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Stimulation of Intestinal Na^+/D-Glucose Cotransport by Monoclonal Antibodies

Konrad Honold,† Barbara Ludeke,†,* Hans Hengartner,‡ and Giorgio Semenza†
†Laboratory for Biochemistry of the ETH, ETH-Zentrum, CH-8092 Zürich, Switzerland, and ‡Institute for Pathology, Department of Experimental Pathology, University Hospital of Zürich, CH-8091 Zürich, Switzerland

Summary. The small intestinal brush border membrane is endowed with a number of transport systems. Monoclonal antibodies were produced against integral membrane proteins and tested for their ability to bind to such membranes. For this purpose papain-digested, deoxycholate-extracted BBMVs from rabbit small intestine were used to immunize mice. Of the 765 hybridoma supernatants tested, 119 gave a significantly higher extent of binding to the crude antigen preparation as compared with the background. The monoclonal antibodies were also tested for their ability to influence the sodium-dependent uptake of solutes into intact BBMVs. Two monoclonal antibodies clearly showed stimulation of secondary active D-glucose transport, whereas sodium-dependent uptake of L-alanine and L-proline was not affected. Hydrophobically labeled, i.e. intrinsic, membrane proteins of 175, 78 and 65 kilodaltons could be immunoprecipitated by both monoclonal antibodies, the 78 kDa band corresponding in all likelihood to the Na^+/glucose cotransporter.

Key Words brush border membranes · sodium-dependent cotransport · monoclonal antibodies · immunoprecipitation

Introduction

Our knowledge on the structure-function relationship of the Na^+/glucose cotransporter(s) of the brush border membranes is still fragmentary (for a review till 1984, *see* Semenza et al.). The molecular size of the small intestinal cotransporter, or one of its subunits, was demonstrated to be in the 70–80 kDa range by partial negative purification (Klip, Grinstein & Semenza, 1979*a,b*), selective labeling (Hosang et al., 1981; Peerce & Wright, 1984*a,b*), isolation by immunochromatography using inhibiting monoclonal antibodies (mABs)[1] (Schmidt et al., 1983), and very recently by cDNA cloning and sequencing (Hediger et al., 1987). Other molecular sizes have also been reported, i.e. 160–165 kDa (Malathi, Preiser & Crane, 1980; Malathi & Preiser, 1983). Perhaps these larger molecular weights indicate that the cotransporter may occur as oligomer.

The mABs mentioned above were unfortunately lost. We have thus carried out new lymphocyte fusions which have now yielded two mABs which activate Na^+-dependent D-glucose transport. This activation strongly supports the existence of a conformational change as an essential step in the mode of operation of the cotransporter, for which we have earlier produced functional evidence (Toggenburger et al., 1978; Kessler & Semenza, 1983) and for which others have produced evidence based on changes in the fluorescence of a label (Peerce & Wright, 1984*a,b*). For the renal cotransporter Lever's group (Wu & Lever, 1987*b*), have provided evidence for a conformational change on the basis of experiments using mABs. In the following we describe the preparation of our new mABs which specifically activate Na^+-dependent D-glucose transport in small-intestinal BBMVs.

* *Present address:* Laboratory of Neuropathology, University of Zürich, CH-8091 Zürich, Switzerland.

[1] *Abbreviations:* mAB, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DOC, deoxycholate; BBM(V), brush-border membrane (vesicle); EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid; EDTA, ethylenediamine-tetraacetic acid; Tris-HCl, tris-(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kDa, kilodalton; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TID, 3-trifluoromethyl-3-(*m*-iodophenyl)diazirine.

Materials and Methods

Materials

Goat anti-mouse IgM + IgG (heavy and light chain) were from Tago Inc., Burlingame, CA. Goat anti-mouse IgM(F_c) and goat anti-mouse IgG(F_c) horseradish peroxidase conjugates were purchased from Biogenzia Lemania SA, Lausanne, CH. Goat anti-mouse IgM + IgG (heavy and light chain) alkaline phosphatase conjugate was from Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.; mAB MOPC 104E from Litton Bionetics, Kensington, MD.

Preparation of BBMVs

Vesicles were prepared from frozen rabbit small intestine by the method of Schmitz et al. (1973) as modified by Kessler et al. (1978*a*). However, magnesium chloride was used in the precipitation step (Booth & Kenny, 1974) and EGTA included during thawing and homogenization as described by Hauser et al. (1980). Finally, membranes were homogenized in 150 mM KCl, 50 mM Tris-HCl, pH 7.4, and diluted to 4.5 mg/ml. To obtain a higher yield of intact, sealed vesicles a chromatography step on a Sepharose 4B-column followed (Carlsen, Christiansen & Bro, 1983). The membranes were stored in liquid nitrogen.

Partial Purification of BBMs

BBMVs were subjected to controlled papain digestion followed by extraction with DOC as described by Klip et al. (1979*a*).

Immunization

Female, six-week-old BALB/c mice were immunized intraperitoneally with partially purified BBMs exhibiting specific phlorizin binding activities of 16 to 24 pmol/mg protein.

For fusion A, mice received 1.1 mg membrane protein in 200 μl BSS emulsified in an equal volume of Freund's complete (day 0) or incomplete adjuvant (day 23). The final booster, given on day 44, contained 0.5 mg protein in 300 μl Hank's balanced salt solution.

For fusion B, mice received 200 μg membrane protein in 170 μl balanced salt solution emulsified in 170 μl Freund's complete (day 0) or incomplete adjuvant (days 14, 26, 45).

The sera of the mice were tested for antibodies binding to the antigen preparations by ELISA. Mice with the highest serum titers were boosted on day 49 with antigen preparations emulsified in Freund's incomplete adjuvant, and served as spleen donors four days later.

Detection of mAB Binding to BBMVs

Mouse sera and hybridoma supernatants were tested for the presence of antibodies against partially purified BBMVs in a solid-phase ELISA. Microtiter plates were precoated with 50 μg/ml poly L-lysine (average molecular weight: 100,000) in H_2O. After washing with water, 100 μl containing 5 or 12.5 μg membrane protein in 100 mM sodium carbonate, pH 9.6 were added to each well. For this and the following steps PBS was used for washing. After blocking with 0.5% (wt/vol) BSA in the same buffer, plates were incubated with culture supernatants or serum samples diluted (if necessary) in PBS containing 0.1% (wt/vol) BSA. The wells were then washed and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin, directed against the heavy and light chains of IgM and IgG, diluted in 0.1% BSA in PBS was added. After washing with PBS containing 0.05% Tween 20, *p*-nitrophenyl phosphate was added at 1 mg/ml in 50 mM sodium carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$. *p*-nitrophenol was measured at 405 nm on an ELISA reader (Virion, Rüschlikon, CH).

Hybridoma Preparation

Lymphocytes derived from spleens were fused with cells from myeloma lines F0 (De St. Groth & Scheidegger, 1980) or X63-Ag8.653 (Kearney et al., 1979). Fusions were carried out at a spleen cell/myeloma cell ratio of 1 or 2 using polyethylene glycol 4000 as fusing agent as described (Galfré et al., 1977; Galfré & Milstein, 1981). Cells were plated in microtiter wells containing a feeder layer of 10^4 X-irradiated (2000 rad, 5 min) peritoneal macrophages. Half-replacement with selective HAT-medium was performed 24 hr after fusion. Aminopterin was omitted from the medium after two weeks. The cells were then slowly weaned from hypoxanthine and thymidine.

Test for Antibody Class and Determination of Antibody Concentration by ELISA

Microtiter plates were coated with 3 μg goat anti-mouse immunoglobulin (H- and L-chain) antibodies per ml corresponding to 0.21 μg anti-IgG and 0.089 μg anti-IgM per well. After washing with PBS containing 0.05% Tween 20, the samples were added neat or diluted in PBS containing 0.05% Tween 20 and 0.1% (wt/vol) BSA or ovalbumin. Commercially available immunoaffinity purified mABs MOPC 104 E (IgM λ1) and UPC 10 (IgGκ) were used as standards for quantitation. BSA or ovalbumin served as blanks. Class specific goat anti-mouse Fc antibodies coupled to horseradish peroxidase were used as second antibodies. 0.1 mg/ml ABTS [2,2'-azino-di(3-ethyl-benzthiazoline)sulfonate] and 1.2% H_2O_2 in 100 mM sodium phosphate buffer, pH 4.0 were added to start the enzyme reaction, which was terminated by adding 50 μl 50 mM sodium fluoride in 0.5 mM EDTA and 5 mM NaOH prior to reading absorbance at 405 nm. The method used for quantitation is based on the observation that the concentration of a sample shifts the symmetrical sigmoidal curve of the response plotted as a function of the logarithm of concentration along the x-axes without altering its shape. Calculations were performed using a logit-log transformation (Ritchie, Nickerson & Fuller, 1983) to linearize the data.

Purification and Concentration of mABs from Culture Supernatant

For large scale purification of mAB, a 50% saturated ammonium sulfate precipitation of hybridoma supernatants was performed before further handling. Culture supernatants containing antibodies of the IgM-class were concentrated in Amicon filtration cells using XM 300 filters with a cutoff of 300 kDa. The retentate was washed four times with 80 mM KCl, 10 mM phosphate, pH 7.4, to remove low molecular weight components. For maximal

possible antibody concentration the volume was further reduced using Centricon 30 Microconcentrators (cutoff, 30 kDa; Amicon).

Screening Method to Identify mABs that Influence Solute Uptake into BBMVs

115 μg Sepharose 4B-purified BBMVs were added to the partially purified antibody in buffer T (183 mM Mannitol, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.02% potassium azide, 80 mM KCl, 10 mM phosphate, pH 7.4) and incubated for 2 hr at room temperature. The antibody incubation was terminated by collecting the vesicles at 30,000 × g for 30 min at 4°C. After resuspension with buffer T, the vesicles were kept on ice.

Transport experiments were performed according to Kessler, Tannenbaum & Tannenbaum (1978*b*). 10 μl of membrane suspension (10 μg protein) were incubated with 5 μl of buffer T containing 300 mM NaSCN and 500 μM ^{3}H-labeled substrate (3 μCi D-glucose/point, 4.5 μCi L-alanine/point or 7 μCi L-proline/point). Uptake was measured at the times at which the maximal overshoot occurs. Under our conditions, this is the case after 15 sec for sugar transport, and for the amino acids after 1 min. At the chosen incubation times, 2.5 ml ice-cold stop solution (250 mM KCl in 1 mM Tris-HCl, pH 7.0) was injected into the test tubes. Membranes were collected on nitrocellulose filters prewetted in aqueous poly L-lysine solution (10 mg/ml, MW 109,000; Franzusoff & Cirillo, 1983) and rinsed once with 5 ml stop solution. The filters were dissolved in toluene/Triton X-100/acetic acid 20:10:1 (vol/vol/vol) containing 3.5 g/liter Permablend III and counted in a Beckman Liquid Scintillation Counter.

Hydrophobic Labeling of DOC-Extracted BBMV

[^{125}I]TID was prepared as described by Brunner and Semenza (1981). BBMVs were extracted with DOC according to Klip et al. (1979*a*). Membranes were resuspended to 1.5 mg/ml in 250 mM KCl, 10 mM HEPES-Tris, pH 7.4, and flushed with a gentle stream of N_2 for 30 min at 0°C. 450 μCi/ml [^{125}I]TID was added to the vesicle suspension and equilibrated for 30 min at 4°C. The sample was photolyzed according to Spiess, Brunner and Semenza (1981). Labeled vesicles were washed four times with 183 mM mannitol, 1 mM $MgCl_2$, 80 mM KCl, 10 mM phosphate, pH 7.4 containing 1% ovalbumin and once with ovalbumin-free buffer.

Immunoprecipitation of Solubilized BBM-Proteins

1.5 mg DOC-extracted, [^{125}I]TID-labeled BBMV were incubated with mABs partially purified and concentrated from 40 ml culture supernatant for 2 hr at room temperature as described for the AB-incubation preceeding the transport assay. Unbound material was removed and the vesicles were washed twice in buffer S (80 mM KCl, 10 mM phosphate, 183 mM mannitol, 1 mM $MgCl_2$, pH 7.4). Membranes were solubilized with Triton X-100 (1%, detergent/protein = 4:1 (wt/wt), 1 hr at room temperature). After a 100,000 × g centrifugation for 1 hr, an appropriate amount of goat anti-mouse antibodies coupled to Sepharose 4B (according to the manufacturer's instructions) was added to the supernatant and incubated for 12 hr at room temperature in the presence of 1% ovalbumin. Sepharose-bound material was sedimented in an Eppendorf centrifuge and washed twice in solution S containing 1% Triton, twice in a similar solution containing 500 mM KCl, once in solution S containing 1% Triton and finally, twice in 100 mM LiCl, 183 mM mannitol, 1 mM $MgCl_2$, 1% Triton. The last pellet was dissolved in sample buffer for PAGE (containing 2% SDS, 5% glycerol, 5.8 mM Tris-HCl, pH 6.8, 10 mM EDTA and 0.002% bromphenol blue). After boiling for 2 min, insoluble material was removed by centrifugation and the supernatant was analyzed on a 10% slab gel (Laemmli, 1970) under nonreducing conditions. Dried gels were exposed to Kodak SO-282 films at −80°C.

Protein Determination

Protein was determined according to Lowry et al. (1951).

Results

Antigen Preparation

As starting material for the antigen preparation we used BBMVs from rabbit small intestine prepared by a Mg^{2+}-precipitation method, as described in Materials and Methods. Controlled papain digestion followed by deoxycholate extraction (Klip et al., 1979*a*) allowed a partial negative purification of glucose transporter. This procedure resulted in a significantly altered band pattern in silver-stained SDS-polyacrylamide gels. For example, two of the major bands, i.e., those of the sucrase-isomaltase complex, a stalked ectoenzyme of this membrane, were removed to a very large extent (*data not shown*). To quantitate the enrichment of Na^+-dependent glucose transporter we measured the binding of phlorizin, a competitive inhibitor of active sugar uptake. Binding was measured after equilibrating membranes in 100 mM NaCl or 100 mM KCl, 300 mM mannitol and 10 mM HEPES-Tris, pH 7.0. Specific phlorizin binding to protein-depleted membranes, expressed as difference between Na^+- and K^+-dependent phlorizin binding, was in the range of 17–23 pmol/mg protein, i.e., two- to threefold higher than to untreated membranes.

Binding of mABs to Partially Purified BBMS

In a first screening, hybridoma supernatants were assayed for binding to the antigen preparations in a solid-phase ELISA. For this purpose papain-digested, deoxycholate-extracted BBMVs were immobilized on microtiter plates. After incubation with culture supernatants, bound mABs were visu-

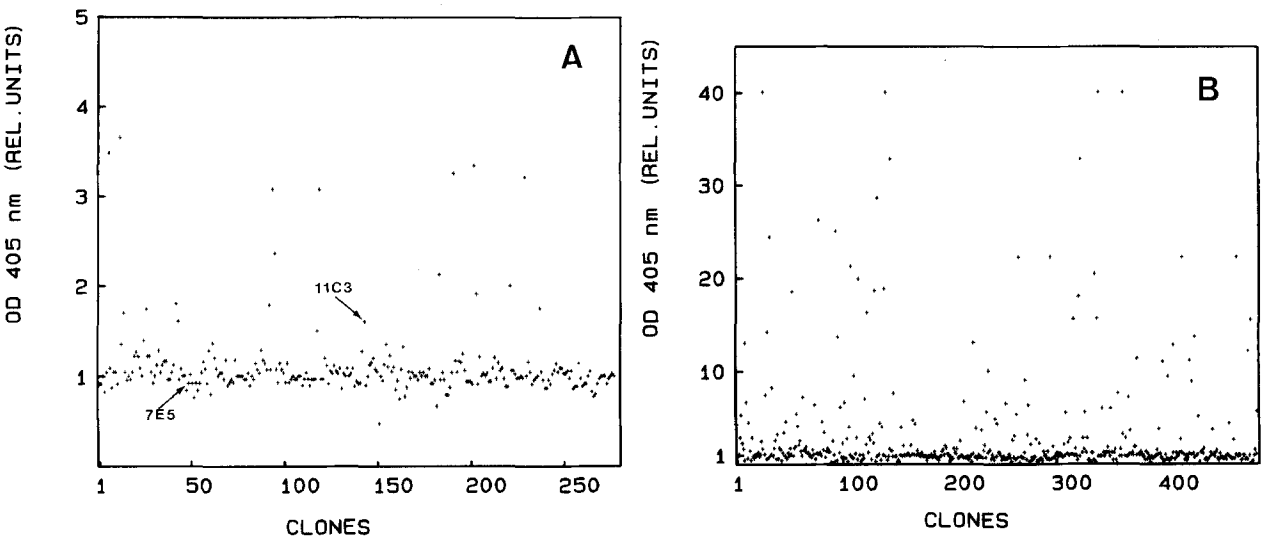

Fig. 1. Binding of mABs to the antigen preparation in solid-phase ELISA. Microtiter wells were coated with 12.5 μg (panel *A*) or 5 μg (panel *B*) membrane protein per well. Supernatants were collected from wells containing hybrids at 10% confluence or more. The binding of the mAB was measured as described in Materials and Methods. The Y axes show the response relative to the mean response of those supernatants, which gave a response similar to that of myeloma supernatants. The clones tested in the two panels originated from two different fusions

alized using alkaline phosphatase conjugated goat anti-mouse antibodies. A large fraction of the hybridoma supernatants gave a response similar to that of supernatant obtained from nonproducing myeloma cells. The mean response of this group of hybridomas was determined graphically and defined as background. Figure 1 shows the binding capacity of the mABs normalized to background. Panel *A* shows the binding of mABs obtained from fusion A. Of 277 culture supernatants tested, 28 exhibited binding activity clearly above background (i.e., >1.3 × background). 88 hybridomas produced IgM but only 17 produced IgG. Panel *B* shows the results for hybridomas generated in fusion B. Of 488 culture supernatants tested, 91 showed significant binding to the papain-digested, DOC-extracted membranes (>3 × background). Again, the number of IgG producers was low: only 29 hybridomas produced immunoglobulins of this class whereas 176 produced IgM. (Note that the scales are different in panels *A* and *B*, as different amounts of membrane protein were used for coating the microtiter plates, resulting in different background levels.)

Transport Experiments

For further characterization of mABs we expanded all hybridoma cells producing antibodies showing binding to BBMs in solid-phase ELISA, all IgG producers, and approximately 10% of those exhibiting no detectable binding to BBMs. We attempted to test as many mABs as possible in transport experiments, as one would expect only a small number of the antibodies binding to BBMs in the first screening to have an effect on the Na^+-dependent transport systems tested. Furthermore, some of the mABs may have failed to bind to membranes in the ELISA test, as epitopes might have been destroyed during the manipulations (e.g. the alkaline treatment, incubation in detergent) necessary for this assay.

Uptake of D-glucose, L-alanine and L-proline was measured as described in Materials and Methods. Partial purification of mABs was required, since crude hybridoma supernatants impaired the effective adsorption of BBMV to the nitrocellulose filters. The mAB/phlorizin binding sites ratio in the transport assays was adjusted to one or higher.

Purified, concentrated mixtures of up to eight mABs were preincubated with BBMVs before transport assays. The results obtained with five antibody mixtures are shown in Fig. 2. Taking the mean uptake value of the other four incubation mixtures in the assay as reference (100%), mixture 3 caused a 44% increase in the rate of sodium-dependent glucose uptake. Stimulation was specific for sugar transport as sodium-dependent uptake of alanine and proline was not influenced. The stimulation by mixture 3 was concentration dependent (Fig. 3), a 1000-fold dilution produced no detectable activation. This stimulation of D-glucose uptake occurred via the Na^+-dependent D-glucose cotransporter, as shown by the fact that 1 mM phlorizin, or substitution of K^+ for Na^+ depressed D-glucose up-

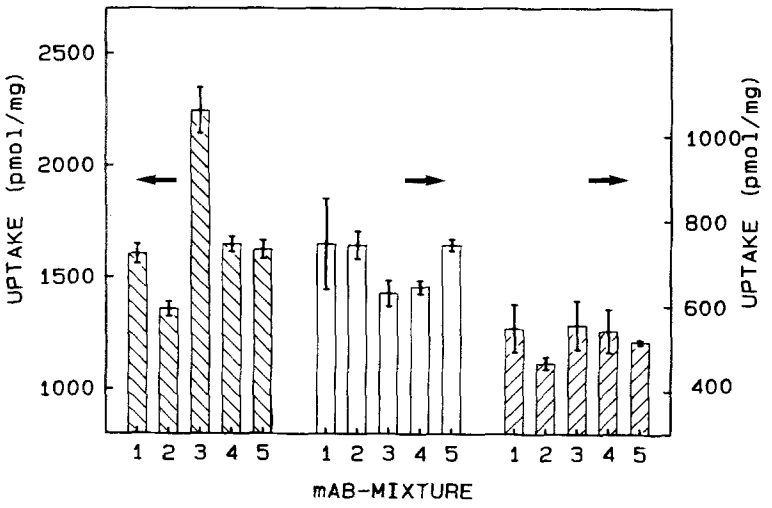

Fig. 2. The influence of mAB on sodium-dependent uptake of D-glucose (▨), L-alanine (□) and L-proline (▨) into BBMV. Vesicles were preincubated with mixtures of partially purified and concentrated (100-fold) antibodies. After removing the unbound material, solute uptake was measured with, initially, 100 mM NaSCN and 166 μM tritium-labeled solute at the outside of the vesicles, by using a rapid filtration technique. Glucose transport was terminated after 15 sec, amino acids after 60 sec. Each point reflects the mean of three measurements (±SD). Nonspecific binding to the poly L-lysine-coated filters was subtracted

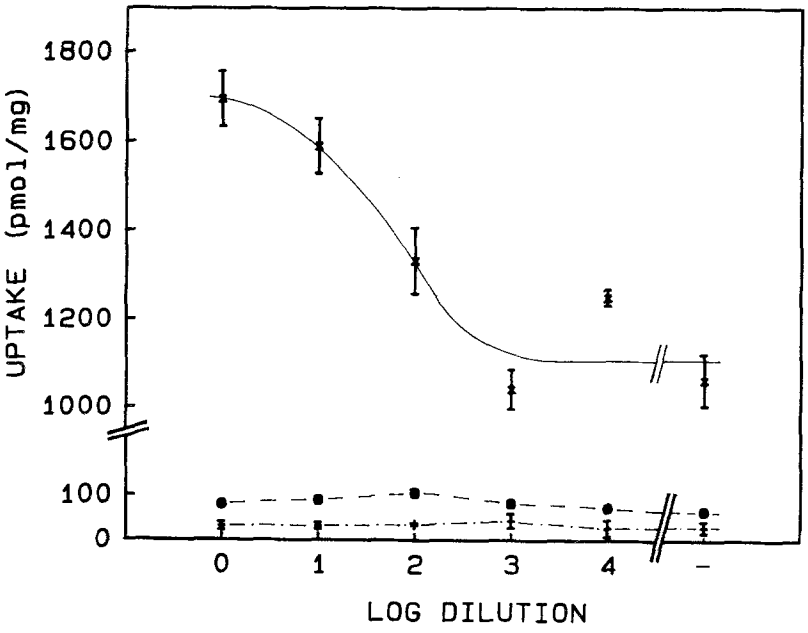

Fig. 3. Concentration dependence of the antibody-induced increase in sodium-dependent glucose transport. Vesicles were preincubated with different concentrations of antibody mixture 3 (*cf.* Fig. 2). The undiluted sample contained 140 μg IgM. Sugar uptake was measured under initially the same conditions as described for Fig. 2 (—), in the presence of 1 mM phlorizin (---) and in the presence of K^+ instead of Na^+ (·····). (–) reflects uptake in the absence of antibodies

take to the basal level, which was not increased by mixture 3.

The clones whose products comprised antibody mixture 3 were analyzed separately for the production of immunoglobulins capable of stimulating Na^+/glucose cotransport. Supernatants of two clones, 7E5 and 11C3, were shown to stimulate sugar transport relative to the others, which were treated similarly (*not shown*). Partially purified and concentrated 7E5 and 11C3 mABs were diluted in serial tenfold steps and preincubated with BBMVs. Na^+/glucose cotransport in these vesicles was measured as described for the initial screening. Also, the amount of immunoglobulin bound to these vesicles was measured in an ELISA. Both antibody binding and stimulation of transport were concentration dependent. However, at high antibody concentrations a decrease in the effect on transport was observed (Fig. 4). Thousandfold and higher dilutions of the concentrated antibody solutions of both 7E5 and 11C3 led to transport rates similar to those measured in the absence of antibodies. This result most likely indicates that maximal stimulation is achieved by multivalent binding of the antibodies and that the decrease at high mAB concentrations is probably due to monovalent binding.

Assuming 10 pmol phlorizin binding sites/mg membrane protein (Toggenburger et al., 1978), a rough estimation suggests that at the mAB concentration leading to maximal stimulation of transport,

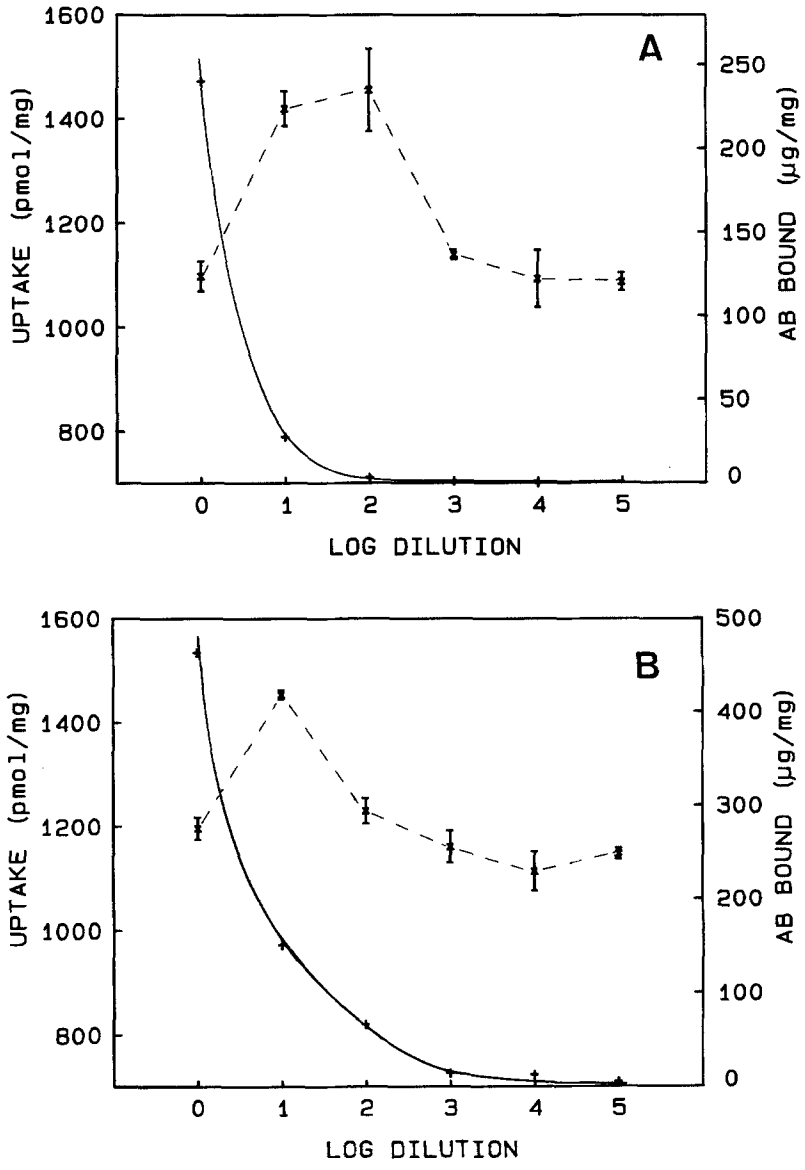

Fig. 4. Effect of 7E5 (*A*) and 11C3 (*B*) on glucose transport (---) and binding to BBMV (—). Vesicles were preincubated with mAB at different dilutions (7E5: 152 µg undiluted, 11C3: 506 µg undiluted). Sugar transport was measured as described for Fig. 2. The amount of bound antibody was measured by incubating aliquots of the AB-preincubated vesicles for 24 hr at 4°C in wells of microtiter plates which were coated with goat anti-mouse antibodies. This incubation was performed in the presence of 0.05% Tween 20. Bound antibodies were quantified as described in Materials and Methods

the molar ratio of antibody/phlorizin binding sites is about 15 : 1 in the case of 11C3, and 3 : 1 to 0.4 : 1 for 7E5 (*see* Fig. 4).

We compared the effect of these antibodies on the cotransporter to the effect of $\Delta\tilde{\mu}_{Na^+}$. Increasing the initial NaSCN (out) concentration from 100 mM (as used throughout for the transport experiments shown here) to 200 mM did not significantly increase D-glucose uptake. Values measured after 15 sec were 1104 (±88) and 1183 (±27) pmol/mg, respectively (n = 3).

7E5 and 11C3 Recognize Different Epitopes

The mABs 11C3 bind to papain-digested and deoxycholate-extracted BBMs immobilized on microtiter plates, whereas binding by 7E5 antibodies were not detected (Fig. 1). There are two possible explanations why 7E5 showed no binding in this initial screening: (1) The mAB concentration in the culture supernatant may have been much lower than in supernatants used for the purification of antibodies for the functional screening. (2) Especially in the case of protein-depleted BBMVs, the epitope to which 7E5 binds may be very sensitive to the manipulations necessary for the solid-phase ELISA assay.

As Fig. 4*B* shows that the epitope to which 7E5 binds must be intact in native BBMVs (as this mAB showed stimulation of Na^+-dependent glucose transport and binding to the vesicles), an ELISA assay was performed using microtiter plates coated with native BBMs such as used for transport experiments. 11C3 and 7E5 antibodies were purified and

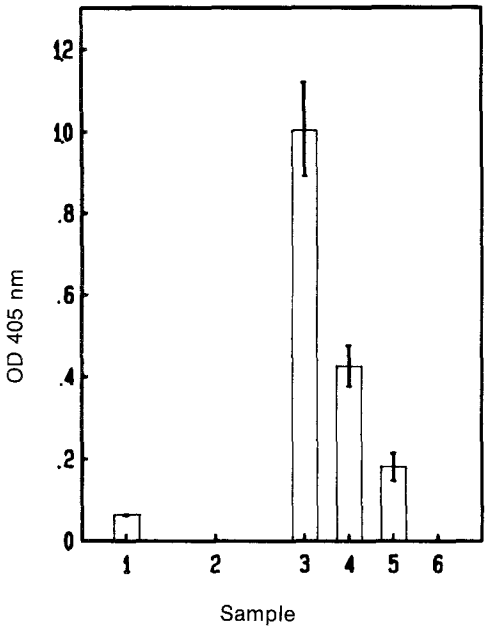

Fig. 5. Binding of 7E5 and 11C3 to BBMs immobilized on microtiter plates. Microtiter plates were coated with native BBMs (5 μg membrane protein/well). The following amounts of antibodies were tested: No. 1: 1 μg 104E (mouse myeloma protein, control), No. 2: 3.5 μg 7E5, No. 3: 11.3 μg 11C3 in the first well followed by serial 10-fold dilutions (numbers 4 through 6). Bound antibodies were visualized using alkaline phosphatase conjugated anti-mouse antibodies

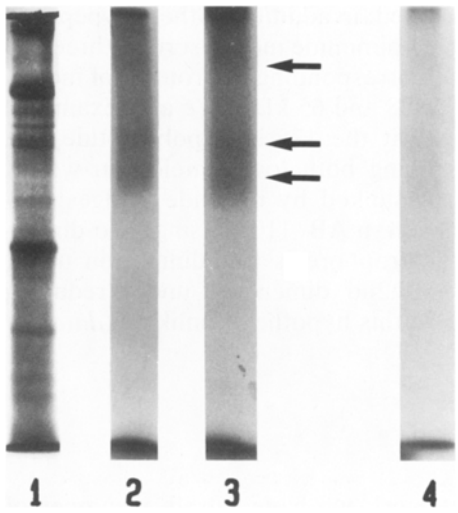

Fig. 6. Immunoprecipitation of Triton X-100 solubilized BBM proteins. DOC-extracted, [^{125}I]TID-labeled membranes (lane 1, Coomassie blue staining) were incubated for 2 hr with concentrated 11C3 and 7E5 culture supernatant (690 and 330 μg immunoglobulin, respectively). Culture supernatant from a clone which produces only light chains served as control. The vesicles were washed once and solubilized with 1% Triton X-100. The 100,000 × *g* supernatant was immunoprecipitated by goat anti-mouse antibodies coupled to Sepharose 4B. After extensive washing, Sepharose 4B-bound material was removed by adding SDS-PAGE sample buffer and boiling for 2 min. After electrophoresis in 10% acrylamide gels under nonreducing conditions, the dried gels were exposed to Kodak SO-282 film for 9 days (lane 2: 7E5, lane 3: 11C3) or 19 days (lane 4: control). Bands corresponding to molecular weights of 175, 78 and 65 kDa are indicated by arrows (top to bottom). (These bands are more evident in the original fluorogram and are present in the Western blot also.)

concentrated before use. The result of the solid-phase ELISA is shown in Fig 5. 11C3 antibodies showed concentration-dependent binding, whereas for 7E5 no binding could be shown even at the highest immunoglobulin concentration tested under these conditions (antibody/phlorizin binding sites ≈70). This result is in agreement with the initial screening, and demonstrates that the epitope for 7E5 but not for 11C3 is destroyed in ELISA assays as performed here. Thus the epitopes to which 11C3 and 7E5 bind are different.

This conclusion also agrees with the different patterns of immunoblots produced by mAB 11C3 and 7E5 (*not shown*). Whereas the former recognizes three polypeptides in addition to those recognized by nonimmune sera (apparent molecular weights 175, 78 and 65 kDa), the latter does not (which probably indicates again that the epitopes of mAB 7E5 are labile and do not survive the manipulations of this technique either).

Immunoprecipitation of Solubilized BBM Proteins by 7E5 and 11C3

Membrane transporters are expected to be transmembrane proteins. This was shown to be the case for the small intestinal Na^+/glucose cotransporter by Klip et al. (1979*c*), and agrees with the numerous hydrophobic stretches found in the amino acid sequence (Hediger et al., 1987). With the goal of labeling the intrinsic membrane proteins without modifying their extramembranous domains (which are presumably involved in the formation of antigen-antibody complexes), we used the hydrophobic photoreagent [^{125}I]TID (Brunner & Semenza, 1981) to label DOC-extracted BBM. After incubation with partially purified mABs, they were washed and solubilized with Triton X-100. Antigen-antibody complexes were precipitated by goat anti-mouse IgM antibodies coupled to Sepharose 4B and washed extensively. Immunoprecipitates were analyzed on SDS-PAGE followed by autoradiography (Fig. 6). Both 7E5 and 11C3 precipitated radioactively labeled BBM proteins of approximately 175, 78 and 65 kDa (lanes 2 and 3, respectively), whereas the control shows only very little staining in these regions (lane 4).

The results obtained by immunoprecipitation were confirmed by immunoblotting. As noted

above, 11C3 stained, in addition to the polypeptides also labeled by nonimmune mouse serum, three further bands also corresponding to proteins of molecular weight 175, 78 and 65 kDa. We also examined the possibility that the 175 kDa polypeptide is a multimer containing both lower molecular-weight components crosslinked by disulfide bridges: Immunoblotting with mAB 11C3 using two-dimensional SDS-gelectrophoresis (1st dimension under nonreducing and 2nd dimension under reducing conditions) made this hypothesis unlikely (*data not shown*).

Discussion

In the present work we have raised a number of mABs against papain-digested, deoxycholate-extracted small intestinal BBMs. In these negatively purified membranes the sodium/glucose cotransporter is enriched approximately twofold. We found two mABs that stimulated sodium-dependent glucose uptake into intact vesicles.

The mABs developed by us did not stimulate or inhibit other membrane transport systems tested, i.e. sodium-dependent L-alanine and L-proline uptake. Thus their action must be fairly specific: for example, it can be ruled out that this activation is brought about by these antibodies via a hypothetical stabilization of the initial $\Delta\tilde{\mu}_{Na^+}$. The complex patterns in immunoprecipitation and in Western blots (*not shown*) does not allow us to rule out the possibility that more than one polypeptide is involved in the stimulatory effect caused by 7E5 and 11C3 antibodies. Yet the simplest hypothesis is that these antibodies stimulate the Na^+/glucose cotransporter by interacting directly with it.

Previous work from our laboratory has indicated a likely mode of operation of the small intestinal Na^+/D-glucose cotransporter (Kessler & Semenza, 1983): in the absence of a $\Delta\tilde{\mu}_{Na^+}$ across the membrane, the sodium and the D-glucose binding sites would be more available from the cytosolic than from the luminal side; a $\Delta\Psi$ (negative inside) would induce a conformational change in the cotransporter making these substrate binding sites more easily available to Na^+ and to D-glucose or phlorizin from the luminal side. A conformational change in the Na^+/glucose cotransporter was also suggested by Peerce and Wright (1987) based on the effect of sodium on the fluorescence of a fluorophor bound to the cotransporter.

The activating effect of mABs 7E5 and 11C3 strongly supports the notion that the operation of this cotransporter involves a conformational change. More specifically, we propose that these mABs stabilize the more active (or "better accessible") form of the cotransporter. If this is the case, then the rate-limiting step in the cyclic operation of the intestinal Na^+/glucose cotransporter should be located (in the absence of mAB) at some point in the re-exposure of the substrate binding sites onto the luminal surface. Or, to express it in a slightly different way, mABs 7E5 and 11C3 stabilize a conformational form in the Na^+/glucose cotransporter in which the slowest step in D-glucose out-in transport is accelerated without inhibiting the translocation of the Na^+/glucose-loaded carrier.

At the present stage of our knowledge it would be futile to try to localize more precisely the individual step(s) which is (are) accelerated by these mABs. It appears as if these antibodies render the cotransporter more responsive to $\Delta\tilde{\mu}_{Na^+}$ and/or affect it over and above $\Delta\tilde{\mu}_{Na^+}$ itself: In fact, in the presence of a NaSCN gradient, 100 mM (out) to zero (in), the addition of these mABs accelerates Na^+-dependent D-glucose uptake by some 40%. In contrast, increasing the initial NaSCN gradient to 200 mM (out) to zero (in) does not lead to increased uptake.

Whatever the exact mechanism of the mAB-triggered stimulation of the Na^+/glucose cotransporter, the epitopes responsible for this effect must be located at the outer, luminal side of the membrane, because the membrane vesicles are sealed and right-side out (Tannenbaum et al., 1977). The binding of 7E5 and 11C3 to the membranes is concentration dependent. In contrast, the stimulation of the sugar transport is abolished at the highest concentrations of both clones. Multivalent binding of antibody may be necessary to stimulate uptake, and in the presence of excess antibody competition between monovalent and multivalent binding seems to occur. Therefore, the stimulation of Na^+-dependent glucose uptake into vesicles might be established by the stabilization of hypothetical homo- or heteromultimers.

Inhibition of transport systems has been previously demonstrated by poly- or monoclonal antibodies (e.g. this Na^+/glucose cotransporter: Schmidt et al., 1983; lactose permease of *Escherichia coli:* Carrasco et al., 1984; the glucose transporter of human erythrocytes: Boyle et al., 1985; the iodothyronine transporter of rat liver cells; Mol et al., 1986). On the other hand it is known that antibodies are able to enhance, e.g., the affinity of other antibodies (Chamat et al., 1986; Sawutz, Sylvestre & Homey, 1987). Also, binding of Cytochalasin B to the glucose transporter of human erythrocytes is activated upon preincubation of membranes with a mAB raised against the purified carrier (Allard & Lienhard, 1985). A transport sys-

tem known to be gated by monoclonal antibodies is described by Pecht et al. (1987): Using micropipette-supported bilayers formed from membranes of the human T-cell line REX, they demonstrated opening of ligand-gated ion channels selective for Ca^{2+}.

Lever's group (Wu & Lever, 1987*a*) have recently reported the production and characterization of mABs directed against the renal Na^+/glucose cotransporter, one of which stimulates phlorizin binding by approximately 25%. The properties of these mABs appear to be somewhat different from ours, because they are reported neither to stimulate nor to inhibit sodium-dependent D-glucose uptake by renal BBMV. However, their mAB significantly prevented transport inhibition by bound phlorizin. As judged by the changes in fluorescence of a fluorophoric affinity label covalently bound to the cotransporter, this antibody may also act by stabilizing an active conformation of the cotransporter (Wu & Lever, 1978*b*). How far the differences are related to the antibodies themselves and/or to the different Na^+/glucose cotransporters investigated cannot be said at the moment. It is known that the typical renal Na^+/glucose cotransporter (that of the outer cortex) has different kinetic properties than the carrier prevailing in the small intestine; in particular it has a much higher affinity for phlorizin (Turner & Moran, 1982*b*; reviewed by Semenza et al., 1984).

In immunoprecipitation both 7E5 and 11C3 were shown to form complexes with solubilized membrane components of 175, 78 and 65 kDa molecular weight (Fig. 6). These proteins were labeled by [^{125}I]TID, indicating that these components are intrinsic membrane proteins, as the Na^+-dependent D-glucose transporter is (Klip et al., 1979*c*; Hediger et al., 1987).

Antibody 7E5 did not react with brush border membranes immobilized on microtiter-plates, whereas 11C3 reacted significantly (*see* Figs. 1 and 5). Binding of 7E5 to membranes could only be demonstrated using intact BBMV (Fig. 4). The epitopes recognized by the two mABs must differ greatly in their stability. 7E5 might recognize an epitope comprised of secondary and/or tertiary protein structures which is very sensitive to the manipulations necessary during solid phase ELISA assay, whereas the 11C3 epitope is much more resistant. Immunoblotting also confirmed that the 7E5 epitope is very labile (*not shown*).

Numerous polypeptides have been suggested to be involved in sodium-dependent glucose transport. For the renal cotransporter, target size inactivation has led to molecular weights of 110 kDa for the phlorizin receptor (Turner & Kempner, 1983), and 240 and 345 kDa for the functional cotransporter (Lin et al., 1984). Purification/reconstitution experiments and affinity labeling have led to numerous polypeptides of molecular weights between approximately 50 and 100 kDa (e.g., Koepsell et al., 1983, 1986; Silverman & Speight, 1986; Neeb, Kunz & Koepsell, 1987). For the intestinal transport system, polypeptides mainly in the range of 70–75 kDa have been suggested to be (at least a part of) the Na^+/glucose cotransporter. By photoaffinity labeling (Hosang et al., 1981) and by immunoadsorption using specific, inhibiting mABs (Schmidt et al., 1983) we have produced evidence for this transporter (or a subunit thereof) having an apparent molecular weight of approximately 75 kDa. This molecular size also corresponds to that of the bands indicated by other criteria (negative purification, Klip et al., 1979, 1980; selective labeling, Peerce and Wright, 1984*a,b*). Hediger et al. (1987) recently reported expression cloning and sequencing of the intestinal Na^+/glucose cotransporter cDNA, leading to a single peptide of 662 amino acids, of a calculated molecular weight of 73,080. In immunoprecipitation, two of our mABs also reacted with a band of approximately 78 kDa,[2] which is consistent with the results indicated above, and also with the idea that these mABs activate Na^+-dependent D-glucose uptake by acting directly on the cotransporter itself. Of course, it is quite possible that the larger and the lower molecular weight components co-precipitated by our mABs are the result of crossreactivity with unrelated proteins or nonspecific co-precipitation (although the latter was certainly minimized by repeated extended washings with detergent and high ionic strength solutions). It is also possible that the 65 kDa band may have arisen from the 78 kDa band by limited proteolysis.

Recently Wu and Lever (1987*c*) have isolated (a part of) the renal Na^+/D-glucose cotransporter with an apparent molecular weight of 75 kDa. This cotransporter has the characteristics of the "renal" or "outer cortex" type (e.g., high-affinity phlorizin binding). The identical molecular size reported for the two kindred cotransporters (for the functional differences between the two, *see* Semenza et al., 1984) may indicate (i) identical proteins, the differences in function resulting from modulation by the microenvironment (e.g. membrane lipids), additional subunit(s) (perhaps the two additional bands precipitated with 11C3 and 7E5) or other factors; (ii) related but not identical proteins. Clearly, more information is needed to solve this problem.

[2] This band is rather broad, perhaps due to glycosylation.

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