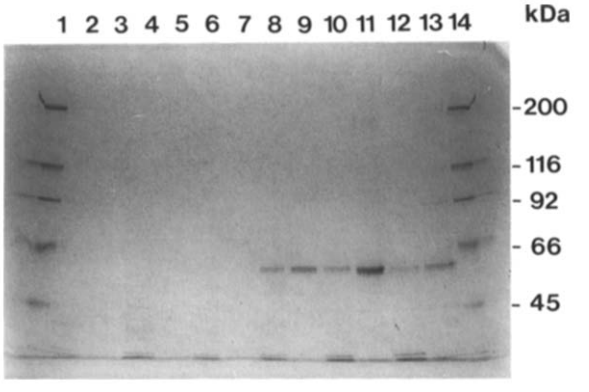

Fig.3. Effect of NaCl on protein binding to the phlorizin affinity column as judged by SDS-PAGE of 100 μl aliquots of the fractions eluted with phlorizin. Lanes 1 and 14, M_r markers; lanes 2–5, column wash; lanes 6–13, elution with phlorizin. Even-numbered lanes: 125 mg of protein was applied onto the phlorizin column in the absence of NaCl. Odd-numbered lanes: identical experiment, but protein was applied in the presence of 100 mM NaCl.

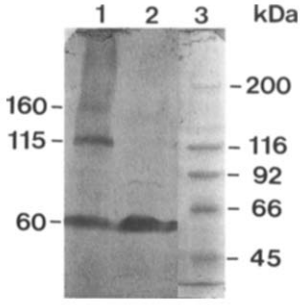

Fig.4. Cross-linking reaction and Western blot analysis of membrane proteins from pig kidney BBM using rabbit anti-60 kDa protein serum. Lane 1, BBM treated with DMS; lane 2, untreated BBM; lane 3, molecular mass standards stained with Ponceau S.

experiment using a phloretin column, three proteins of 40, 55, and 60 kDa were bound and could be eluted with phlorizin (fig.1A). However, the amount of the 60 kDa protein eluted with phlorizin was lower, whereas the amount of the 55 kDa protein was significantly higher.

Protein cross-linking experiments with DMS and the proteins eluted with phlorizin suggest that the 60 kDa protein, solubilised in Triton X-100, can exist as a dimer or trimer, as after cross-linking protein bands at 115 and 160 kDa could be observed in SDS-PAGE. In identical experiments using BBMs three protein bands corresponding to 60, 115, and 160 kDa reacted with polyclonal antibodies against the 60 kDa protein in a Western blot analysis (fig.4). The 115 and 160 kDa protein

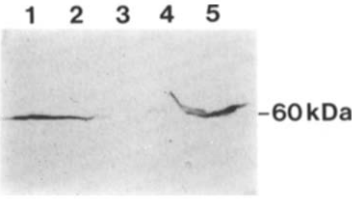

Fig.5. Western blot analysis of membrane proteins from LLC-PK_1, rabbit medulla and rabbit kidney cortex using rabbit anti-60 kDa protein serum. The reaction of antibodies with protein transferred to a nitrocellulose sheet was visualized by an HRP-protein A conjugate color reaction. Lane 1, 3 μg pig kidney 60 kDa protein plus 170 μg LLC-PK_1 membrane proteins; lane 2, 3 μg pig kidney 60 kDa protein; lane 3, 550 μg rabbit medulla membrane proteins; lane 4, 170 μg LLC-PK_1 apical membrane proteins; lane 5, 300 μg rabbit kidney cortical BBM proteins. Due to the high content of lipids in the samples of lanes 3–5, protein bands were distorted.

bands were absent in BBMs which were not treated with DMS.

The 60 kDa protein purified by HPLC was used as antigen to obtain polyclonal antibodies from rabbits. In Western blot analysis, the polyclonal antibodies reacted not only with the purified 60 kDa protein but also with a 60 kDa protein in apical membranes of LLC-PK_1 cells which exhibit Na^+/D-glucose cotransport activity and in rabbit renal BBM, whereas there was no reaction with membrane proteins derived from rabbit medulla which are devoid of Na^+/D-glucose cotransporter (fig.5).

The polyclonal antibodies inhibit by 44% phlorizin binding to pig kidney BBM, whereas there was only 35% inhibition with polyclonal antibodies and rabbit kidney BBM. A monoclonal antibody (11C2), raised against total rabbit kidney BBM proteins, also reacted with the 60 kDa protein purified from pig kidney BBM in Western blot analysis. This monoclonal antibody inhibits phlorizin binding to pig and rabbit kidney BBM by 60% and 75%, respectively.

4. DISCUSSION

We have purified a 60 kDa protein which we assume to be the Na^+/D-glucose cotransporter, as it specifically binds to phlorizin agarose. This binding was found to be dependent on 50 mM Na^+, but other monovalent cations could replace Na^+ at higher concentrations (100 mM K^+ or NH_4^+). Binding of phlorizin to BBM was previously found to be dependent on Na^+ [2], but Na^+ could be replaced by K^+ at higher concentrations (200 mM) (Koepsell, H., personal communication). The preferential specificity of the Na^+/D-glucose cotransporter for Na^+ may be lowered after solubilisation in Triton X-100.

Phlorizin presumably interacts with the binding site for D-glucose of the Na^+/D-glucose cotransporter [2]. However, D-glucose failed to inhibit binding of the 60 kDa protein to phlorizin agarose. This may be due to the lower affinity of D-glucose ($K_m \sim 1$ mM) to the Na^+/D-glucose cotransporter compared to phlorizin ($K_i \sim 1\ \mu M$) [3].

Phloretin, the aglycon of phlorizin, also inhibits the Na^+/D-glucose cotransporter, although at higher concentrations [21]. Similarly, the amount of the 60 kDa protein bound to phloretin agarose is smaller than the amount of the 60 kDa protein bound to phlorizin agarose under identical experimental conditions. In contrast, a higher amount of a 55 kDa protein binds to the phloretin column than to the phlorizin column. This 55 kDa protein may be the Na^+-independent D-glucose carrier which has a higher affinity to phloretin and a similar molecular mass [21].

The assumption that the 60 kDa protein is the Na^+/D-glucose cotransporter is further strengthened by the fact that antibodies directed against the 60 kDa protein inhibit Na^+-dependent phlorizin binding to pig and rabbit kidney BBM. Inhibition of phlorizin binding to rabbit small intestinal and pig kidney BBM by antibodies has previously been reported. These antibodies recognized a 72 and a 75 kDa protein, respectively [1,22].

The 60 kDa protein presumably exists as a dimer or trimer in the solubilised and membrane-bound state. It is interesting to note that by radiation inactivation experiments 110, 230 and 345 kDa have been obtained for the native Na^+/D-glucose cotransporter [23,24], which roughly represent multiples of 60.

Although the molecular mass of the rabbit small intestinal Na^+/D-glucose cotransporter has recently been determined by DNA sequencing to be 73 kDa [25], this does not rule out the possibility that the described 60 kDa protein, isolated from pig kidney outer cortex, is the Na^+/D-glucose cotransporter. Membrane proteins often have been found to migrate according to a much lower M_r in SDS polyacrylamide gels, compared to the M_r determined for them by DNA sequencing or amino acid sequencing [26–29].

Furthermore, in vitro translation of size-selected mRNA encoding the rabbit intestinal Na^+/D-glucose cotransporter revealed mainly two proteins of 57 and 63 kDa [30]. In addition, the kinetic properties of the renal and small intestinal cotransporters are different [2]. Whether this difference is due to organ-specific gene rearrangement, mRNA splicing, protein processing, or lipid microenvironment remains to be determined.

Current investigations are aimed at determining the amino acid sequence of the 60 kDa protein. Preliminary experiments suggest that the amino terminus of the 60 kDa protein is blocked.

Therefore, peptides have been isolated and are sequenced. Thus, it will be possible to compare sequence data of the 60 kDa protein with the sequence data predicted for the Na^+/D-glucose cotransporter from rabbit small intestine.

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