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Immunological recognition of sodium/D-glucose cotransporter from renal brush border membranes by polyclonal antibodies

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Antisera prepared in rabbit to a D-glucose-inhibitable phlorizin binding component of the pig kidney brush border membrane precipitated more than 90 percent of the D-glucose-inhibitable phlorizin binding activity from a Triton extract. These antibodies also stimulated D-glucose uptake by native brush border membranes at low D-glucose concentrations (1 mM) and inhibited it at higher D-glucose concentrations. Immunoblotting was used to locate polypeptide subunits of the glucose transporter in polyacrylamide gels of proteins extracted from the brush border membranes. The antibodies labelled the M_r 70000 phlorizin-binding component in both reducing and non reducing conditions. Two additional polypeptides with relative molecular mass of 120000 and 45000 were also recognized under the same conditions; they might correspond, respectively, to another Na^+/D-glucose cotransport unit and to a post mortem degradation product.

Introduction

Considerable experimental evidence indicates that phlorizin competes with glucose for the binding site on the brush border membrane transporter [1,2]. Moreover transport and binding data suggest that transporter populations are different in kidney and intestine in regard to D-glucose and phlorizin affinity and capacity and sodium-glucose stoichiometry [3].

Whereas the intestinal sodium/D-glucose cotransporter was early identified as a M_r 72000 polypeptide [4,5] and more recently cloned by injection of poly A-RNA from intestinal mucosa in *Xenopus laevis* [6], the molecular weight determination of renal glucose transporter have led to ambiguous results, when using monoclonal antibodies generated by immunization of mice with kidney brush border membranes enriched in sodium-dependent phlorizin-binding or apical membranes from LLC-PK_1 renal epithelial cell line [7,8]. The discrepancies noted in these results could be due to the presence of more than one Na^+/D-glucose cotransport unit in renal tissue as previously reported [9,10] and very recently confirmed [11]. Here we report a different approach to obtain antibodies by immunization of rabbits with a M_r 70000 polypeptide, previously identified as the phlorizin-binding moiety of sodium/glucose cotransporter from pig kidney brush borders [12].

Abreviations: PMSF, phenylmethanesulfonyl fluoride; MSH, 2-mercaptoethanol; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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Materials and Methods

General procedures

Brush border membranes isolated from pig kidney cortex and suspended in 100 mM NaCl, 1 mM Tris-Hepes (pH 7.4) buffer (NaCl medium) were extracted by Triton X-100 at 1% final concentration (Triton extract). A transporter-enriched brush border sample was obtained from this extract by removal of Triton and solubilization of the insoluble material in NaCl medium supplemented with 1% Triton X-100 and 0.1% SDS [13] then the transporter enriched sample was applied on a 5–15% acrylamide linear gradient in the presence of 0.25% Triton X-100, 0.1% SDS and M_r 70000 protein fraction electroextracted as previously described [12].

Preparation of antisera

Antibodies against the M_r 70000 polypeptide fraction were obtained by injecting subcutaneously a rabbit

with antigen (100–200 μg) emulsified with an equal volume of Freund's complete adjuvant. Additional injections of the antigen in Freund's incomplete adjuvant were given 3 weeks and one month later. The rabbit was bled 8 days after the last injection and the resultant antiserum was stored at −30°C. Preimmune serum was obtained from the same rabbit prior to the first injection.

Dot-immunobinding procedure

Nitrocellulose membrane (NC) was placed in a 96-well dot-blot apparatus (Hybridot Manifold, BRL). Increasing dilutions of pure antigen or detergent extracts in PBS (100 μl) were added to each well. Free binding sites on the membrane were blocked with PBS containing 5% fat-free milk powder (45 min at 37°C) with constant shaking. Antiserum or preimmune serum (100 μl appropriately diluted in PBS) were then applied to the NC and incubated for 1 h at 37°C or overnight at +4°C with constant shaking. The membrane was washed free of unreacted sera with PBS containing 0.05% Tween 20 and incubated for 1 h at 37°C with ^{125}I-labeled protein A (250000 cpm/ml) in PBS containing 5% fat free milk powder. The NC was washed with PBS-Tween, then exposed to Kodak Trimax XD film overnight at −80°C for autoradiography.

Western immunoblotting procedure

Protein samples were separated on a 5–15% acrylamide linear gradient as previously described [12] in the presence of 0.25% Triton X-100, 0.1% SDS or 0.1% SDS only. The proteins thus separated were electroblotted onto a NC filter by the method of Towbin et al. [14] using a horizontal transfer electrophoresis unit (Biolyon, France). Subsequent steps were the same as those described for the dot-immunobinding method. Sera were diluted 1/100.

Other methods

Reconstitution of detergent extracted proteins into liposomes was carried out as previously described [13] using one part of protein and five parts of dried egg yolk L-α-phosphatidylcholine. Specific D-glucose-inhibitable phlorizin binding was calculated by substracting the binding of 3 μM radioactive phlorizin in the presence of 20 mM D-glucose from the binding in the presence of 20 mM mannitol [12].

Chemicals

D-[U^{14}C]Glucose (258 m Ci/mmol) and [^{3}H]phlorizin (60.7 Ci/mmol) were purchased from New England Nuclear. L-α-Phosphatidylcholine from egg yolk, Tween 20, PMSF and protein A-Sepharose were obtained from Sigma (St. Louis, MO), Triton X-100 and sodium dodecyl sulfate from Merck (Darmstadt, F.R.G.). Nitrocellulose membranes were purchased from Millipore. Protein A was iodinated by V. Bayer from Biophysical Laboratory of Medicine Faculty (Nice). All other chemicals were of the highest grade.

Results

The immunoreactivity of rabbit polyclonal antiserum obtained by immunization with the brush border membrane M_r 70000 polypeptide was tested by the dot-blot method. An immunological response was obtained up to 1/250 dilution of antiserum using one microgram of the antigen (Fig. 1A). By contrast, bovine serum albumin did not react with the antiserum and the M_r 70000 antigen did not react with the preimmune rabbit serum (not shown). Triton X-100 extract and a glucose transporter-enriched brush border sample also showed immunoreactivity (Fig. 1B and C). Other plasma membranes purified from human erythrocytes or rat hepatocytes cells, which contain facilitated diffusion systems for D-glucose transport did not react in the dot-blot with the rabbit antiserum against the M_r 70000 polypeptide.

In a previous study, this electroextracted M_r 70000 polypeptide fraction reconstituted into liposomes was designed as a functional molecular protein entity of the Na^+/D-glucose cotransport [12] and shown to be homogeneous in two-dimensional electrophoresis (unpublished results). Experiments were carried out to test the influence of rabbit polyclonal antisera on sodium dependent D-glucose uptake by purified brush border vesicles. Below 1 mM D-glucose concentration a slight stimulation close to 15% of D-glucose transport was observed when compared to a brush border membrane control treated with rabbit preimmune serum (Fig. 2,

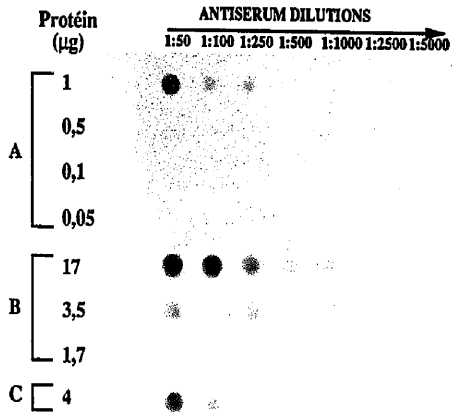

Fig. 1. Dot-blot immunobinding of the M_r 70000 protein fraction (A), 1% Triton extract (B) and transporter enriched brush border sample (C) with the antiserum against the M_r 70000 protein fraction. Immunobinding procedure was detailed in Materials and Methods.

inset). It can be noted that phlorizin failed to inhibit the uptake of 1 mM D-glucose by brush border membranes only when these membranes were treated with the rabbit antiserum against the M_r 70000 polypeptide; this result is similar to that found by others [7]. In contrast

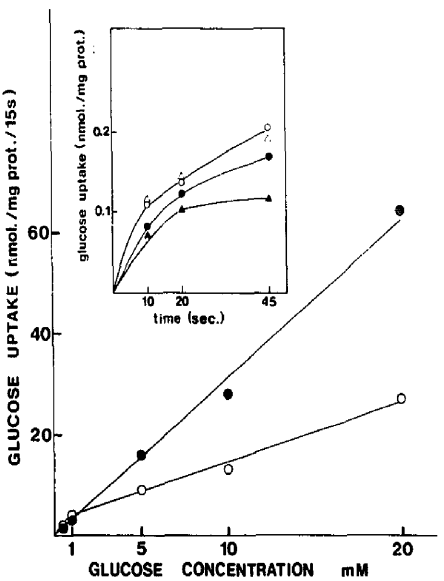

Fig. 2. Effect of anti M_r 70000 protein serum on Na^+-dependent D-glucose transport at various D-glucose concentrations by native brush-border vesicles. Native brush border membranes (10 mg) were preincubated in 100 mM NaCl, 100 mM mannitol, 1 mM Hepes-Tris (pH 7.4) buffer for 15 h at +4°C in the presence of 300 µl of preimmune rabbit serum (dark symbols) or immune anti M_r 70000 protein serum (open symbols). Membranes were diluted in a large volume of 300 mM mannitol, 1 mM Hepes-Tris (pH 7.4) buffer and centrifuged for 20 min at 100000× g. This washing is repeated twice. Final pellets were suspended in the same medium at 5 mg/ml final protein concentration. Sodium-dependent D-glucose transport was initiated at 25°C by addition of 30 µl of preteated membranes (150 µg) to a 60 µl volume of 150 mM NaSCN (or 150 mM KSCN), 100 mM mannitol, 1 mM Hepes-Tris (pH 7.4) buffer containing various D-glucose concentrations to obtain final concentrations between 0.1 to 20 mM (0.2 µCi of labelled sugar per assay). Transport was stopped after 15 s of incubation with 2 ml of a cold stopping solution containing 100 mM NaCl (or KCl), 1 mM Hepes-Tris (pH 7.4) buffer and the mixture was applied to presoaked membrane filter (0.65 µm Sartorius filter), then the filter was washed with 2 ml of the stopping solution. The radioactivity retained on the filter was measured using Picofluor 30 scintillator (10 ml) in a Packard 2000 CA TRI CARB Spectrometer. Inset represents kinetics of 1 mM radioactive D-glucose transport by brush border membranes treated as above using equilibrium isotope exchange procedure: 30 µl of pretreated membranes (150 µg) were preincubated for 1 h at 25°C in 60 µl of 1 mM. Hepes-Tris (pH 7.4) buffer containing 100 mM NaCl (or KCl), 1.5 mM of unlabelled D-glucose with (triangle symbols) or without (circle symbols) 0.15 mM phlorizin; the isotope exchange was initiated by addition of 15 µl of 1 mM radioactive sugar in the same medium (0.2 µCi of labelled sugar per assay). Transport was stopped as mentioned above.

TABLE I

Effect of anti M_r 70000 protein serum on D-glucose-inhibitable phlorizin binding by native brush border vesicles

Native brush border membranes (10 mg) preincubated in the presence of 300 µl of preimmune rabbit serum or immune anti M_r 70000 protein serum and washed as mentioned in Fig. 2 were suspended in 100 mM NaCl, 1 mM Hepes-Tris (pH 7.4) buffer. The D-glucose-inhibitable phlorizin binding was measured as previously described [12]. Each value represents the mean of three separate experiments. The errors indicated are standard deviations.

Additions	D-Glucose-inhibitable phlorizin binding (pmol/mg protein)	% Stimulation
Pre-immune serum	26 ± 0.9	-
Anti 70 kDa protein serum	30 ± 1.2	15

transport experiments with higher D-glucose concentrations led to an inhibition of initial D-glucose uptake by brush border membrane vesicles (Fig. 2). Moreover, this inhibition was specific for the D-glucose substrate, as proven by the absence of stimulation or inhibition in L-glucose uptake whatever was its concentration (data not shown). On the other hand D-glucose-inhibitable phlorizin binding was measured at 3 µM phlorizin concentration: a slight increase in the phlorizin binding (15%) was also shown, when the membranes were treated with the rabbit antiserum (Table I). Taking together, the results indicate that the antibodies do not affect the phlorizin binding, but uncouple Na^+/D-glucose cotransport from inhibition by bound phlorizin, probably by stabilizing an active conformation of the cotransporter [15].

In order to demonstrate the specific recognition of sodium-dependent D-glucose transporter by polyclonal antibodies, the immunoprecipitation of D-glucose-inhibitable phlorizin binding was carried out using brush border membranes extracted with 1% Triton. Treatment with antiserum against the M_r 70000 polypeptide and removal of the resultant immune complex with protein A-Sepharose, after reconstitution into liposomes, resulted in a preparation which gave less than 10% of the binding as compared to an identical preparation treated with preimmune rabbit serum (Table II).

After gel electrophoresis in the presence of Triton X-100, western blot analysis of the electroextracted M_r 70000 polypeptide fraction indicated that the antiserum specifically recognized this protein. In Triton X-100 extract or a glucose transporter-enriched brush border sample, the antibodies against the M_r 70000 recognized two additional bands with approximate relative molecular mass of 120000 and 45000 (Fig. 3). These bands were not detected with the preimmune rabbit serum. The M_r 70000 polypeptide does not appear to represent a subunit derived from the M_r 120000 protein, and the M_r 45000 polypeptide does not appear to be a subunit

TABLE II

Immunoprecipitation of phlorizin binding activity from brush border 1%-Triton extract

1.5 mg proteins of 1%-Triton extract was incubated with 0.1 ml of anti M_r 70000 protein rabbit serum (1 h at 25°C) and immune complexes precipitated by 12 mg of protein A-Sepharose suspended in 100 mM NaCl, 1 mM Tris-Hepes (pH 7.4) buffer for 1 h at 25°C. After centrifugation for 30 min at 50000×g, proteins of the supernatant were reconstituted into liposomes and the D-glucose-inhibitable phlorizin binding measured as previously described [12]. A control was realized with a rabbit preimmune serum in the same conditions. Each value represents the mean of three separate experiments. The errors indicated are standard deviations.

Additions	D-Glucose-inhibitable phlorizin binding in detergent extract after immunoprecipitation (pmol/mg protein)	% Phlorizin binding activity precipitated
Pre-immune serum	35±3	-
Anti 70 kDa protein serum	2±2	94

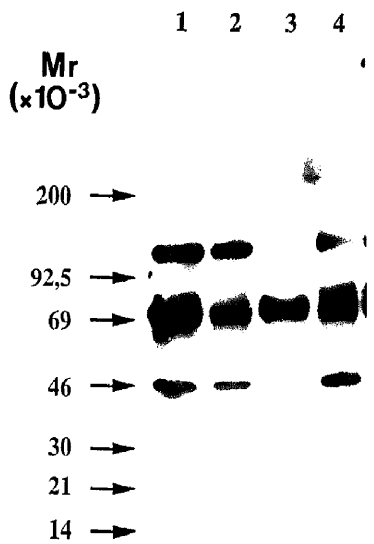

Fig. 3. Immunoblot analysis of brush border 1%-Triton extract with the anti M_r 70000 protein serum. Brush border membranes purified in the presence of protease inhibitor PMSF (lane 1) were extracted by Triton X-100 at 1% final concentration (lane 2) and compared to the Triton extract from membranes purified in the absence of PMSF (lane 4). Aliquots of each sample (100 μg) and the M_r 70000 protein fraction (5 μg) in lane 3 were run on a 0.1% SDS 5–15% linear gradient acrylamide gel at 16 mA per plate for 15 h at 14°C [12]. All samples were applied after boiling for 90 s in the presence of 2% SDS, 5% MSH. Immunoblotting was carried out as described in Material and Methods with a 1/100 diluted antiserum. The protein markers used were myosin (200000), phosphorylase *b* (92500), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000) and trypsin inhibitor (21500).

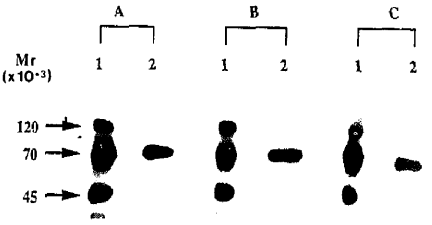

Fig. 4. Immunoblot analysis of Triton extract and M_r 70000 protein fraction in reducing or non reducing conditions. Aliquots of 1%-Triton extract (45 μg) and M_r 70000 antigen (2.5 μg) were run as previously described [12] on 10% acrylamide mini slab gels containing 0.1% SDS (A and B) or 0.25% Triton X-100. 0.1% SDS (C) at 200 in for 1 h. Samples were applied after boiling for 90 s in the presence of 2% SDS, 5% MSH (A) or without boiling in the presence of 2% SDS (B) or 0.25% Triton X-100, 0.1% SDS (C). Immunoblotting was described in Materials and . ethods using a 1/100 diluted antiserum.

of the M_r 70000 polypeptide because no differences were observed in the electrophoretic patterns obtained under reducing (SDS + MSH) and non-reducing (Triton X-100 + SDS or SDS only) conditions (Fig. 4).

Discussion

Phlorizin is a competitive inhibitor of Na^+/D-glucose cotransport at the luminal side of the renal proximal brush border membranes [16]. Quantitative precipitation of D-glucose-inhibitable phlorizin binding activity from Triton extracts of kidney brush border membranes by rabbit polyclonal antiserum shows that the antiserum contain antibodies against (part of) the glucose transporter, and that the M_r 70000 polypeptide represents the phlorizin binding moiety of the Na^+/D-glucose cotransporter. Moreover, the exposure of native kidney brush border membranes to a polyclonal antibody raised against the M_r 70000 polypeptide showed a differential effect on Na^+-dependent D-glucose transport depending on the glucose concentration used in uptake measurements; a slight activating effect on uptake was observed at low glucose concentrations (1 mM), while a more effective inhibition was seen at higher glucose concentration.

It has been well documented that two Na^+/D-glucose cotransporters are present on renal proximal tubule with different affinities for D-glucose and phlorizin, one of them displaying high phlorizin and low D-glucose affinities and the other one low phlorizin and high glucose affinities, the former should be present on outer renal cortex and the latter on outer renal medulla of rabbit kidney [9,10] or both on the outer cortex as well as the outer medulla of pig kidney [11]. Our experiments carried out exclusively on brush border membranes isolated from pig kidney outer cortical slices agree with this latter hypothesis.

Lever's group [7] has recently described a monoclonal antibody purified from an hybridoma obtained by fusion of apical membranes from LLC-PK1 cells, which seems to express only the Na^+/D-glucose cotransporter with the low phlorizin affinity (K_d = 2–4 μM). This antibody stimulated the phlorizin-binding activity, but neither enhanced nor inhibited sodium D-glucose uptake by pig renal brush border membranes. In our experiments we find also a slight increase in D-glucose-inhibitable phlorizin binding (15%) by brush border membranes using a 3 μM final phlorizin concentration. As previously pointed out by Semenza [3] the LLC-PK1 cotransporter is similar to the typical low phlorizin high glucose affinities Na^+/D-glucose cotransporter of small intestine, which is specifically activated by monoclonal antibodies obtained with papain digested, desoxycholate-extracted small intestinal brush border membranes [5]. Finally, in the two cases a protein with a M_r 72000–75000 was identified by the monoclonal antibodies.

From the Western blot analysis, it appears that our polyclonal antibodies not only label the M_r 70000 protein fraction, but also two other proteins with approximate relative molecular mass of 120000 and 45000. The molecular weight of the M_r 45000 polypeptide is close to that found for facilitated diffusion glucose transporters; it has been reported that some of monoclonal antibodies directed against brush border membranes Na^+/D-glucose cotransporter cross reacted at the basal-lateral [17] and erythrocyte [18] membranes, where the facilitated diffusion system for D-glucose is present. However, the M_r 45000 polypeptide immunologically detected here does not represent a contaminating glucose transporter from the basal lateral or erythrocyte membrane, because no immunological cross reactivity was observed with our antibody against erythrocyte or hepatocyte membranes. It is more likely that M_r 45000 polypeptide represents a proteolytic degradation product because, when brush border vesicles were isolated in the presence of a proteinase inhibitor, the intensity of this protein band decreased. A similar result has been recently reported using monoclonal antibodies and had been explained by post mortem degradation of the glucose transporter during membrane preparation [8].

As to the M_r 120000 protein our experiments did not show any direct relationship between this protein and the M_r 70000 polypeptide. However, these two proteins contain common epitopes and are both immunoprecipitated by polyclonal antibodies (data not shown).

It has been shown, using the cloned M_r 73000 polypeptide from small intestinal brush border, that only a single protein species is required for transport activity [6]. It is then likely that the M_r 70000 polypeptide represents the renal low affinity phlorizin Na^+/D-glucose cotransporter (see above) and contributes to the slight increase in D-glucose uptake by brush border membranes at low glucose concentrations observed in our experiments after treatment with our polyclonal antibodies. The Na^+/D-glucose transport inhibition observed in the range of 1 to 20 mM glucose concentration with the same antibodies could be due to the presence of another Na^+/D-glucose cotransporter with M_r 120000. This is consistent with a previous observation that (i) two populations of Na^+/D-glucose cotransporters with low and high affinity phlorizin-binding sites are present at the ratio of 1:1 in porcine outer cortex [11] and (ii) a phlorizin binding component of M_r 110000 with high affinity for phlorizin (K_d approx. 0.5 μM) is yielded in radiation-inactivation experiments [19].

In conclusion the use of polyclonal antibodies allows to prove the presence of two different Na^+/D-glucose cotransporters in kidney outer cortical slices as previously demonstrated by kinetic measurements. These two kinds of transporters could complement one another in the glucose reabsorption by the kidney; one of them with low affinity, but high capacity for D-glucose could achieve the most of the work and the other with high affinity but low capacity take up the remaining substrate.

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