Isolation of (a subunit of) the Na^+/D-glucose cotransporter(s) of rabbit intestinal brush border membranes using monoclonal antibodies

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Carrier (from glucose, isolation from small intestine) Cotransporter (for Na^+/glucose, isolation from small intestine) Monoclonal antibody (for isolation of a carrier) Brush border (small intestinal) Small intestine (glucose/Na^+-cotransport).

1. INTRODUCTION

The identification and isolation of the Na^+/D-glucose cotransporter(s) from either small-intestinal or renal brush border membranes has proven to be a formidable task. A number of approaches have been used in the past, including solubilization-reconstitution photoaffinity and differential labelling but these techniques have been of limited success. We here report the successful use of a monoclonal antibody for the purification of the Na^+/D-glucose cotransporter from rabbit small-intestinal brush border membranes.

2. MATERIALS AND METHODS

D-[1-^{3}H]glucose (7 Ci/mmol) was obtained from Amersham, [^{3}H]phlorizin (42.5 Ci/mmol), Formula-963 and Rabbit-anti mouse [^{125}I]IgG were obtained from New England Nuclear.

Abbreviations: SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer (Na,K, phosphate, pH 7.4), saline

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Dedicated to Professor F. Leuthardt, Zürich, on the occasion of his 80th birthday

2.1. *Antigen used*

Brush border membrane vesicles were prepared from rabbit small intestine by the Ca^{2+}-precipitation method [1], as described in [2]. The vesicles were subjected to controlled papain digestion followed by deoxycholate extraction (0.5 mg.mg protein^{-1} protein) [3], which resulted in a 3.4-fold enrichment of phlorizin-binding sites, as compared to the original membrane vesicles.

2.2. *Immunization and cell hybridization*

As described in [4], BALB/c mice received intraperitoneal injection of the antigen indicated above. Booster intraperitoneal injections were administered 4 weeks later, and mice were sacrificed 3–4 days after the booster immunization. Mouse spleen cells were fused with mouse myeloma SP2/0-Ag 14 (SP2) cells as in [4]. At about day 21 after the fusion, supernatants from the wells containing hybridomas were assayed for anti-brush border membrane antibodies (see below). Cells from the positive wells were grown as ascites tumors in BALB/c mic primed with Pristane.

2.3. *Hybridoma screening and characterization*

A solid phase radioimmunoassay was utilized in the initial screening of hybridomas for monoclonal antibodies against rabbit intestinal brush border membranes. Membranes, immobilized on (poly)-

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L-lysine-coated PVC microtiter plates, were incubated with culture medium from hybridomas followed by detection of membrane-bound mouse immunoglobulins with ^{125}I-labelled rabbit anti-mouse IgG as in [5].

Hybridomas, which were positive in the initial screening assay, were further screened for monoclonal antibodies specific for the Na^+/D-glucose cotransporter based on the ability of culture medium to inhibit phlorizin binding to brush border membrane vesicles. Vesicles were pre-incubated with culture medium followed by determination of phlorizin binding using Kessler's short-time incubation apparatus [6].

Monoclonal antibodies specific for the Na^+/D-glucose cotransporter (as determined by inhibition of pholirizin binding) were further characterized with regard to their effects on D-glucose uptake into brush border membrane vesicles.

2.4. *Purification of Na^+/D-glucose cotransporter with monoclonal antibody affinity columns*

Monoclonal antibody IM11 was purified from ascites fluid by ammonium sulfate precipitation (50% saturation) followed by dialysis against 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.5). IM11 immunoglobulin and control mouse immunoglobulin at identical protein concentrations were coupled to CNBr-activated Sepharose 4B (1.5–3.5 mg protein . g wet gel^{-1}). Immunoaffinity columns were prepared in 1.1 × 5 cm ISCO polypropylene columns. Brush border membrane vesicles or deoxycholate-extracted membrane fragments were solubilized with 0.5% digitonin in phosphate-buffered saline (PBS) pH 7.4)/0.2 mm EGTA (10 mg membrane protein in 5 ml solution) for 5 min at 22°C, centrifuged for 20 min at 40 000 × *g* and the supernatant was immediately applied to the monoclonal antibody affinity column. After washing the column with PBS, adsorbed proteins were eluted as follows: 10 mM D-mannose in PBS; PBS; 10 mM D-glucose in PBS; 1 M D-glucose in PBS. Alternatively the following sequence was used: 10 mM D-mannose in PBS, 15 μM phlorizin in PBS; 100 μM phlorizin in PBS. The eluates were concentrated over a Diaflo ultrafiltration membrane YM10 and analyzed by polyacrylamide gel electrophoresis (PAGE) (8.4 × 2.7) in 1% sodium dodecylsulfate (SDS). Gels were stained with either Coomassie blue or silver stain [7].

Na^+-dependent D-glucose uptake into the vesicles and Na^+-dependent phlorizin binding were determined as in [8,9].

3. RESULTS

Monoclonal antibodies specific for the Na^+/D-glucose cotransporter were identified with two screening assays:

(i) An initial solid-phase radioimmunoassay using papain-digested, deoxycholate-extracted brush border membranes [3]; followed by

(ii) A second screening assay based on the ability of culture medium to inhibit phlorizin-specific binding to brush border membrane vesicles.

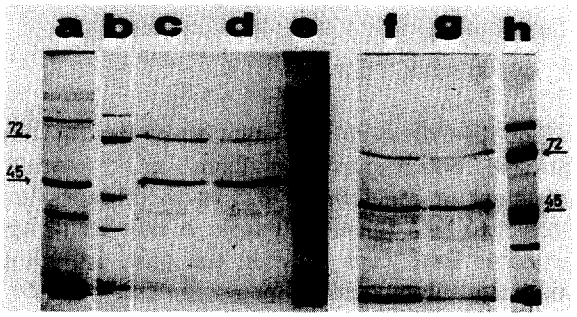

Fig.1. SDS–PAGE of brush border membrane proteins and of chromatographic fractions from the immunoadsorption. Brush border membrane vesicles (10 mg protein) were solubilized with 0.5% digitonin, 0.2 mM EGTA in phosphate-buffered saline (PBS) for 5 min at 22°C, centrifuged for 20 min at 40 000 × *g* and the supernatant was immediately applied to the immunoaffinity column. After extensive washing with PBS, 10 mM D-mannose in PBS, and again with PBS, elution was carried out with either D-glucose (first 10 mM (lane c) and then 1 M (lane d)) or, alternatively, with pholorizin (first 15 μM (lane f), then 100 μM (lane g)). The major bands in lanes, c, d, f and g had app. M_r-values of 72 and 45 kDa. Lane a is from the digitonin extract originally applied to the column; lane e is from the digitonin extract originally applied to the column; lane e is from the initial intact brush border membrane vesicles. Lanes b,h are from the protein standards (from the top: muscle glycogen phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; chicken ovalbumin, 43 kDa; carbonic anhydrase, 30.1 kDa; trypsin inhibitor, 20.1 kDa). The SDS–PAGE's were silver-stained. For more details, see text.

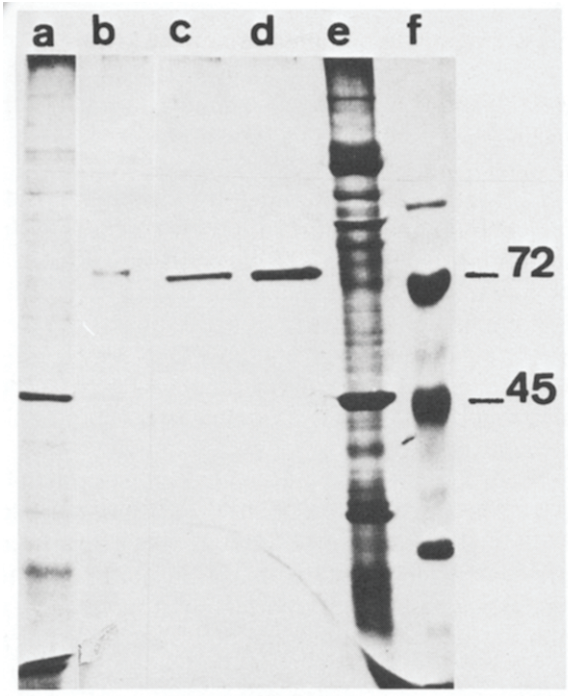

Fig.2. SDS–PAGE of deoxycholate-extracted brush border membrane fragments and of chromatographic fractions from their immunoadsorption. Same conditions as in fig. 1, except that protease inhibitors were present throughout (preparation of the brush border vesicles, extraction with deoxycholate, with digitonin, immunoadsorption and elution). The protease inhibitors were: aprotinin, bacitracin, soya bean trypsin inhibitor (and, from the deoxycholate extraction onwards, benzethonium Cl, pepstatin and PMSF). Lane: (a) pellet from the deoxycholate extraction; (b) digitonin extract therefrom: (c) phlorizin (15 μM) eluate; (d) second phlorizin (100 μM) eluate; (e) initial, intact brush border membrane vesicles; (f) protein standards, as in (b) and (h) of fig.1. The SDS–PAGE's were silver-stained.

Out of a total of 528 hybridomas, one (IM11) secreting a monoclonal antibody specific for the Na^+/D-glucose transporter was selected for further study. It was determined to be of the IgM isotype.

Monoclonal antibody IM11 was immunoaffinity purified from ascites fluid, and it was tested for the ability to inhibit Na^+-dependent G-glucose transport into small-intestinal brush border membrane vesicles and Na^+-dependent phlorizin binding. The concentrations of monoclonal antibody IM11 yielding 50% inhibition of [^{3}H]phlorizin binding was 1 μM and glucose transport was 0.44 μM. As the assay design involved preincubation of the vesicles with antibody IM11 in the absence of Na^+ (i.e., in K phosphate buffer, pH 7.5, 0.14 M KCl, 0.1% ovalbumin), washing the vesicles free of antibody and then testing them for Na^+-dependent D-glucose transport and phlorizin binding, the interaction of the cotransporter with monoclonal antibody IM11 clearly did not require the presence of Na^+.

The specific inhibition of Na^+/D-glucose transport into small-intestinal brush border membrane vesicles and of Na^+-dependent phlorizin binding to vesicles strongly suggested that monoclonal antibody IM11 was likely to be an ideal reagent for the affinity purification of the rabbit intestinal brush border Na^+/D-glucose cotransporter(s). Thus, immunoaffinity chromatography of digitonin-solubilized brush border membranes was carried out on an immunoadsorbent prepared by coupling monoclonal antibody IM11 to Sepharose 4B (see section 2).

Because the antigenic determinant on the Na^+/D-glucose cotransporter recognized by antibody IM11 is likely to be involved with the sugar binding region of the molecule, ligand-specific elution of adsorbed protein was attempted with both D-glucose and phlorizin. Moderate concentrations of either D-glucose (10 mM) or phlorizin (15 μM) eluted one polypeptide of 72 kDa as determined by SDS–PAGE (fig. 1, 2). This protein was considerably enriched as compared to the starting material – indeed it was purified to apparent homogeneity when deoxycholate–extracted membrane fragments were used, rather than whole vesicles. The 72 kDa band did not elute if D-mannose, rather than D-glucose, was used as the eluant. Non-immune mouse immunoglobulin affinity column control experiments demonstrated the specificity of the IM11 column. A second band of apparent M_r of 45 kDA was also evident in D-mannose-, D-glucose- and phlorizin-eluates from both IM11 and control mouse immunoglobulin affinity columns. This band on SDS-polyacrylamide gels co-migrated with actin (very conspicuous in the original brush border membranes, fig. 1). It could be eliminated from D-glucose eluates of IM11 immunoaffinity columns if deoxycholate-extracted brush border membrane fragments were

used as a source of cotransporter. Deoxycholate extraction of brush border membranes has been shown in [3] to considerably reduce the amount of membrane-associated actin.

4. DISCUSSION

The monoclonal antibody used here (IM11) has been shown to inhibit Na^+-dependent D-glucose uptake into brush border membrane vesicles and Na^+-dependent phlorizin binding onto these vesicles and onto deoxycholate-extracted membrane fragments prepared therefrom. (a complete description and characterization of the monoclonal antibodies will be found in: Schmidt, U.M., Eddy, B., Fraser, C.M., Semenza, G. and Venter, J.C.; in preparation.) These data indicate that monoclonal antibody IM11 is directed in all likelihood to a determinant in the sugar binding region of the Na^+/D-glucose cotransporter(s). When this monoclonal antibody (coupled to Sepharose 4B) was used as an immunoadsorbent for digitonin-solubilized small-intestinal brush border membranes, or for deoxycholate-extracted, digitonin-solubilized fragments of brush border membranes, a band of 72 kDa (by SDS–PAGE) was enriched to near homogeneity, or to homogeneity, respectively.

Several lines of evidence strongly suggest that the 72 kDa protein isolated by immunoaffinity chromatography and SDS–PAGE represents (a part of) the small intestinal Na^+/D-glucose cotransporter.

(i) It interacts with a monoclonal antibody directed against this membrane component, as shown by it being retained by the immunoadsorbent prepared from this antibody (fig.1,2);

(ii) It is eluted by moderate concentrations of D-glucose (10 mM) the apparent K_m-values for D-glucose transport in the presence of Na^+ ranges between about 0.1 and 2 mM, depending on the experimental setup [6,9,10];

(iii) It is eluted (fig.1,2) by moderate concentrations of the well-known inhibitor of this system, phlorizin [9,11] (15 μM) the apparent K_d- and K_i-values for phlorizin binding and for phlorizin inhibition of D-glucose transport in the presence of Na^+ ranges between about 5 and 9 μM [9,12];

(iv) It is not eluted by D-mannose, a sugar with negligible affinity of this transport system [13];

(v) It is the only band eluted from immunoaffinity columns by phlorizin or D-glucose when digitonin solubilizates of deoxycholate-extracted membrane fragments are used (fig.2). (Deoxycholate-extraction of brush border vesicles yields membrane fragments in which the Na^+/D-glucose cotransporter is enriched 3.4-fold and the actin band at 45 kDa is strongly decreased or totally removed [3]);

(vi) The app. M_r, 72 kDa, of the affinity-purified protein is identical to a protein which has been previously identified as being (a part of) the Na^+/D-glucose cotransporter by the use of a photoaffinity label phlorizin derivative [14], or by semi-selective labelling with $HgCl_2$ [15] or by partial negative purification [3];

(vii) The obviously very low concentration of the 72 kDa band (fig.1,2) is compatible with the known very low concentration of the Na^+/D-glucose cotransporter(s) in these membranes (about 12–14 pmol.mg^{-1} protein; i.e., 0.1–0.4% [9]).

It seems unlikely that the 72 kDa band should arise by limited proteolysis from a higher M_r band. In fact, exactly the same size was obtained on SDS–PAGE when protease inhibitors were present in the digitonin solubilizates (fig.1,2).

We cannot state at present whether the 72 kDa protein isolated by immunoaffinity chromatography and SDS–PAGE represents the intact, whole Na^+/D-glucose cotransporter, or only a subunit therefrom. Previous studies on target size analysis [16] and PAGE under non-reducing conditions [17] of *renal* Na^+/D-glucose cotransporter(s) have reported app. M_r-values of 110 and 160 kDa, respectively. However, comparison between the possible molecular mass and subunit composition of the cotransporters of the small-intestinal and renal brush border membranes is made difficult by the difference in species and by the possibility that the two brush border membranes may be endowed with two distinct Na^+/D-glucose

cotransporters [18–24]. It is possible – at least in procaryotes – that the Na^+-dependent transport systems may be heterodimers, the subunit interacting with Na^+ being identical in all of them; mutants of *B. alcaliphilus* [25] and of *Escherichia coli* [26] have been described in which all (but there are only a few) such Na^+-dependent transport systems are simultaneously affected.

Regardless of the entire subunit composition of the native small-intestinal Na^+/D-glucose cotransporter(s), the data presented above unequivocally show that a polypeptide of 72 kDa represents all or part of the rabbit intestinal brush border Na^+/D-glucose cotransporter. The 72 kDa polypeptide isolated here is at least partially functional, since it still interacts with D-glucose and with phlorizin, as documented by it being eluted by these ligands. Work is presently in progress aiming at characterizing the 72 kDa polypeptide.

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