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Oligomeric structure of the sodium-dependent phlorizin binding protein from kidney brush-border membranes

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Immunodetection of solubilized kidney brush-border proteins on Western blots using antibodies against the 70 kDa phlorizin binding component of sodium-glucose cotransporter allows to identify an additional protein band with apparent molecular mass of 120 kDa in the presence of reducing agent dithiothreitol. Antibodies specifically eluted from the 70 kDa protein still recognize the 120 kDa protein on Western blot. The lack of dissociation of the 120 kDa protein from native brush borders or Triton X-100 extract in the presence of dithiothreitol can be improved by an extended incubation at 25°C; this protein is fully dissociated when purified by electroelution from polyacrylamide gel and gives two subunits with apparent molecular masses of 70 and 60 kDa by Coomassie staining and Western blot analysis. The effect of dithiothreitol on the renal brush-border membrane phlorizin binding is studied; a decrease in the number of high-affinity phlorizin binding sites without modification of the affinity to the binding molecule is observed. These data suggest that the high-affinity phlorizin binding moiety of sodium-glucose cotransporter exists in the kidney as a dimeric structure.

Introduction

A number of kinetic studies carried out on kidney brush-border membranes (BBM) demonstrated the presence of two sodium-glucose cotransport systems with different affinities, capacities and stoichiometries [1–4]. The data were obtained from experiments dealing with either the D-glucose transport measured in sodium gradient or equilibrium exchange conditions or the binding of phlorizin, the competitive inhibitor of D-glucose for its binding site on BBM carrier.

The state of the art seems to be more clear for the intestinal sodium-glucose cotransport, since recent results failed to detect any difference in kinetic parameters of sodium glucose cotransporters in jejunal BBM [5]. A 72 kDa protein involved in the sodium glucose cotransport has been previously isolated from small intestinal BBM by immunoaffinity chromatography [6] and more recently the same molecular mass was found for the intestinal sodium glucose transporter SGLT1 by cloning and expression in *Xenopus* oocytes [7–9]. On the contrary the cloned renal SGLT1 was found predominantly in the outer medulla of rabbit kidney [10].

On the other hand several attempts have been made to determine the molecular mass of the transport system(s) in situ without dissociation of the membrane by detergents. In this field the method of radiation inactivation, largely used to study the size and structure of both soluble and membrane bound enzymes [11], has been employed at various irradiation doses. In the intestinal BBM the target size of the cotransporter measured by this method gave the average value of 290 kDa which could correspond to a homotetramer comprised of four 73 kDa SGLT1 peptides [12].

The same method does not give the same results in kidney BBM, although the cloned renal sodium-glucose cotransporter closely resembles that of the small intestine [13,14]. The range of kidney cotransporters molecular sizes estimated by radiation inactivation varied from 110 kDa for the smallest unit to 1000 kDa for the greatest cluster [15–18] and could be related to different configurations in situ. More recently the reported cloning of a possible other subunit of the sodium-glucose symporter adds to the complexity [19], but could explain the differences observed between kidney and small intestine.

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Abreviations: BBM, brush-border membrane; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

In any case no direct evidence has been provided that the sodium-glucose cotransporter is an oligomeric structure, which contains at least the previously cloned component SGLT1 of the cotransporter. The aim of this report is to demonstrate the oligomeric state of the sodium-glucose cotransporter, when solubilized from the kidney BBM by detergents and analyzed by Western blot procedure using polyclonal antibodies directed against a 70 kDa protein [20], previously identified as the phlorizin binding moiety of the sodium-glucose cotransporter [21]. Our data indicate that this phlorizin binding moiety exists as a mixture of 70 kDa and 120 kDa proteins, which could be, respectively, monomeric and dimeric forms in pig renal proximal tubule BBM.

Materials and Methods

Preparation of protein samples

BBM vesicles were prepared from the cortex of pig kidneys provided by the local slaughterhouse using the $MgCl_2$ precipitation method previously described [22]. Vesicles were suspended in 300 mM mannitol, 10 mM Tris-Hepes (pH 7.4) buffer at a protein concentration of 20 mg/ml. Aliquots of vesicles suspension were fast frozen in liquid nitrogen until the use.

Triton solubilization: one volume of BBM vesicles in 300 mM mannitol, 10 mM Tris-Hepes (pH 7.4) was added to one volume of 2% Triton X-100 in 200 mM NaCl medium buffered by 1 mM Tris-Hepes at pH 7.4 and kept for 1 h at 4°C, then centrifuged for 1 h at $180000 \times g$. The resulting clear supernatant represented the detergent extract. Electroextracted 120 kDa and 70 kDa were prepared as previously described [21] with slight modifications. Triton extract was concentrated by treatment with Bio-Beads SM-2 (0.6 g wet weight of beads/ml), followed by removal of beads and sedimentation of insoluble proteins by ultracentrifugation (1 h at $180000 \times g$). The resultant pellet was solubilized in a small volume of the electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine (pH 8.3) containing 0.1% SDS) to a concentration of 10 mg/ml. Electrophoresis of the concentrated detergent extract was carried out according to O'Farrell [23] with the multiphasic buffer system slightly modified as follows: 0.4 M Tris-HCl (pH 8.8) in the lower gradient gel and 0.075 M Tris-HCl (pH 6.8) in the stacking gel. The electrode buffer contained 0.1% SDS, 25 mM Tris-HCl, 192 mM glycine (pH 8.3). A 4–12% acrylamide linear gradient for the separating gel and 4% for the stacking gel were chosen. Membrane proteins (200 μg/lane) were loaded on gel plates (1.5 mm thick, 160 mm wide, 180 mm long) after treatment by 2% SDS without heating; when the 120 kDa fraction was used for phlorizin binding measurements SDS concentration was 0.1%. Running conditions were 10 mA/gel overnight. The acrylamide fractions corresponding to the apparent molecular masses of 120 kDa and 70 kDa identified in comparison with protein markers electrophorized in the same run were separately diced and electroeluted using the Bio-Rad Electroeluter (Model 422) through dialysis membranes with a cut-off of 12–15 kDa. Elution was performed for 3 h at 10 mA/tube and 4°C in electrophoresis buffer. Proteins were recovered in the tube and their concentration measured by BCA (Pierce).

This protocol was slightly modified in the case of Fig. 4. To avoid contamination of the immunoreactive 120 kDa protein by closely migrating proteins, an additional step was added before electroextraction of the 120 kDa band. The concentrated detergent extract was solubilized in PBS buffer (pH 7.4) containing 0.5% CHAPS and applied on a column of IgG against the 70 kDa polypeptide coupled to Sepharose 4B as described by the manufacturer (Pharmacia). The column was equilibrated in PBS containing 0.1% CHAPS. Immunoreactive proteins were eluted by 100 mM glycine-HCl (pH 2.5), 0.1% CHAPS and the eluent neutralized with 1 M Tris base before concentration and applying on electrophoresis gel.

Western immunoblots

Protein samples were separated on 4–12% acrylamide linear gradient in above-mentioned electrophoresis buffer at 200 V for 1 h using the Mini-Protean II Cell (Bio-Rad), then the gel plate equilibrated for 30 min in transfer buffer (25 mM Tris-HCl, 192 mM glycine (pH 8.3) containing 20% methanol). Proteins were electroblotted onto PVDF membrane (Millipore) for 1 h at 100 V using a Mini Trans Blot (Bio-Rad). Subsequent steps were the same as described in a previous report [20] with IgG fraction derived from antisera raised in rabbits against the 70 kDa antigen and purified on protein A-Sepharose (Pharmacia).

Affinity purification of IgG via binding to the 70 kDa antigen

This purification was performed according to Borregaard and al. [24]. A load of 200 μg of 70 kDa polypeptide was run in 4–12% SDS-PAGE, then electrophoretically transferred to PVDF membrane as mentioned above. PVDF was stained with 0.2% (w/v) Ponceau Red in 3% TCA (w/v) and destained with water. The strip of PVDF corresponding to the position of the 70 kDa fraction was cut out, immersed for 1 h at room temperature in blocking solution (PBS containing 0.6% Tween-20), then incubated for 1 h at 37°C in the presence of IgG against the 70 kDa polypeptide (0.1 mg/ml in blocking solution). The excess antibody was eliminated by five washings of 10 min in PBS containing 0.05% Tween-20. IgG bound to the 70 kDa polypeptide was eluted by an incubation in 0.2 M

glycine-HCl (pH 2.8) for 5 min. The paper was removed and the eluant neutralized with 2 M Tris base. The protein concentration of the eluted antibodies was 22 μg/ml as estimated by the absorbance at 280 nm. The antibodies were used half-diluted as primary antibody in Western blotting of the 70 kDa antigen or membrane proteins from Triton extract of BBM.

Phlorizin binding measurements

The measurements were performed with BBM vesicles (7–8 mg of protein per ml) incubated for 2 h at 25°C in the presence of 2 or 20 mM DTT. Phlorizin binding was carried out by adding [^{3}H]phlorizin at final concentrations ranging from 0.1 μM to 8 μM to membrane vesicles (100–200 μg protein) in 100 mM NaSCN, 10 mM Hepes-Tris (pH 7.4) in the absence (total binding) or presence of 1 mM unlabeled phlorizin (non specific binding). The total volume of mixture was 90 μl. After 15 min of incubation at 25°C each sample was applied to 0.65 μm Sartorius filter, rapidly washed with ice-cold stop solution containing 150 mM NaCl, 10 mM Hepes-Tris (pH 7.4) and counted for the radioactivity. Specific Na^+-dependent phlorizin binding was calculated by subtracting binding in the presence of 1 mM phlorizin from binding in the absence of unlabeled phlorizin.

As to the phlorizin binding to the electroextracted 120 kDa protein, the acrylamide fraction corresponding to the apparent molecular mass of 120 kDa was electroeluted as mentioned above. Aliquots (5 μg) in 0.4 ml of 50 mM Tris-Hepes (pH 7.4) were layered on a presoaked nitrocellulose membrane (Millipore) in 10 wells of a 96-well dot-blot apparatus (Hybriblot Manifold, BRL) using a minimal vacuum speed. Then [^{3}H]phlorizin at 3 μM final concentration in 0.1 ml of 100 mM KCl, 10 mM Hepes-Tris (pH 7.4) for 5 wells or 100 mM NaCl, 10 mM Hepes-Tris (pH 7.4) for the 5 other wells was added and incubated for 5 min at 25°C. After sucking up and washings, the nitrocellulose corresponding to each well was cut up and counted for the radioactivity. Phlorizin binding measurements were repeated with another sample of electroextracted 120 kDa protein.

Results and Discussion

In a previous paper we have shown that polyclonal antibodies against a 70 kDa polypeptide identified as the phlorizin binding moiety of sodium-glucose cotransporter recognized an additional 120 kDa polypeptide by Western blot analysis of native BBM. This 120 kDa fraction was resistant to the action of the reducing agent β-mercaptoethanol (5% final concentration) after boiling 3 min in 4% SDS [20]. Similar results are obtained with dithiothreitol (50 mM final concentration) under the same conditions. The 120 kDa protein

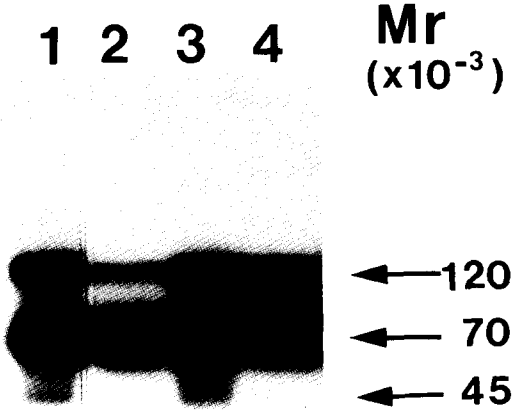

Fig. 1. Immunoblot analysis of membrane proteins from BBM treated or untreated by 1% Triton X-100 in reducing conditions. Purified BBM (6 mg/ml) suspended in 300 mM mannitol, 10 mM Hepes-Tris (pH 7.4) (lanes 1 and 2) or solubilized by 1% Triton X-100 (lanes 3 and 4) were treated for 2 h at 25°C in the presence of 2 mM (lanes 1 and 3) or 20 mM DTT (lanes 2 and 4). Aliquots (5 μg) were applied in 4–12% SDS polyacrylamide gradient gel after boiling for 3 min in the presence of 2% SDS and run for 1 h at 200 V and +4°C. Immunoblotting was described in Materials and Methods using antibodies directed against the 70 kDa protein. The protein markers used were thyroglobulin subunit (330 kDa), ferritin (220 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), catalase subunit (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa).

is not confined to pig kidney BBM, since it was found from other kidney species (rat, human). It can be detected in LLC-PK1 cells by Western blot analysis demonstrating its epithelial localization.

Increasing the time of incubation at a lower temperature (25°C) resulted in a partial dissociation of the 120 kDa protein. Triton X-100 at 1% final concentration on kidney BBM during 2 h at +4°C in the presence of 20 mM DTT did not lead to increased dissociation of the 120 kDa protein (Fig. 1). This paradoxal behaviour has been previously described when studying multimeric species of human facilitated glucose transporter (GLUT 1) in detergent extracts from erythrocyte membranes and related to the possible presence of peripheral membrane proteins [25]. On the other hand the 120 kDa protein fraction electroeluted from polyacrylamide gel as previously described [21] was completely dissociated by 50 mM DTT with concomitant appearance of a 70 kDa polypeptide when analyzed by Western blot procedure (Fig. 2).

Therefore it appears that the 120 kDa protein would contain epitopes belonging to the 70 kDa subunit previously identified as the D-glucose inhibitable phlorizin binding moiety of sodium-glucose cotransporter after reconstitution in liposomes [21]. As an additional piece of evidence, affinity purification of the polyclonal antibodies was performed by elution from the 70 kDa antigen as described in Materials and Methods. These antibodies still recognized the 120 kDa protein in BBM

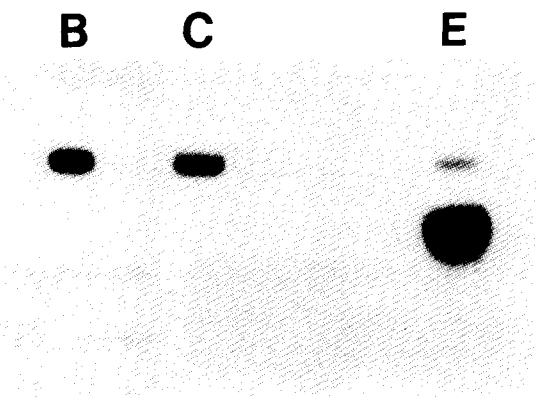

Fig. 2. Immunoblot analysis of electroextracted 120 kDa in reducing or non reducing conditions. The 120 kDa protein was electroextracted as described in Materials and Methods. Aliquots (2 μg) were applied in 4–12% SDS-polyacrylamide gradient gel after addition of 4% SDS without (lane B) or with boiling for 3 min (lanes C and E) in the absence (lanes B and C) or the presence of 50 mM DTT (lane E). The slab gel was run at 200 V and 4°C for 1 h. Immunoblotting of the protein was performed with antibodies directed against the 70 kDa protein (see Materials and Methods). Proteins markers (as in Fig. 1) were applied in lanes A and D.

by Western blot procedure. In addition the 120 kDa protein displayed a sodium-dependent phlorizin binding capacity; indeed the electroextracted 120 kDa protein layered on nitrocellulose membrane as described in Materials and Methods bound 120 ± 12 and 30 ± 10 pmol of phlorizin/mg of protein, respectively, in the presence of sodium and potassium ions. Finally the apparent molecular mass of 120 kDa is close to the molecular size of 110 kDa estimated for the high-affinity phlorizin binding moiety of the renal BBM D-glucose transport protein by radiation inactivation method [15].

The Na^+-dependent phlorizin binding at various concentrations was measured using kidney BBM vesicles incubated for 2 h at 25°C in the presence of 2 and 20 mM DTT. As can be seen in Fig. 3, Scatchard plots showed a decrease in the number of high-affinity phlorizin binding sites in membrane vesicles treated with 20 mM DTT. The number of low-affinity sites was not modified. Thus the loss of high-affinity phlorizin binding sites after DTT treatment, also previously reported [26], would be due to a decrease of the 120 kDa protein. The dissociation constant K_d of high-affinity phlorizin binding was not modified by DTT treatment at 20 mM (Fig. 3 inset); this result confirms that the effect of DTT on high-affinity phlorizin binding is not due to a decrease in the phlorizin affinity for the sodium-glucose cotransporter, but only to a loss of binding sites, probably related to the dissociation of the 120 kDa in protein subunits. In a previous work we have demonstrated that the thiol reagent *N*-ethylmaleimide inhibited the sodium-dependent glucose transport by kidney BBM; this inhibition was partially prevented by the presence of D-glucose [27]; using DTT similar data were reported for the sodium phlorizin binding by kidney BBM [26]. These observations provide evidence for the existence of (at least) two disulfide bonds important to the functioning of glucose transporter(s); one of them could be involved in the association of two subunits to give the dimer of 120 kDa.

At present it may be asked whether this protein is a homodimer (two identical 70 kDa subunits associated

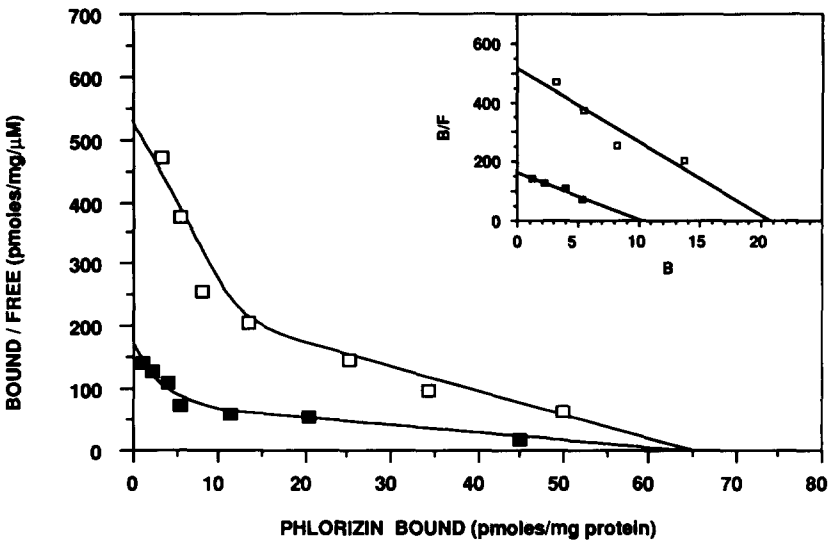

Fig. 3. Scatchard analysis of sodium-dependent phlorizin binding to kidney BBM vesicles incubated in the presence of 2 mM and 20 mM DTT. Vesicles (7–8 mg/ml) were preincubated in the presence of 2 mM (□) or 20 mM DTT (■) in 300 mM mannitol, 10 mM Hepes-Tris (pH 7.4) buffer for 2 h at 25°C. Aliquots (100–200 μg) of vesicles were then incubated in 100 mM NaSCN, 10 mM Hepes-Tris (pH 7.4) buffer with different phlorizin concentrations for 15 min at 25°C. The measurements were corrected for nonspecific binding as described under Materials and Methods. The results represent the average of triplicate sample points of a representative experiment. Standard deviations are too small to illustrate in the figure.

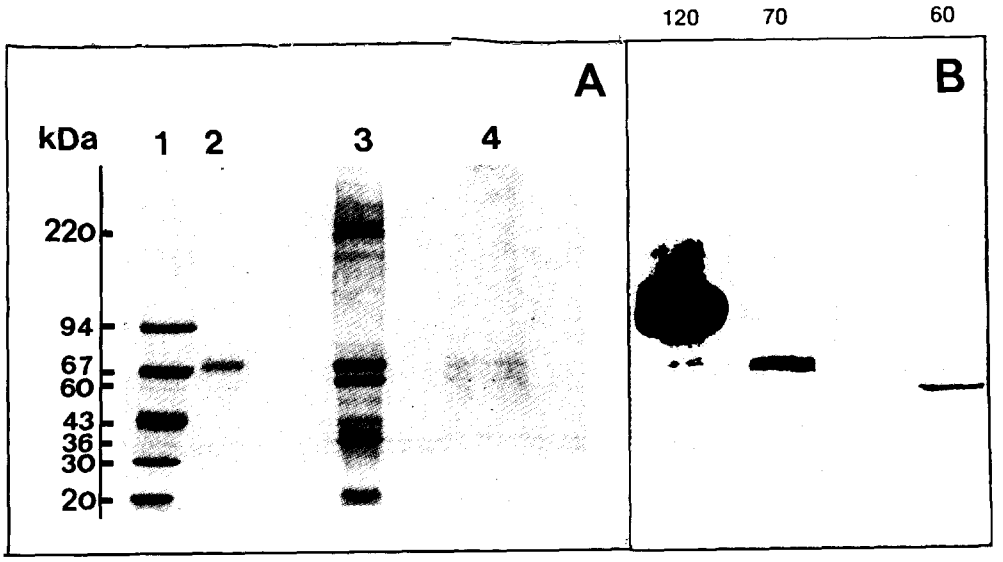

Fig. 4. Staining and immunoblot analysis of subunits derived from the 120 kDa protein. The 120 kDa protein was electroextracted as described in Materials and Methods in the protocol including the immunoaffinity step, then boiled for 3 min in the presence of 50 mM DTT, 3% SDS. (A) An aliquot (lane 4) was electrophorized on a 4–12% acrylamide linear gradient mini-gel before Coomassie staining. Control of electroextracted 70 kDa (lane 2) and protein markers (as in Fig. 1) (lanes 1 and 3) were applied on the same gel. (B) Another aliquot was layered on a 4–12% acrylamide preparative gel to separate and electroelute the protein bands seen in the stained gel. Then these protein bands were separately analyzed on a 4–12% acrylamide linear gradient mini-gel by Western immunoblotting as described in Fig. 2. Control of electroextracted 120 kDa was applied on the same gel.

by disulfide bonds) or a heterodimer of a 70 kDa subunit with another protein subunit. This latter hypothesis has been suggested by others to explain the binding of monoclonal antibodies directed against the pig kidney sodium-glucose cotransporter to the 75 kDa and 47 kDa polypeptide components [4]. In our hands, Western blot analysis of electroextracted 70 kDa and 120 kDa proteins submitted to 50 mM DTT did not reveal the 47 kDa polypeptide; this species was sometimes seen in Western blot of native BBM in our experiments; in all likelihood it could be a proteolytic product of other immunoreactive polypeptides. In support of the hypothesis of a heterodimeric structure for the renal sodium-glucose cotransporter, a cDNA clone P20 has been recently identified from a cDNA library, which expressed a polypeptide with an apparent molecular mass of 68 kDa; when P20 cRNA was coinjected in *Xenopus* oocytes with SGLT1 cRNA in stoichiometric amounts, the authors found two apparent sites with low and high affinity for methyl α-D-glucopyranoside [19].

From the data shown in Fig. 2, it seems that the dissociation of the 120 kDa protein give an unique spot focused at 70 kDa, in favour of a homodimeric structure; nevertheless the presence of two immunoreactive bands merging in a single large plot as seen in Fig. 2 or another non immunoreactive protein associated with the 70 kDa protein cannot be ruled out. To select from these hypotheses, the bulk of detergent solubilized proteins recognized by antibodies against the 70 kDa antigen was separated on immunoaffinity column, then the retained proteins treated by 2 mM DTT were electrophorized and the 120 kDa fraction electroeluted from the acrylamide gel. This fraction was treated by 50 mM DTT. After electrophoresis two bands with respective apparent molecular masses of 70 and 60 kDa were seen on the gel stained by Coomassie dye (Fig. 4A). Both bands electroeluted from a preparative acrylamide gel and separately electrophorized were recognized by polyclonal antibodies against the 70 kDa protein (Fig. 4B).

Finally, from our experiments it seems that the 120 kDa species represents a heterodimeric protein involved in the high-affinity Na^+-dependent phlorizin binding; this protein could therefore correspond to the low-affinity high-capacity Na^+-D-glucose cotransporter, since it is likely that the units capable of phlorizin binding are also the units capable of BBM D-glucose transport; this is true for the binding of the inhibitor cytochalasin B to the facilitated D-glucose transporter GLUT 1 of the red cell ghost membranes [25].

In conclusion since we have previously demonstrated that electroeluted 70 kDa fraction binds phlorizin after reconstitution in proteoliposomes, the 120 kDa and 70 kDa proteins could represent, respectively, dimeric and monomeric forms of the sodium-dependent phlorizin binding protein.

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