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EFFECT OF ANTISERUM TO A 99 kDa POLYPEPTIDE ON THE UPTAKE OF TAUROCHOLIC ACID BY RAT ILEAL BRUSH BORDER MEMBRANE VESICLES

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A 99 kDa polypeptide in rat ileal brush border membrane (BBM), regarded as a component of the active bile acid transport system on account of photoaffinity labeling, has been purified by affinity chromatography and preparative gel electrophoresis and utilized as an immunogen for raising polyclonal antibody. Immune serum, but not preimmune serum, specifically recognized a single band of 99 kDa protein on immunoblots of ileal and renal BBM. In contrast, no reactivity was observed with proteins in jejunal BBM. This polyclonal antibody, compared with preimmune serum and anticytosolic bile acid binding protein (14 kDa) serum, significantly inhibited the Na^+ dependent uptake of [^{3}H] taurocholate by BBM vesicles ($\rho < 0.01$). [^{14}C] D-glucose uptake by BBM vesicles was not influenced by the immune serum ($\rho < 0.01$). Thus, these studies provide further support for the specific role of a 99 kDa protein in ileal BBM bile acid transport.

The bile acids are a family of acidic sterols whose physical properties determine their physiological role in the micellar solubilization of cholesterol and phospholipid in bile and lipolytic products of digestion in the intestinal lumen. In order to maintain adequate concentrations for micellarization, bile acids undergo an enterohepatic circulation. Bile acids are synthesized from cholesterol in the liver, secreted in bile and stored in the gallbladder. Upon the ingestion of food, the gallbladder contracts, emptying bile into the proximal small intestine where bile acids facilitate lipid absorption. Bile acids are absorbed by passive diffusion throughout the small intestine and in addition, by a carrier-mediated transport system in the ileum prior to their return to the liver via the portal circulation (1).

The physiological properties of the ileal bile acid transport system have been well described in vivo (2-3) and in vitro using whole intestinal segments (4-5), isolated epithelial cells (6-7) and brush border membrane (BBM) vesicles (8-11). The studies indicated that bile acids are transported against an electrochemical gradient, and the energy for this active transport is derived from the Na^+ gradient

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across the BBM. The coupling of active bile acid transport to the Na^+ gradient is believed to occur via a BBM protein, however, the transport protein has not been identified. The photolysis of BBM in the presence of a radiolabeled, photolabile derivative of a natural bile acid, followed by SDS-polyacrylamide gel electrophoresis, has revealed that the highest amount of radioactivity from the photoprobe is incorporated into a 99 kDa polypeptide. Photolysis in the presence of unlabeled taurocholate inhibited the binding suggesting that the photolabile derivative was bound to the same polypeptide as the natural bile acid (12). An approach to confer transport function to a membrane binding protein is the specific inhibition of transport by antibody to this protein (13). In the present report, studies were performed with antiserum raised against purified 99 kDa polypeptide which provide further support for the role of this protein in ileal BBM bile acid transport.

MATERIALS AND METHODS

Supplies: [^{3}H] taurocholic acid (2.10 Ci/mmol) and [^{14}C] D-glucose (4.43 mCi/mol) were purchased from New England Nuclear (Boston, MA). Complete and incomplete Freunds' adjuvants were from DIFCO Laboratories (Detroit, MI); nitrocellulose membrane was obtained from Bio-Rad Laboratories (Richmond, CA).

Preparation of membrane vesicles: Brush-border membrane vesicles (BBMV) were prepared from the jejunum, ileum, and kidneys of male 180-220g Sprague-Dawley rats by the Mg^{2+} precipitation method (14,15). Protein was determined by the method of Lowry et al (16) after precipitation with ice cold 10% trichloroacetic acid using bovine serum albumin (BSA) as a standard. The enrichment in the specific activity (final vesicle over starting homogenate) of leucine aminopeptidase (E.C.3.4.11.2) was 10-16 fold.

Purification of 99 kDa protein: BBMV from ileum were solubilized in buffer containing 0.5% n-octylglucoside, 5% glycerol, 5 mM mercaptoethanol and 10 mM Hepes-Tris, pH 7.4, by constant shaking overnight. The solubilized BBMV were centrifuged at 50,000 x g for 1 hour. The supernatant was diluted 5-fold with equilibration buffer containing 0.1% n-octylglucoside, 5% glycerol, 5 mM mercaptoethanol, 1M NaCl and 10 mM Hepes-Tris, pH 7.4. The diluted supernatant (8-10 mg of protein) was applied to a lysylglycocholate-Sepharose 4B affinity column which had been prepared according to von Dippe et al (17). The column was washed with 15 ml of equilibration buffer to remove non-absorbed material. The protein that remained bound to the column was eluted with 12 ml of equilibration buffer plus 3% n-octylglucoside. The eluate which yielded 0.3 mg of protein was dialysed overnight against equilibration buffer to remove the n-octylglucoside. The eluted fraction was subjected to preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with total acrylamide concentration of 7% at a ratio of acrylamide:bisacrylamide of 97.2:2.8. The 99 kDa band was selectively excised from the gel using the side-strip method (18). The protein in the gel slice was electroeluted with a S & S Elutrap Electro-Separation System (Schleicher and Schuell, Inc., Keene, NH) at 100 volts for 8 h. The electroeluted protein (100-200 μg) was used as an antigen for raising polyclonal antibody.

Preparation of polyclonal antibody: 50 μg of the 99 kDa electroeluted protein was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into a female New Zealand rabbit. A second injection of the same amount of the protein with incomplete Freund's adjuvant was performed two weeks later. The booster injections (50 μg protein each) without Freund's adjuvant were given at two week intervals. After 6 months, the rabbit was bled by ear vein. The titer and specificity of the antiserum were determined by immunoblotting.

Immunoblotting procedure: 150 μg aliquots of solubilized BBMV from jejunum, ileum and kidney were subjected to SDS-PAGE followed by transblotting to nitrocellulose membrane (0.45 μm) in 0.2M glycine, 20% methanol, adjusted to pH 8.3 with Tris, using the Bio-Rad Trans-Blot cell. The transfers were performed at 70 volts for 4 h. For immunoblotting, blotted sheets were incubated with 3% BSA in PBS containing 10 mM sodium phosphate, pH 7.5, and 0.9% saline, washed and then incubated with 1:500

dilution of the antiserum at 25°C for 45 minutes. After three washes, the sheets were incubated with 1:200 dilution of biotinylated anti-rabbit IgG, and Avidin and biotinlylated-horseradish peroxidase complex according to directions of the Vectastain ABC reagent kit (Vector Laboratories, Burlingame, CA). The blots were developed by the addition of the peroxidase substrate solution containing 0.02% hydrogen peroxide, 0.1% diaminobenzidine tetrahydrochloride in 0.1 M Tris buffer, pH 7.2.

Transport studies: Uptake of [^{3}H] taurocholate and [^{14}C] D-glucose was measured by the membrane filtration technique (19). BBMV were suspended in preincubation media containing pre-immune or immune sera at 25°C for 45 minutes. The transport reaction was initiated by adding 20 µl aliquots of preincubated BBMV to 100 µl of incubation buffer kept in a water bath at 37° C. The compositions of the preincubation and incubation media are provided in the legends to the figures. At desired time intervals, the transport reaction was terminated by the addition of 1 ml of ice-cold stop solution that had the same composition as the incubation buffer, plus unradiolabeled 0.05 mM taurocholate or 0.1 mM D-glucose to decrease nonspecific binding of labeled substrates, respectively, to the filters. The entire contents were pipetted onto the middle of a pre-washed, pre-chilled filter (cellulose nitrate, 0.45 µm pore size; Sartorius Filters, Inc. Hayward, CA), kept under suction. The filter was rinsed immediately with 5 ml of ice cold stop solution and then solubilized in 10 ml of Filter-Count cocktail (Packard Instrument Co., Meriden, CT). The radioactivity remaining on the filter was counted with a Packard 1900 TR liquid scintillation analyzer. After correction for radioactivity bound to filters in the absence of membrane vesicles, the uptake of radiolabeled substrate was calculated and expressed as pmol/mg protein. All experiments were performed in triplicate with two or more freshly prepared membranes.

Statistical analysis: Analysis of the data for significant difference ($p < 0.05$) was according to the Student's test for unpaired data (20).

RESULTS AND DISCUSSION

Initial studies were carried out to purify a 99 kDa polypeptide from ileal BBMV that had been identified previously by photoaffinity labeling studies as a putative bile acid transport protein (12,21). Solubilized ileal BBMV were applied to a lysylglychocholate-Sepharose 4B affinity column and eluted from the column with 0.5% n-octylglucoside. When the eluted fraction was subjected to SDS-PAGE, partial purification was seen when compared to starting membrane material (Figure 1, compare middle and right lanes). The 99 kDa protein band was then cut from the gel and electroeluted. The electroeluted fraction was analyzed by SDS-PAGE . As shown in Figure 2, a single protein band of 99 kDa was observed.

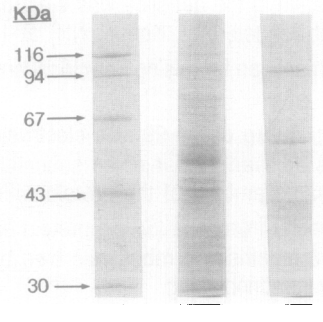

FIGURE 1. Affinity chromatography of n-octylglucoside-solubilized brush border membranes. Molecular weight protein standards (left lane), 25 µg of the solubilized brush border membrane (middle lane) and 7 µg of the adsorbed protein fraction (right lane) eluted from the lysylglycocholate-Sepharose 4B affinity column with 0.5% n-octylglucoside were subjected to SDS-PAGE (minigel) and Coomassie brilliant blue R-250 staining.

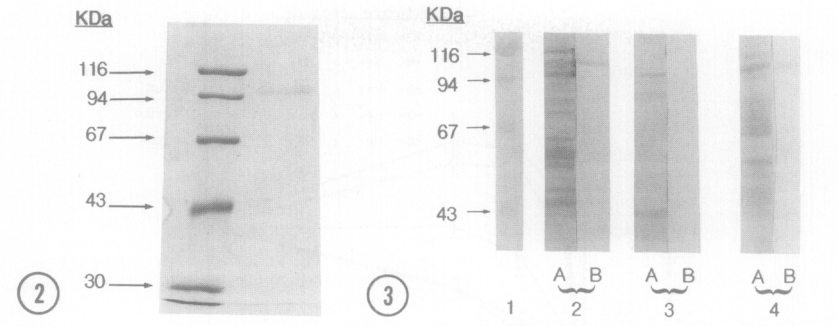

FIGURE 2. Preparative gel electrophoresis of the 99kDa protein. Molecular weight protein standards (left lane) and 3 μg of 99 kDa protein electroeluted from the preparative gel slice (right lane) were subjected to SDS-PAGE (minigel) and Coomassie brilliant blue R-250 staining.

FIGURE 3. Immunoblotting analysis of brush border membranes from jejunum, ileum, and kidney using the rabbit antserum against 99 kDa bile acid-binding protein. 1. Molecular weight protein standard. 2. Ileum. 3. Jejunum. 4. Kidney. A. Coomassie brilliant blue stain. B. Immunoblot with 1:500 dilution of 99 kDa antiserum. The antiserum specifically reacted with a 99kDa protein on immunoblots of ileum and kidney, but not jejunum.

Previous studies have indicated that bile acids are transported across the BBM of the ileum (2,4,6,9-10) and renal proximal tubule (22), but not jejunum (2,6), by a Na^+-dependent active transport system. Moreover, photoaffinity labeling with a radiolabeled 7,7 azo derivative of taurocholic acid resulted in a predominant incorporation of radioactivity into a 99 kDa polypeptide from ileal (12) and renal (23), but not jejunal (12), BBMV. These observations suggested that ileal and renal active bile acid transport was mediated via a common transport protein. This possibility was explored with immunoblotting studies. As shown in Figure 3A, solubilized BBMV from ileum, jejunum, and kidney were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Proteins from identically run gels were transferred to nitrocellulose and cut into strips. The strips were incubated with rabbit antiserum against the purified ileal 99 kDa polypeptide. The antiserum specifically reacted with protein on immunoblots of ileum (Figure 3, B2) and kidney (Figure 3, B4), but not jejunum (Figure 3, B3). These findings suggested that the 99 kDa polypeptides from ileum and proximal tubule are immunologically identical.

In order to provide further support for the role of the 99 kDa polypeptide in the active ileal transport of bile acids, antibody inhibition studies were performed. As shown previously (9-10), the imposition of an extravesicular to intravesicular Na^+ gradient resulted in the stimulation of taurocholate uptake by brush border membrane vesicles isolated from rat ileum. Therefore, the uptake of [^{3}H] taurocholate into ileal brush border membrane vesicles was studied in the presence and absence of a Na^+ gradient. As shown in Figure 4, the 20, 60, and 120 second uptake of [^{3}H] taurocholate was stimulated in the presence of the Na^+ gradient ($[Na^+]_{out} > [Na^+]_{in}$) as compared to the absence of the sodium gradient ($[Na^+]_{out} = [Na^+]_{in}$). Antiserum raised against purified 99 kDa polypeptide inhibited significantly ($p < .05$), the 20, 60, and 120 second uptake of Na^+ gradient-dependent uptake but had no effect on the uptake of taurocholate in the absence of the Na^+ gradient. Moreover, preimmune serum had no effect on the Na^+ gradient dependent uptake of taurocholate. To further test whether the inhibition of taurocholate by anti-99 kDa was selective, two additional experiments were performed. First, the effect of the antiserum to the 99

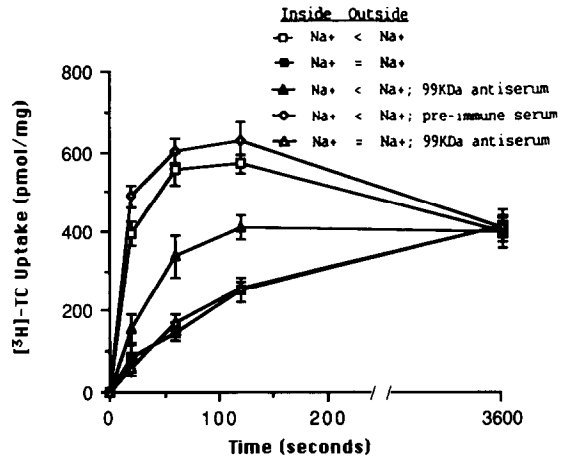

FIGURE 4. Effect of 99 kDa antiserum on taurocholate uptake by ileal BBMV. BBMV (1.2 mg protein) were suspended in medium A ([Na^+] inside vesicles < [Na^+] outside) consisting of 100 mM mannitol, 100 mM choline Cl, 20 mM HEPES, adjusted to pH 7.4 with Tris, and then preincubated for 45 min at 25°C with 0.9 mg of preimmune serum (◊), 99 kDa antiserum (▲) or without serum (❑). Meanwhile, the same amount of BBMV, suspended in medium B ([Na^+] inside vesicles = [Na^+] outside) consisting of 100 mM mannitol, 100 mM NaCl and 20 mM HEPES, adjusted to pH 7.4 with Tris, was preincubated for 45 min at 25°C with 0.9 mg 99 kDa antiserum (Δ) or without serum (■). Vesicles were incubated in medium consisting of 100 mM mannitol, 100 mM NaCl, 20 mM HEPES, adjusted to pH 7.4 with Tris, and 0.05 mM [^3H] taurocholate at 37 °C. Each point represents the mean ± S.E. for 6 determinations.

kDa brush border membrane bile acid binding protein was tested on D-glucose uptake by ileal BBMV. As shown in Figure 5, the presence of an internally directed Na^+ gradient resulted in stimulation of D-glucose uptake when compared to uptake in the absence of a Na^+ gradient. As expected, the addition of the

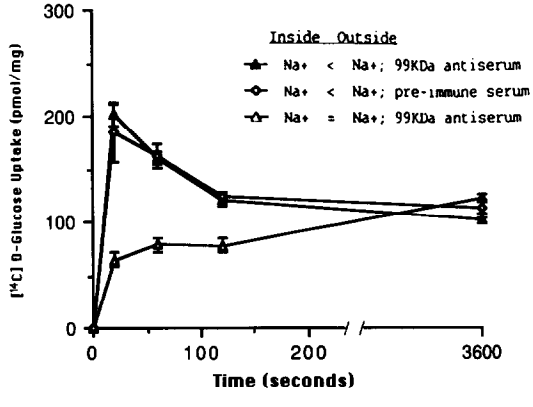

FIGURE 5. Lack of effect of 99 kDa antiserum on D-glucose uptake by ileal BBMV. BBMV (1.2 mg protein) suspended in medium A ([Na^+] inside vesicles < [Na^+] outside) were preincubated with 0.9 mg of 99 kDa antiserum (▲) or pre-immune serum (◊), or in medium B ([Na^+] inside vesicles = [Na^+] outside) with 99 kDa antserum (Δ) for 45 min at 25°C. The compositions of preincubation and incubation media were as described in the legend to Figure 4 with the exception that vesicles were incubated in medium consisting of 0.1 mM [^{14}C] D-glucose, rather than [^3H] taurocholate, at 37°C. Each point represents the mean ± S.E. for 6 determinations.

antiserum had no effect on Na^+ gradient-dependent D-glucose uptake when compared to D-glucose uptake in the presence of preimmune serum. Second, previous studies have shown that a major cytosolic bile acid binding protein has a molecular weight of 14,000 (24). Thus, similar experiments were performed with antibody to this polypeptide. No significant difference was seen between the Na^+ gradient dependent uptake of taurocholic acid when measured in the presence or absence of this antibody to cytosolic binding protein (data not shown). From the present study, it appears that an identical 99 kDa BBM protein is selectively involved in ileal and renal Na^+ - bile acid co-transport.

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