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Monoclonal Antibodies That Bind the Renal Na^+/Glucose Symport System. 1. Identification†

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ABSTRACT: Phlorizin is a specific, high-affinity ligand that binds the active site of the Na^+/glucose symporter by a Na^+-dependent mechanism but is not itself transported across the membrane. We have isolated a panel of monoclonal antibodies that influence high-affinity, Na^+-dependent phlorizin binding to pig renal brush border membranes. Antibodies were derived after immunization of mice either with highly purified renal brush border membranes or with apical membranes purified from LLC-PK_1, a cell line of pig renal proximal tubule origin. Antibody 11A3D6, an IgG_{2b}, reproducibly stimulated Na^+-dependent phlorizin binding whereas antibody 18H10B12, an IgM, strongly inhibited specific binding. These effects were maximal after 30-min incubation and exhibited saturation at increased antibody concentrations. Antibodies did not affect Na^+-dependent sugar uptake in vesicles but significantly prevented transport inhibition by bound phlorizin. Antibodies recognized a 75-kDa antigen identified by Western blot analysis of brush border membranes, and a 75-kDa membrane protein could be immunoprecipitated by 18H10B12. These properties, taken together with results in the following paper [Wu, J.-S. R., & Lever, J. E. (1987) *Biochemistry* (following paper in this issue)], provide compelling evidence that the 75-kDa antigen recognized by these antibodies is a component of the renal Na^+/glucose symporter.

Transporters localized in apical membranes of renal proximal tubule and intestinal epithelial cells catalyze the coupled translocation of glucose and Na^+ in a symport (cotransport) mechanism (Crane, 1977). Renal and intestinal glucose symporters share many functional similarities including substrate specificity and inhibition by the nontranslocated, fully competitive ligand phlorizin. However, certain differences in functional properties have been noted, and there is no evidence that they are identical molecular species [reviewed in Semenza et al. (1984)]. Furthermore, renal and intestinal epithelia may each contain more than one type of Na^+/glucose symporter differing in Na^+/glucose stoichiometries and affinity for phlorizin (Turner & Moran, 1982; Kaunitz et al., 1982).

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The identification and isolation of the proteins that catalyze Na^+/glucose symport have proven difficult and inconclusive. A number of approaches have been attempted, including solubilization and reconstitution (Crane et al., 1976; Ferguson & Burton, 1977; Im et al., 1982; Koepsell et al., 1983; Malathi & Preiser, 1983; Kano-Kameyama & Hoshi, 1983), negative purification and selective thiol labeling (Klip et al., 1979), photoaffinity labeling with phlorizin derivatives (Gibbs et al., 1982; Hosang et al., 1981), and alkylation (Arita & Kawanami, 1980), but these studies did not permit unequivocal identification of the symporter. Schmidt et al. (1983) reported the preliminary characterization of a monoclonal antibody (MAb)[1] that recognized a 72-kDa polypeptide in intestinal brush border membranes, which could be eluted from an immunoaffinity column by Na^+ and glucose, but more direct evidence for the identification of this polypeptide as the glucose transporter was not obtained.

In this study, we describe a panel of monoclonal antibodies derived after immunization with renal membranes and identified by screening for effects on phlorizin binding. These MAb's recognize a 75-kDa polypeptide in renal brush border membranes. The specific effects, both stimulatory and inhibitory, of individual MAb's in this panel on high-affinity, Na^+-dependent phlorizin binding in renal brush border membranes strongly implicate this polypeptide as a component of the renal Na^+/glucose symporter. This interpretation is further strengthened by evidence that these antibodies specifically prevent Na^+-coupled glucose transport in vesicles from inhibition by phlorizin. In the following paper in this issue (Wu & Lever, 1987), we present evidence that these MAb's stabilize a conformation of the symporter that is induced by the cotransported substrates Na^+ and glucose.

Materials and Methods

Materials. [*phenyl*-3,3′,5,5′-^{3}H,*propyl*-3-^{3}H]Phlorizin, 50 Ci/mmol, and [^{14}C]methyl α-D-glucopyranoside, 275 mCi/mmol, were purchased from New England Nuclear. ^{125}I-Protein A was from ICN. Kidneys from domestic hogs were obtained through the generosity of Texas A&M University, College Station, TX. Rabbit anti-mouse (IgG + IgM + IgA) antibody and its peroxidase conjugate were from Zymed. Hybridoma screening kits based on β-galactosidase conjugates were from BRL or Zymed.

Cell Culture. The LLC-PK_1 pig kidney epithelial cell line (ATCC CL101) was grown as described previously (Lever, 1982). Hybridoma cell lines were grown in Iscove's modified Dulbecco's medium (Gibco) plus 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 50 μM β-mercaptoethanol, 100 units/mL penicillin, and 100 units/mL streptomycin (HAT medium). HT medium refers to HAT medium without aminopterin.

Generation of Hybridoma Cell Lines. BALB/c mice were immunized by intraperitoneal injection of 100 μg of either apical membranes from LLC-PK_1 cells or depleted brush border membranes from pig kidney cortex (as specified) emulsified with an equal volume of Freund's complete adjuvant. Booster immunizations of 50 μg of membrane protein were given at 4-week intervals for 2–3 months. At 3 weeks after the last injection, serum was titered by immunoassay and by assay of inhibition of [^{3}H]phlorizin binding. The highest titer mice were hyperimmunized by intravenous injections of 10 μg of membranes in 20 μL of phosphate-buffered saline (PBS) in the tail vein for 3 consecutive days, rested for 1–2 days, and then sacrificed and splenectomized. Splenocytes were fused with P3-X63-Ag8.653 myelomas in a ratio of 3:5 splenocytes to myeloma cells using PEG-1540 or 3400 as described by De St. Groth and Schiedegger (1980). After 14 days of maintenance in HAT medium, hybridomas were switched to HT medium and then screened for antibody secretion.

Hybridomas generated after immunization with apical membranes from LLC-PK_1 cells were screened by the protein A binding assay. Hybridomas generated after immunization with renal brush border membranes were screened by immunoassay using brush border membranes immobilized on nitrocellulose as antigen. Positive wells were expanded and then cloned by serial dilution. Positive monoclonal lines were retested by immunoassay and subtyped by using commercially available subtyping kits (Zymed, Dynatech, Boehringer-Manheim).

Ascites tumors were produced by injection of $\sim 10^7$ hybridoma cells in 0.5 mL of serum-free media into the peritoneal cavity of syngenic BALB/c mice primed 5–7 days earlier by injection with 0.5 mL of Pristane (Aldrich). The ascited fluids were harvested after 10–20 days, centrifuged, and frozen immediately at −85 °C.

Detection of MAb Binding to LLC-PK_1 Cells. LLC-PK_1 cells were plated in 96-well polystyrene microtiter plates (Falcon) at 10^4 cells per well and maintained 1–2 weeks at a confluent density with medium change twice per week before the screening assay. Monolayers were fixed with 0.5% glutaraldehyde in PBS-A (0.137 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 15 min. All steps were carried out at room temperature. Wells were washed 4 times with 2% nonfat dry milk in PBS-A, and this blocking solution was used in all subsequent wash steps and antibody incubations. Then 100 μL/well of hybridoma supernatant was added for 2 h. Wells were washed 4 times, followed by addition of 50 μL/well of rabbit anti-mouse (IgG + IgM + IgA) at 1:2000 dilution for 1 h. After 4 washes, 50 μL containing 7.5×10^4 cpm of ^{125}I-protein A was added for 1 h. Plates were washed 4 times and air-dried, and then wells were exposed to a sheet of Kodak X-Omat X-ray film clamped between the bottom of the microtest plate and a Cronex intensification screen in the dark. After 15 h at −80 °C, film was developed.

Nitrocellulose Filtration ELISA Assay To Detect MAb's That Bind Renal Brush Border Membranes. Brush border membranes (40 μg/well) were immobilized on nitrocellulose in a Bio-Dot 96-well filtration apparatus (Bio-Rad), and immunoassay was carried out by using sheep anti-mouse (IgG, IgM, IgA) conjugated to β-galactosidase (BRL hybridoma screening reagent) as second antibody according to the manufacturer's instructions.

Preparation of Renal Brush Border Membranes. Brush border membranes were prepared from frozen cortices from pig kidney as described by Malathi et al. (1979) except that $MgCl_2$ replaced $CaCl_2$ in the membrane precipitation step. The final membrane pellet was resuspended in either buffer B (0.25 M sucrose, 10 mM $MgCl_2$, 20 mM K^+-HEPES, pH 7.2) for the [^{3}H]phlorizin binding assay or in buffer I (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) for preparation of depleted membranes. The membrane preparation

[1] Abbreviations: MeGlc, methyl α-D-glucopyranoside; HMBA, *N*,*N*′-hexamethylenebis(acetamide); MAb, monoclonal antibody; kDa, kilodalton(s); ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; FITC, fluorescein isothiocyanate.

exhibited a 5–6-fold increase in the specific activity of trehalase, 25% recovery of total trehalase activity, and 5% recovery of protein compared to the initial homogenate.

Preparation of Depleted Membranes. Renal brush border membranes in buffer I (about 2.5 mg/mL) were incubated with 7 volumes (v/v) of ice-cold 0.1 M EDTA (pH 11.2) at 4 °C for 15 min and then centrifuged at 43000*g* for 30 min. The pellet was resuspended in buffer I and centrifuged again. This wash step was repeated. Finally, the membranes were washed with buffer B-1 (0.05 M sucrose, 2 mM $MgCl_2$, 4 mM K^+-HEPES, pH 7.2) and resuspended in buffer B.

Assay of Phlorizin Binding to Membranes. Phlorizin binding activity of 0.12-mg aliquots of renal brush border membranes or apical membrane vesicles from LLC-PK_1 cells was assayed as described previously (Lever, 1984). Samples were incubated for 5 min at 21 °C in 100-μL volumes with 0.2 μM (or the indicated concentration) [^{3}H]phlorizin (0.5 μCi), 0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K^+-HEPES, pH 7.2, and either 100 mM NaCl or 100 mM choline chloride.

Screening Method To Identify MAb's That Inhibit Phlorizin Binding. Since the amount of glucose in hybridoma growth medium was sufficient to completely prevent [^{3}H]-phlorizin binding to membranes, it was necessary to first fractionate each hybridoma supernatant before testing for inhibition. Columns containing 1 mL of preswollen Sephadex G-50 (fine) were centrifuged at 100*g* for 2 min. Then 100 μL of fresh supernatants from hybridoma cultures was applied to each column and centrifuged in test tubes at 100*g* for 2 min. Aliquots (20 μL) of renal brush border membranes (120 μg) were preincubated with 50 μL of Sephadex-treated hybridoma supernatants in the presence of 100 mM NaCl for 1 h at room temperature, and then the [^{3}H]phlorizin binding assay was initiated as described above. Control experiments established that Sephadex G-50 treatment removed over 99% of glucose from supernatant samples with 100% recovery of antibodies as assayed by ELISA (not shown). Sephadex-treated supernatants from control hybridomas did not inhibit phlorizin binding.

Preparation of Apical Membranes from LLC-PK_1 Cells. Monolayers were grown in plastic roller bottles in the presence or absence of 5 mM HMBA and harvested by scraping. Typically, cultures maintained at confluence for 2 weeks in the presence or absence of inducer were used. Apical membranes were prepared as described previously (Lever, 1982). Membranes were stored in liquid N_2 in 1-mL aliquots in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.2.

Assay of Transport Activity in Vesicles. Na^+-dependent glucose transport activity of apical membrane vesicles from LLC-PK_1 cells was assayed with the analogue [^{14}C]methyl α-D-glucopyranoside (MeGlc) as substrate, as described previously (Lever, 1982).

Gel Filtration of IgM Antibodies on Bio-Gel P-200. IgM MAb's were fractionated on Bio-Gel P-200 columns equilibrated with 5 mM $MgCl_2$ and 10 mM K^+-HEPES, pH 7.2, as described by Pretzman et al. (1985).

Purification of IgG Antibodies on Protein A–Sepharose. MAb's of the IgG subtype were purified on protein A–Sepharose (Bio-Rad, MAPS) as described by the manufacturer's instructions. Typically, a yield of 7–8 mg of purified IgG/mL of ascites fluid was obtained. The IgG fraction was then dialyzed against 10 mM K^+-HEPES, pH 7.2, and 5 mM $MgCl_2$, and aliquots were stored at −80 °C.

Gel Filtration on PD-10 Columns. Aliquots (2.5 mL) of ascites fluid were applied to PD-10 Sephadex G-25 (Pharmacia) columns (9.1-mL bed volume and 5-cm bed height) and eluted in equilibrating buffer (10 mM K^+-HEPES, pH 7.2, 5 mM $MgCl_2$).

Western Blot Analysis. Renal brush border membranes, 825 μg, were resolved by SDS–PAGE using a 7.5% acrylamide running gel and a 3% stacking gel. Proteins were electrophoretically transferred to nitrocellulose sheets for Western blot analysis by the method of Towbin et al. (1979) as modified by Yoneyama and Lever (1987). Molecular weight markers were localized on the filter by staining with Amido Black. Filters were incubated with the indicated monoclonal antibody at 4 °C for 24 h, washed, and then incubated with peroxidase-conjugated rabbit anti-mouse (IgG + IgA + IgM) at a 1:200 dilution at room temperature for 2 h. Filters were washed, and then peroxidase activity was visualized according to the intensification protocol described by Adams (1981).

Immunoprecipitation. Renal brush border membranes (3 mg/mL) were solubilized with 5% Triton X-100 in 0.05 M sodium phosphate, 0.125 M sucrose, 5 mM $MgCl_2$, and 10 mM K^+-HEPES, pH 7.2. Insoluble material was removed by centrifugation at 100000*g* for 10 min in a Beckman airfuge. Solubilized material from 0.25 mg of membranes was incubated for 60 min at 4 °C in a final volume of 400 μL containing 0.1 M sodium phosphate, pH 7.3, and 1% Triton X-100 with 0.15 mg of the indicated monoclonal antibody (prepared by gel filtration on PD-10 columns). Then 5 μg of rabbit anti-mouse (IgG + IgM + IgA) was added and the incubation continued for another 20 h at 4 °C. The incubation was terminated by addition of 2 mL of 0.1 M sodium phosphate, pH 7.3, and samples were centrifuged at 3000*g* for 30 min. The immunoprecipitate was washed once with 2 mL of this buffer before analysis of three pooled immunoprecipitates by SDS–PAGE.

Protein Determination. Protein was determined by the method of Lowry et al. (1951).

Results

Enrichment of Phlorizin Binding Sites in Protein-Depleted Brush Border Membranes. Two different strategies were utilized to generate Mab's. In one approach, apical membranes purified from highly differentiated LLC-PK_1 cultures were used as antigen. This cell line from pig kidney exhibits properties of renal proximal tubule in culture, including Na^+/glucose symport activity, and is a more homogeneous cell population than the corresponding tissue. The second approach utilized protein-depleted brush border membranes from pig kidney cortex as an enriched source of the symporter. Depleted membranes were prepared from renal brush border membranes by treatment with EDTA at pH 11.2. This treatment resulted in a significant reduction in the number of protein bands, with enrichment of several proteins as visible by SDS–PAGE analysis (not shown). Depleted membranes exhibited a 2-fold enrichment of total high-affinity, Na^+-dependent phlorizin binding sites/mg of protein, with 90% recovery, compared with the original membranes (Table I). Total high-affinity phlorizin binding sites/mg of protein of depleted brush border membranes were 87-fold increased compared with apical membranes from uninduced LLC-PK_1 cells maintained 2 weeks at confluence (4.9 pmol/mg) and 6-fold increased compared with membranes from confluent cells treated for 2 weeks with 5 mM HMBA, which induces this symporter (not shown).

Production and Screening of Monoclonal Antibodies to the Renal Na^+/Glucose Symporter. Table II summarizes the properties of several MAb's used in this study. Analysis of three independent fusions after immunization of mice with depleted brush border membranes from pig kidney yielded 470

Table I: [^{3}H]Phlorizin Binding to Brush Border Membranes from Pig Kidney Cortex

fraction	n	protein recovery (%)	B_{max} (pmol/mg)	K_d (μM)	phlorizin bound: recovery (%)	phlorizin bound: relative sp act.
kidney homogenate	3	100	32.0 ± 12.9[a]	1.60 ± 0.83	100	1
brush border membranes	3	4.5 ± 0.4	236.6 ± 75.3	2.14 ± 0.39	34.6 ± 13.3	7.9 ± 2.4
depleted brush border membranes	3	2.1 ± 0.4	426.1 ± 132.7	3.69 ± 0.73	31.4 ± 16.6	14.6 ± 5.8

[a] Values are mean ± SE.

Table II: Summarized Properties of MAb's

name	subtype	immunogen	immunization (months)	effect on phlorizin binding
MAb's That Recognize a 75-kDa Antigen				
11A3D6	IgG_{2b}	LLC-PK$_1$[a]	5	stimulation
15G8	IgM	KBBM[b]	1.5	inhibition
15E1.2	IgM	KBBM	1.5	inhibition
18H10B12	IgM	KBBM	1.5	inhibition
Control MAb's				
2H7B4	IgG_2	LLC-PK$_1$	1	none
7F10B9	IgM	LLC-PK$_1$	3	none
3B6C8	IgG_2	LLC-PK$_1$	1	none
11F7B2	IgM	LLC-PK$_1$	5	none
MOPC 141 tumor line (Sigma mouse IgG)	IgG_{2b}			none
				none
TEPC183 tumor line (Sigma mouse IgM)	IgM			none

[a] LLC-PK$_1$ refers to apical membranes prepared from confluent LLC-PK$_1$ cell cultures. [b] KBBM refers to depleted brush border membranes from pig kidney cortex.

antigen-specific hybridomas (out of 1248 viable hybridomas). Of these, 21 significantly inhibited phlorizin binding to brush border membranes. All of these subtyped as IgM's. These MAb's also bound LLC-PK$_1$ cells (not shown).

Positive hybridomas identified by the phlorizin binding screening assay were recloned by limiting dilution. Hybridomas producing antibody to the intial antigen were retested for inhibition of phlorizin binding. Results shown in Figure 1 indicate that a high frequency (about 50%) of the subclones produced MAb's that inhibited phlorizin binding. One antibody, 18H10B12, was selected for further study on the basis of its strong inhibition of phlorizin binding.

From three independent fusions using spleens from mice immunized with apical membranes from LLC-PK$_1$ cells, a total of 504 hybridomas (out of 1144) were identified that produced MAb's to LLC-PK$_1$ monolayers. These MAb's also bound renal brush border membranes (Lever et al., 1986). Of these, 195 were recloned by limiting dilution, yielding 235 antibody-secreting subclones, and supernatants were then screened for inhibition of Na^+-dependent phlorizin binding to renal brush border membranes. One MAb, 11A3D6, subtyped as an IgG_{2b}, was identified that inhibited phlorizin binding by 36 ± 7%, averaged from five separate determinations.

Effect of MAb's on High-Affinity Phlorizin Binding. Although initially identified in hybridoma supernatants by its inhibition of phlorizin binding, MAb 11A3D6, purified from ascites fluid by affinity chromatography on protein A–Sepharose followed by dialysis against 10 mM K^+-HEPES, pH 7.2, and 5 mM $MgCl_2$, reproducibly stimulated high-affinity Na^+-dependent phlorizin binding to renal brush border membranes. Stimulation of phlorizin binding by 11A3D6 (25%) was maximal after a 25-min incubation of membranes with antibody at room temperature (Figure 2A). Stimulation increased as a function of antibody concentration, reaching a plateau of 15% stimulation at 8 μg/mL (Figure 3A). By

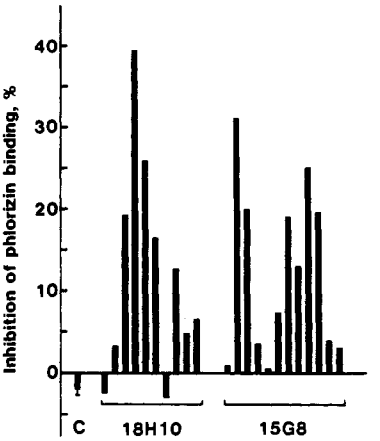

FIGURE 1: Inhibition of phlorizin binding by MAb's: stability of phenotype after recloning by limiting dilution. The hybridomas 18H10 and 15G8 were recloned by limiting dilution, and clones producing antigen-specific MAb's were tested for inhibition of phlorizin binding by the standard screening assay. Inhibition of Na^+-dependent phlorizin binding (in percent) is shown after subtraction of Na^+-free binding. (C) Average of 47 control hybridomas.

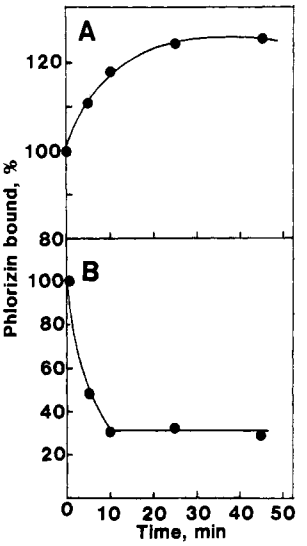

FIGURE 2: Effect of time of preincubation of membranes with MAb's on Na^+-dependent phlorizin binding: (A) 11A3D6 (25 μg); (B) 18H10B12 (70 μg). Renal brush border membranes (0.12 mg) were preincubated for the indicated time with MAb's and then assayed for phlorizin binding. Data are shown after subtraction of Na^+-free binding. Preincubation times shown include the 5-min time of incubation with [^{3}H]phlorizin.

contrast, a control MAb, 3B6C8, an IgG_2 isolated and purified in parallel, exhibited specificity for binding brush border membranes, shown by ELISA assay, but did not affect phlorizin binding. Similarly, a control mouse IgG_{2b} MAb derived by growth of the MOPC 141 tumor line in ascites and purified on protein A–Sepharose as above had no effect.

Interestingly, 11A3D6 in ascites fluid purified by gel filtration on Sephadex G-25 inhibited high-affinity phlorizin binding to brush border membranes (not shown), yet the same batch stimulated phlorizin binding after purification on protein

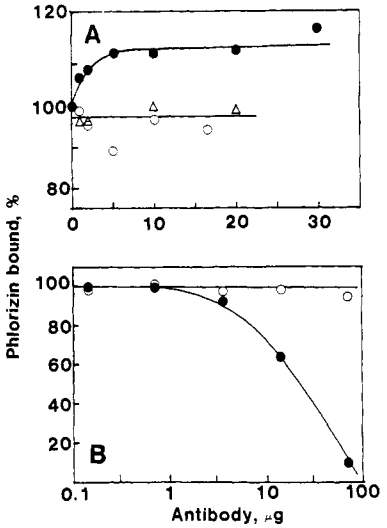

FIGURE 3: Na^+-dependent phlorizin binding by brush border membranes from pig kidney cortex as a function of MAb concentration. (A) Stimulation of phlorizin binding: (●) 11A3D6; (○) mouse IgG_{2b} from myeloma MOPC 141; (△) 3B6C8. (B) Inhibition of phlorizin binding: (●) 18H10B12; (○) 11F7B2. Renal brush border membranes (0.12 mg) were incubated for 40 min at room temperature with the indicated MAb and then assayed for phlorizin binding activity in the continued presence of MAb and 0.2 μM [^{3}H]phlorizin (0.5 μCi). One hundred percent represents 20.4 pmol of phlorizin bound/mg, observed in membranes incubated in the same solution without MAb addition. Data are shown after subtraction of Na^+-free binding (16% of total binding in presence of Na^+). All IgG-type MAb's were purified by protein A–Sepharose followed by dialysis against 10 mM K^+-HEPES, pH 7.2, and 5 mM $MgCl_2$. All IgM-type MAb's were fractionated by gel filtration on PD-10 columns equilibrated with the same solution. Values are means of at least four estimates.

A–Sepharose. By contrast, control IgG MAb's ascites similarly treated by gel filtration did not affect phlorizin binding.

These observations are consistent with the interpretation that 11A3D6 exerts specific effects on phlorizin binding sites which are modified in the presence of other components of ascites fluid or hybridoma growth medium. These component(s) would interact synergistically with the binding of specific MAb to the cotransporter, resulting in inhibition of phlorizin binding. No inhibition was observed after incubation of membranes with control ascites fluid or hybridoma supernatants containing MAb's that bind other membrane proteins. Furthermore, the putative factor is not low molecular weight since it was not removed by gel filtration on Sephadex G-25.

We have tested the possibility that these factors are proteases. In this view, a protease would nick the transporter, triggering a conformational change such that MAb binding would result in inhibition of phlorizin binding. Treatment of membranes for 15 min at 4 °C with concentrations of trypsin from 0.5 to 6.25 μg/mL did not affect phlorizin binding activity or prevent its stimulation by MAb 11A3D6. However, Western blot analysis indicated that the antigen recognized by 11A3D6 was cleaved in trypsin-treated membranes (not shown). These results indicate that cleavage of the 75-kDa antigen by trypsin does not cause loss of phlorizin binding activity, presumably because the active site conformation is maintained in the membrane environment. Similar observations of retention of activity after trypsin cleavage have been reported in the case of the human erythrocyte glucose transporter (Cairns et al., 1984).

In another experiment, we attempted to reconstruct the inhibition of phlorizin binding by addition of 20 μg of purified 11A3D6 to 70 μg of control ascites fluid (10F7B2) that had been treated by gel filtration on Sephadex PD-10 columns. Membranes incubated with the mixture exhibited the same 20% stimulation of phorizin binding observed with purified 11A3D6 alone, yet unpurified 11A3D6 ascites fluid inhibited phlorizin binding; control ascites fluid had no effect (not shown). The nature of this synergy of 11A3D6 with other factors in ascites fluid remains to be elucidated.

Binding of the IgM MAb 18H10B12 to renal brush border membranes strongly inhibited Na^+-dependent phlorizin binding. The time course of inhibition shown in Figure 2B indicates that 20-min incubation of membranes with 18H10B12 was sufficient to observe maximal inhibition. A similar time course was observed with a 4-fold higher titer of antibody, but in this case 100% inhibition of Na^+-dependent phlorizin binding was achieved (not shown). Inhibition of phlorizin binding by 18H10B12 increased as a function of antibody concentration, reaching a plateau of 80–90% inhibition at 70 μg of protein (ascites fluid) (Figure 3B). In contrast, a control IgM MAb, 11F7B12, isolated in parallel, bound renal brush border membranes with a similar titer but did not affect phlorizin binding (Figure 3B). These experiments used ascites fluid that had been treated by gel filtration on Sephadex G-25 (PD-10 columns) to remove low molecular weight contaminants.

After fractionation on Bio-Gel P-200 under conditions where components of molecular mass less than 200 kDa are retained, IgM MAb's were quantitatively recovered in the excluded volume as shown by ELISA assay. After this additional fractionation step, 18H10B12 ascites fluid was still active in inhibiting phlorizin binding (not shown). After purification by HPLC using a Mono-Q column (Pharmacia), 18H10B12 retained the specific inhibitory effects on phlorizin binding.

Similar effects of MAb's on phlorizin binding to apical membrane vesicles from LLC-PK_1 cells were observed. MAb 11A3D6 stimulated phlorizin binding by 34% when apical membrane vesicles from confluent cell cultures were used, whereas control IgG MAb 2H7B4 had no significant effect. MAb 18H10B12 inhibited phlorizin binding to apical vesicles from either HMBA-induced or uninduced confluent cultures by 25% (not shown).

Identification of Antigen Recognized by MAb's as a 75-kDa Protein. Western blot analysis of brush border membranes after resolution by SDS–PAGE indicated that both 11A3D6 (Figure 4) and 18H10B12 (not shown) each recognized an antigen of apparent molecular mass 75 kDa. This band was not recognized either by control MAb's such as 3B6C8 which had no effect on phlorizin binding but recognized other antigens in brush border membranes or by MAb's of matched subtype isolated from nonspecific myelomas. The dark band of apparent molecular mass 200 kDa is visible before initiation of the peroxidase reaction and is also visible with some control antibodies, suggesting that it may be due to a substance in the tissue extract. Addition IgM MAb's, 15E1.2 and 15G8, identified from the same fusions by our screening as positive for inhibition of phlorizin binding (Table II), also recognized the 75-kDa antigen (not shown). These results indicate that, whether isolated after immunization with depleted brush border membranes or apical membranes from LLC-PK_1 cells, in each case MAb's that specifically influenced Na^+-dependent phlorizin binding recognized an antigen with the same apparent molecular mass. The association of the 75-kDa antigen with the Na^+/glucose symport system is further strengthened by the close correspondence of this apparent molecular mass with previous estimates from other approaches [reviewed in Semenza et al. (1984)].

Immunoprecipitation of a 75-kDa Protein by MAb 18H10B12. Additional evidence that the antigen recognized

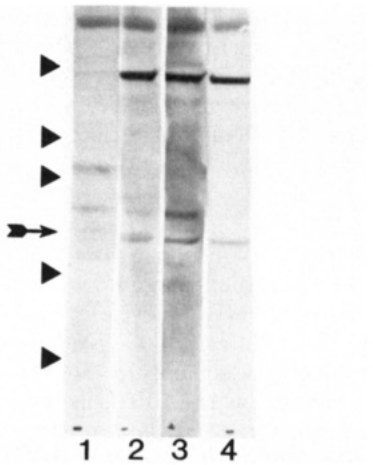

FIGURE 4: Identification of antigen recognized by MAb's. Renal brush border membranes, 0.825 mg, were resolved by SDS–PAGE and transferred to nitrocellulose for Western blot analysis as described under Materials and Methods. Lane 1, mouse IgG (Sigma); lane 2, 11A3D6, hybridoma supernatant; lane 3, 11A3D6, ascites fluid; lane 4, 11A3D6, purified IgG. Nitrocellulose strips were incubated with the indicated MAb at the following concentrations: 25 μg/mL in the case of purified IgG's; 1:40 dilution in the case of ascites fluid; undiluted in the case of hybridoma supernatants. Molecular mass markers of 200, 130, 92.5, 66, and 45 kDa, respectively, are indictated by the filled triangles. The arrow indicates the position of the 75-kDa antigen.

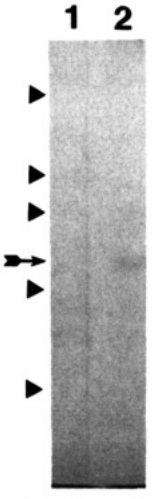

FIGURE 5: Immunoprecipitation of a 75-kDa a protein by 18H10B12. Immunoprecipitation was performed as descried under Materials and Methods. Lane 1, 18H10B12 minus rabbit anti-mouse second antibody; lane 2, 18H10B12 plus rabbit anti-mouse second antibody. MAb's were fractionated by gel filtration on PD-10 columns. Filled triangles indicate the positions of molecular mass markers as described for Figure 4. The arrow indicates the position of the 75-kDa antigen.

by 18H10B12 is a 75-kDa species was provided by the immunoprecipitation experiment shown in Figure 5. Renal brush border membranes solubilized with 5% Triton X-100 were incubated for 1 h with either 18H10B12 or the control MAb, and then rabbit anti-mouse (IgG + IgM + IgA) was added for an additional 20 h at 4 °C. Washed immunoprecipitates were analyzed by SDS–PAGE. The 75-kDa protein immunoprecipitated by 18H10B12 was not immunoprecipitated by the control IgM 11F7B2 (not shown).

Effect of Na^+ Concentration. MAb 18H10B12 specifically inhibited the Na+-dependent component of phlorizin binding associated with the Na^+/glucose cotransporter. In the experiment shown in Figure 6, MAb's from ascites fluid were recovered from the excluded volume of PD-10 columns and then incubated with membranes in the presence of concen-

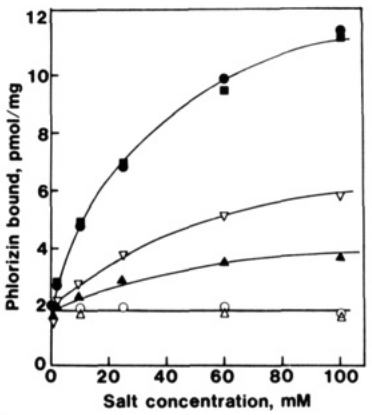

FIGURE 6: Na^+ dependence of phlorizin binding component inhibited by 18H10B12: (●) no addition, NaCl; (○) no addition, choline chloride; (■) 11F7B12, 70 μg, NaCl; (▲) 18H10B12, 70 μg, NaCl; (▽) 18H10B12, 35 μg, NaCl; (△) 18H10B12, 70 μg, choline chloride. Renal brush border membranes (0.12 mg) were incubated with MAb's in the presence of the indicated concentration of NaCl or choline chloride and then assayed for [^{3}H]phlorizin binding.

trations of NaCl or choline chloride from 0 to 100 mM. At 70 μg of 18H10B12, 80% inhibition of the Na^+-dependent component was observed at each Na^+ concentration, but Na^+-free binding was not affected. By contrast, control MAb 11F7B2 (70 μg) had no effect on Na^+-dependent binding. At a lower concentration (35 μg), 18H10B12 inhibited the Na^+-dependent component of phlorizin binding by 60% at each Na^+ concentration in this range.

MAb's Have No Effect on Na^+-Dependent Hexose Uptake in Vesicles. No effect on either Na^+-dependent or Na^+-independent transport activity of the Na^+/glucose cotransporter was observed after incubation of apical membrane vesicles isolated from LLC-PK_1 cells with saturating amounts of either 11A3D6 purified by protein A–Sepharose or 18H10B12 purified by PD-10 treatment (not shown). In each case, activity of the antibody was confirmed by measurement of effects on Na^+-dependent phlorizin binding. Nonspecific transport inhibition was observed if unpurified antibody preparations were used.

MAb's Diminish Transport Inhibition by Phlorizin. Incubation of apical membrane vesicles from LLC-PK_1 cells with purified MAb 11A3D6 resulted in significantly decreased sensitivity of Na^+/glucose symport activity to inhibition by phlorizin (Figure 7). K_i values for transport inhibition by phlorizin, calculated from these Dixon plots, were 1.0 μM in the presence of MAb 11A3D6 and 0.31 μM in control samples. Similar results were also obtained by using vesicles from HMBA-induced cells (not shown). Sensitivity to phlorizin inhibition of untreated control membranes did not differ from that of membranes treated with control MAb's. These observations indicate that interaction of MAb 11A3D6 with the phlorizin binding site uncouples Na^+/glucose symport from inhibition by bound phlorizin, while exhibiting no effect on transport in the absence of phlorizin.

DISCUSSION

In the presence of Na^+, phlorizin binds with high affinity and specificity to Na^+/glucose symporters and interacts competitively with sugar substrates of this system (Frasch et al., 1970). A large number of hybridomas derived after immunizing mice with apical membrane preparations were screened by a specific functional test based on inhibition of phlorizin binding in order to identify monoclonal antibodies that bind the renal Na^+/glucose symport system. The selectivity of our approach is underscored by the observation that

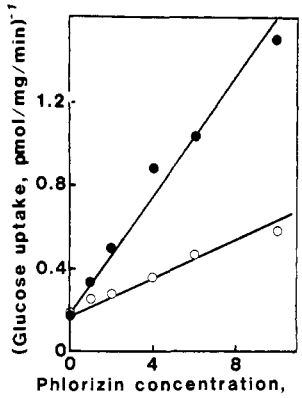

FIGURE 7: MAb's decrease inhibition of cotransport activity by phlorizin. Apical membrane vesicles, 0.2 mg, prepared from confluent uninduced LLC-PK_1 cultures were incubated with 16 μg of either (○) 11A3D6 or (●) Sigma mouse IgG_{2b} for 30 min at room temperature. Then the indicated concentration of phlorzin was added, and transport assay was initiated by addition of [^{14}C]methyl α-glucopyranoside, 0.5 mM final concentration, and either NaCl or choline chloride, 100 mM final concentration, for 5 min. Values are corrected by subtraction of Na^+-free transport (50% of total) from transport observed in the presence of NaCl. Na^+-dependent transport activity in the absence of phlorizin was 61.20 pmol/(mg·min) for 11A3D6 and 58.62 pmol/(mg·min) for Sigma mouse IgG_{2b}, respectively. Data are shown as analyzed by a Dixon plot. K_i values are calculated by using a K_m value of 10 mM and V_{max} of 5 nmol/(mg·min) for glucose transport (Lever, 1982).

all MAb's identified by effects on phlorizin binding also recognized an antigen in renal brush border membranes of the same apparent molecular mass, 75 kDa, regardless of whether the immunizing antigen was derived from renal brush border membranes or from the apical membranes of a long-term renal cell line. Using a reciprocal approach, we have screened by Western blot analysis over 300 hybridomas generated to this renal membrane preparation; only one MAb was found that recognized a 75-kDa antigen, and this antibody did not affect phlorizin binding. On the basis of the number of high-affinity phlorizin binding sites in pig renal brush border membranes depleted of extrinsic proteins, it can be calculated that Na^+/glucose symporters represent 3.2% of the total protein in the preparation used for immunization; the corresponding estimate for apical membranes from induced LLC-PK_1 cells is 0.5%. These values closely parallel the observed incidences of MAb's that inhibit phlorizin binding obtained from each type of membrane antigen. The phenotype was retained by a high frequency of subclones derived from a second cloning of these positive hybridomas.

Estimates of the molecular masses of components of the renal Na^+/glucose symporter of 52 and 94 kDa (by SDS–PAGE) after purification and reconstruction (Koepsell et al., 1983), 46, 74, and 82 kDa by affinity labeling (Koepsell et al., 1986), 110 kDa by target size inactivation (Turner & Kempner, 1982), and 160 kDa under nonreducing conditions (Malathi et al., 1980) have been reported. Recent studies have suggested a molecular mass of 72 kDa for a component of the intestinal Na^+/glucose symporter on the basis of negative purification and thiol labeling (Klip et al., 1979), photoaffinity labeling (Hosang et al., 1981), and substrate-protectable FITC labeling (Peerce & Wright, 1984).

A screening strategy based on high-affinity ligand binding offers increased sensitivity and reproducibility and less susceptibility to nonspecific inhibition compared with screening by inhibition of transport. We observed nonspecific transport inhibition in the presence of unpurified hybridoma supernatants of ascites fluid which did not persist after antibody purification. Furthermore, a higher frequency of MAb's would be expected to be identified on the basis of recognition of a specific binding site rather by inhibition of function. When purified *lac* carrier of *Escherichia coli* was used as immunizing antigen, only 1 in 60 antigen-positive antibodies inhibited transport (Carrasco et al., 1984). Only 2 out of 9 monoclonal antibodies to the Na^+,K^+-ATPase of rat kidney inhibited transport (Schenk & Leffert, 1983). In the case of the Na^+,K^+-ATPase, observations that a polyclonal antibody to the α subunit did not affect transport have been offered as evidence against a rotating carrier model (Kyte, 1974).

The intimate connection between MAb specificity and Na^+-coupled glucose transport function was revealed by the novel observation that purified MAb 11A3D6 binding specifically prevented this transport inhibition by phlorizin. This effect cannot be explained by simple competition for the phlorizin binding site since purified 11A3D6 did not prevent [^{3}H]phlorizin binding. It appears more likely that MAb binding stabilizes an active conformation of the transporter, as suggested by evidence provided in the following paper (Wu & Lever, 1987).

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Monoclonal Antibodies That Bind the Renal Na^+/Glucose Symport System. 2. Stabilization of an Active Conformation†

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ABSTRACT: Conformation-dependent fluorescein isothiocyanate (FITC) labeling of the pig renal Na^+/glucose symporter was investigated with specific monoclonal antibodies (MAb's). When renal brush border membranes were pretreated with phenyl isothiocyanate (PITC), washed, and then treated at neutral pH with FITC in the presence of transporter substrates Na^+ and glucose, most of the incorporated fluorescence was associated with a single peak after resolution by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The apparent molecular mass of the FITC-labeled species ranged from 79 to 92 kDa. Labeling of this peak was specifically reduced by 70% if Na^+ and glucose were omitted. Na^+ could not be replaced by K^+, Rb^+, or Li^+. FITC labeling of this peak was also stimulated after incubation of membranes with MAb's known to influence high-affinity phlorizin binding, and stimulation was synergistically increased when MAb's were added in the presence of Na^+ and glucose. Substrate-induced or MAb-induced labeling correlated with inactivation of Na^+-dependent phlorizin binding. MAb's recognized an antigen of 75 kDa in the native membranes whereas substrate-induced FITC labeling was accompanied by loss of antigen recognition and protection from proteolysis. These findings are consistent with a model in which MAb's stabilize a Na^+-induced active conformer of the Na^+/glucose symport system.

Secondary active transport of glucose in renal and intestinal cells is mediated by a symport mechanism in which sugar movement across the plasma membrane is coupled to movement of Na^+ down its electrochemical potential gradient (Crane, 1977). Kinetic studies (Kaunitz & Wright, 1984) and inactivation studies (Weber & Semenza, 1983) of substrate translocation in intestinal membranes strongly support a coupling mechanism in which conformational changes in the symporter induced by Na^+ binding induce increased carrier affinity for glucose. Na^+-induced conformational changes in an intestinal brush border membrane protein identified as a good candidate for the glucose symporter were detected by using a substrate-protectable FITC[1] label as a reporter group (Peerce & Wright, 1984, 1985). However, due to difficult progress in the unequivocal identification of the protein(s) that catalyze Na^+/hexose symport, very little is known about the role of conformational changes in transporter function at the molecular level.

As an approach to develop highly specific probes to investigate the structure and function of the renal Na^+/glucose

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[1] Abbreviations: FITC, fluorescein isothiocyanate; PITC, phenyl isothiocyanate; MAb, monoclonal antibody; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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