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Developmentally Regulated 75-Kilodalton Protein Expressed in LLC-PK_1 Cultures Is a Component of the Renal Na^+/Glucose Cotransport System

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Na^+/D-glucose symport is a secondary active glucose transport mechanism expressed only in kidney proximal tubule and in small intestine. A monoclonal antibody that recognized the Na^+/glucose symporter of pig renal brush border membranes also recognized a 75-kD protein in apical membranes isolated from highly differentiated LLC-PK_1 cultures, an epithelial cell line of pig renal proximal tubule origin. The 75-kD antigen was enriched from solubilized LLC-PK_1 apical membranes by means of high-pressure liquid chromatography. The symporter antigen became apparent on the apical membrane surface after the development of a confluent monolayer in correlation with the expression of transport activity. Long-term treatment of cultures with the differentiation inducer hexamethylene bisacetamide was accompanied by a dramatically increased expression of the symporter antigen as detected quantitatively by Western blot analysis and qualitatively by immunofluorescence staining. The number of symporter-positive cells was dramatically increased after inducer treatment as predicted for differentiation-regulated expression. These results identify a 75-kD protein as a component of a developmentally regulated renal Na^+/glucose symporter expressed in cell culture.

Key words: renal epithelial cell cultures, cell differentiation, hexamethylene bisacetamide, immunofluorescence, glucose transport

Renal proximal tubule and intestinal apical membranes contain transporters that catalyze Na^+-coupled active D-glucose transport in a symport (cotransport) mechanism. These symporters are functionally distinct from glucose transporters of nonepithelial cell types, exemplified by the erythrocyte glucose transporter, in that the latter catalyze facilitated diffusion, do not exhibit Na^+ dependence, and are not capable of concentrative uptake of glucose. The two types of glucose transporters are immunologically distinct [1], show no DNA sequence homology [2], and also differ in inhibitor and substrate specificity. Na^+/glucose symporters are relatively resistant to

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inhibition by cytochalasin B or phloretin, which strongly inhibit the facilitative diffusion type of glucose transporter [3], but are sensitive to the high-affinity inhibitor phlorizin, which does not inhibit the diffusional type [4]. The kidney also contains a facilitative diffusion type of glucose transporter in its basolateral membrane, which may be homologous to the HepG2 hepatoma glucose transporter [5].

We have recently identified a subunit of the Na^+/glucose symporter of renal cortical brush border membrane as a 75-kD glycoprotein [6,7], purified this glycoprotein to homogeneity [8], and characterized it as (a subunit of) the symporter by reconstitution of activity and recognition by monoclonal antibodies known to interact functionally with the symporter [8].

To investigate the regulation of Na^+/glucose symporter expression, we have utilized the long-term epithelial cell line LLC-PK_1 derived from pig kidney cortex [9]. Sparse actively dividing cultures express barely detectable levels of Na^+-dependent glucose transport activity. Following the development of a confluent cell density, Na^+/glucose symport activity increases significantly together with other differentiated markers characteristic of renal proximal tubule [10,11]. An additional dramatic enhancement in the levels of Na^+/glucose symport activity as well as other differentiated functions were observed after the addition of the differentiation inducer hexamethylene bisacetamide (HMBA) to confluent cultures [11,12].

In order to explore further the mechanism of increased symporter activity following inducer treatment, specific antibody probes were utilized to identify this transport system in differentiating cell cultures. Results from this approach indicated that induction of transport activity reflected an increase in the number of cells expressing the symporter protein.

MATERIALS AND METHODS

Materials

[*Phenyl*-3,3',5,5'-^{3}H, *propyl*-3-^{3}H]Phlorizin, 50 Ci/mmol, was purchased from New England Nuclear. Rabbit anti-mouse (IgG + IgM + IgA), its peroxidase conjugate, and goat anti-rabbit IgG were from Zymed. n-Octyl glucoside was from Boehringer-Mannheim.

Cell Culture

The LLC-PK_1 pig kidney epithelial cell line (ATCC CL101) was grown in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12, supplemented with 10% fetal calf serum (Sterile Systems, Logan UT) and 2 mM glutamine, as described previously [11]. For membrane preparation, cultures were grown to confluence in 850-cm^2 roller bottles and then induced with 10 mM HMBA for 2 weeks, with two medium changes per week, before harvest.

Monoclonal Antibodies

To provide a specific probe to identify and localize Na^+/glucose symporter expression in cell culture, monoclonal antibodies (MAbs) 11A3D6, an IgG_{2b}, and 15E1.2, an IgM, were prepared by standard procedures [13] after immunization of Balb/c mice with apical membranes isolated from confluent LLC-PK_1 cultures [6]. Hybridomas were screened for effects on Na^+-dependent phlorizin binding to brush

border membranes, recloned, subtyped, and expanded in ascites. MAbs were characterized as specific for the renal Na^+/glucose symporter as described previously [6]. Control antibodies were 3F8E12, an IgG_1 obtained from a similar fusion, and commercial preparations of mouse IgG and IgM derived from myelomas. IgGs were purified by Protein A affinity chromatography, and IgMs were purified by gel filtration on Sephadex G-25 [6].

Membrane Fractionation

Apical membranes were isolated from confluent LLC-PK_1 cultures after 2 weeks induction with 5 mM HMBA as described previously [14]. Typically, ten 850-cm^2 roller bottles yielded 130 mg membrane protein. The purity of these preparations was similar to that described previously [14]. Membranes (48 mg protein) were solubilized in 8 ml of 46 mM n-octyl glucoside (Boehringer Mannheim), 2 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.7 (Buffer A) by stirring for 20 min at 0°C. After centrifugation at 160,000*g* for 1 h, the supernatant was injected onto a Mono Q HR 5/5 column (Pharmacia) through a Model 7125 syringe loading sample injector and eluted with a gradient of 0–1 M NaCl in 50 ml of buffer A at a flow rate of 1 ml per min.

Western Blot Analysis

Strips were incubated in the blocking reagent (1% nonfat dry milk, 0.9% sodium chloride, and 10 mM Tris-HCl, pH 7.5) at 37°C for 1 h and then incubated for 24 h at 4°C with 25 μg/ml of either (+) 11A3D6 or (−) 3F8E12 (control antibody). Strips were washed 5 times with 0.9% NaCl, 0.05% Tween-20, 10 mM Tris-HCl, pH 7.5 over a period of 15 min and then incubated with peroxidase-conjugated rabbit anti-mouse (IgG + IgA + IgM) at 1:200 dilution in the blocking solution for 2 h at room temperature. The strips were washed as described above and soaked in 200 ml of 0.05% diaminobenzidine, 0.025% cobalt chloride, 0.02% nickel ammonium sulfate solution [16] for 15 min at room temperature in the dark. Then, 68 μl of 30% hydrogen peroxide was added, and strips were developed and immediately photographed using Polaroid type 55 film.

Immunofluorescence Histochemistry

Confluent monolayers grown on No. 1, 22 × 22-mm glass coverslips were maintained for 2 weeks in the presence or absence of 5 mM HMBA and then were fixed using 2% formaldehyde in PBS for 30 min at 4°C followed by incubation in 0.1 M glycine in PBS for 15 min. A solution of 2% bovine serum albumin in PBS was used as a blocking medium in all wash steps and antibody incubations. Coverslips were washed three times and then incubated with MAb 15E1.2 at 1:5 dilution in 100 μl for 45 min at 37°C in a humidified chamber. Coverslips were then washed three times and then incubated with rabbit anti-mouse (IgG + IgM + IgA) antibody at 1:2,000 dilution for 45 min at 37°C. After three rinses, FITC-conjugated goat anti-rabbit IgG was added for 45 min at 37°C in the dark. Coverslips were rinsed three times and then mounted in gelvatol containing 0.5 mg/ml p-phenylenediamine to minimize photobleaching. Photomicroscopy was carried out using a Leitz microscope with epifluorescence optics and Kodak Tri-X Pan film. Fluorescence staining by a control, nonspecific IgM MAb was negligible.

RESULTS

High-Performance Liquid Chromatography (HPLC) Fractionation and Western Blot Analysis

MAbs 11A3D6 and 15E1.2 specifically recognized by Western blot analysis a 75-kD glycoprotein purified to homogeneity from renal brush border membranes and identified as the Na^+/glucose symporter [6,8].

We next characterized the antigen recognized by MAb 11A3D6 in LLC-PK_1 cultures. From previous estimates based on ^{3}H-phlorizin binding activity, it was calculated that apical membranes from LLC-PK_1 cultures treated for 2 weeks with 5 mM HMBA contain 14-fold higher levels of the symporter compared with membranes from uninduced confluent cultures [6]. By comparison, renal brush border membranes contain a sixfold higher number of phlorizin binding sites compared with membranes from induced cultures [6]. As predicted from these estimates, while MAb 11A3D6 detected a 75-kD antigen by Western blot of solubilized renal brush border membranes, the antigen recognized by MAb 11A3D6 was not expressed at high enough levels to be detectable by Western blot analysis of LLC-PK_1 apical membranes (600–800 μg) even after HMBA induction (not shown).

Solubilized LLC-PK_1 apical membranes from HMBA-induced cells were fractionated by HPLC in order to enrich the symporter before screening fractions by Western blot analysis. Membranes were solubilized in n-octylglucoside and applied to a Mono-Q column. Figure 1A illustrates the fractionation of proteins after elution using a NaCl gradient; proteins were eluted beginning at NaCl concentrations above 0.3 M. MAb 11A3D6 recognized a 75-kD protein, which was eluted at a NaCl concentration of 0.31 M (Fig. 1B). This is the same apparent molecular weight recognized in renal brush border membranes [6]. No immunoreactivity was observed in any other fraction eluting from the column (not shown). Control MAb 3F8E12 did not recognize any protein eluting in any of the fractions.

By contrast, the 75-kD protein was not detectable after similar fractionation of membranes from confluent uninduced cells (not shown). This observation indicated that HMBA induction triggered a quantitative increase in the symporter protein. It is interesting to note that the 75-kD protein of LLC-PK_1 apical membranes eluted at a lower NaCl concentration (0.31 M NaCl) than that required for elution of the 75-kD protein from pig renal brush border membranes (0.35–0.39 M NaCl) [7]. This may reflect differences in net charge of the symporter from cell culture material compared with that derived from the corresponding renal tissue, despite their similar apparent molecular mass. The symporter is a glycoprotein as shown by glucosamine content of the purified protein from renal brush border membranes [7] and by immunoprecipitation of ^{3}H-glucosamine-labeled material from labeled LLC-PK_1 cell extracts using specific antibody (Wu and Lever, unpublished observations).

Immunofluorescence Histochemistry

In order to explore further the basis of increased Na^+/glucose symporter expression in LLC-PK_1 cultures after differentiation inducer treatment, immunofluorescence staining patterns of control and induced cultures were compared. Confluent cultures plated on glass coverslips were treated for 14 days with 5 mM HMBA with medium change every 2 days and then processed for immunofluorescence staining with symporter-specific MAb 15E1.2. Control confluent cultures were maintained in

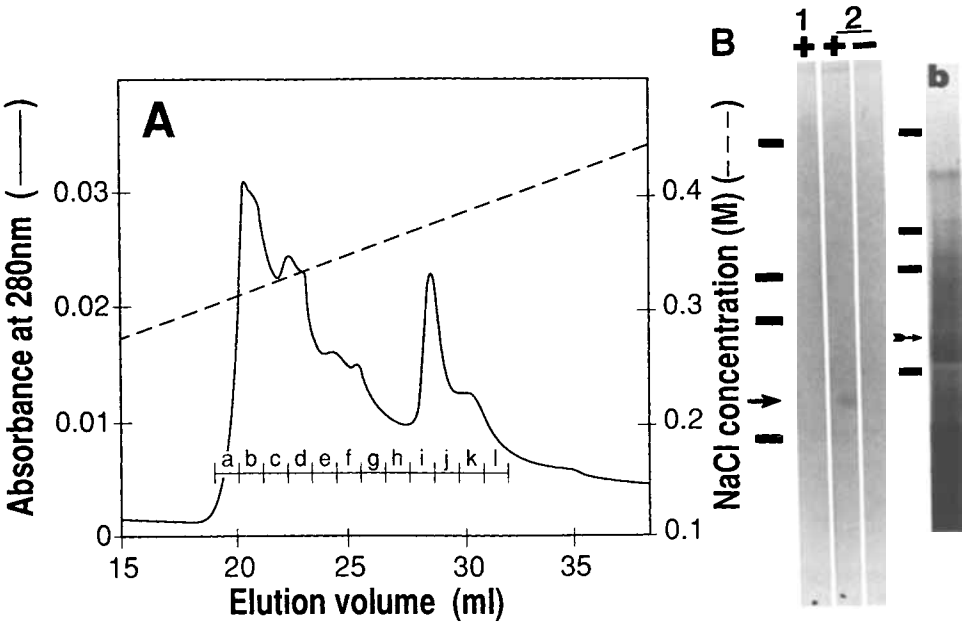

Fig. 1. **A:** Fractionation of solubilized apical membranes by HPLC. Apical membranes (48 mg protein) isolated from confluent LLC-PK_1 cultures after 2 weeks induction with 5 mM HMBA were solubilized with n-octylglucoside and then fractionated on a Mono Q column as described in "Materials and Methods." **B:** Detection of a 75-kD antigen by MAb 11A3D6. Fractions eluted from the Mono Q column were analyzed using 7.5% acrylamide sodium dodecyl sulfate-polycrylamide gel electrophoresis at 17.5 milliamps per gel constant current and transferred to nitrocellulose for Western blot analysis [15]. Molecular weight standards (Bio-Rad) were myosin, 200 kD, β-galactosidase, 130 kD, phosphorylase b, 92.5 kD, and bovine serum albumin, 66 kD, as indicated by the bars. Either (+) purified 11A3D6 or (−) 3F8E12 (control antibody) was used as the first antibody. **Lane 1:** flow-through fraction (500 μg). **Lane 2:** fraction b after Mono Q chromatography (200 μg). The arrow indicates the position of the 75-kD protein. No immunoreactivity was observed in any other fraction eluting from the Mono-Q column. **b:** The protein silver stain pattern of fraction b is also shown.

parallel without inducer treatment. HMBA-treated monolayers exhibited a dramatic increase in the *number* of positively staining cells compared with controls (Fig. 2). This observation indicates that differentiation inducer treatment increases the number of committed cells that express the Na^+/glucose symporter as a proportion of the total cell population. Even under conditions of maximal induction, a proportion of cells in the population remain symporter-negative. Transporter-positive cells often occurred in clusters slightly raised from the plane of focus [17]. Similar results have been obtained with MAb 11A3D6, but staining was more diffuse, presumably because of the lower binding affinity of this MAb (not shown).

DISCUSSION

The facilitative type of glucose transporter is increased after viral transformation [18] or oncogene expression [19], an effect mimicked by tumor promoters. By contrast, expression of the cotransport type is differentiation-specific, tissue-specific,

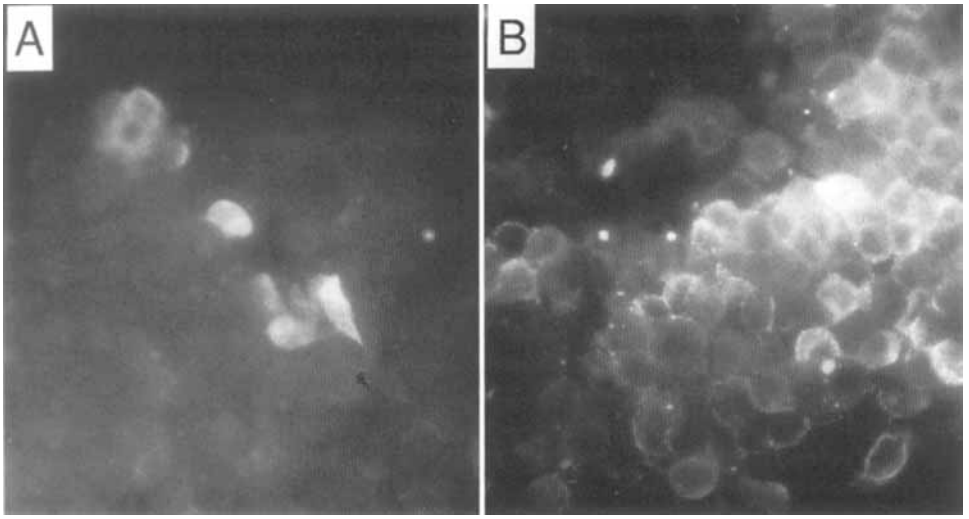

Fig. 2. Immunofluorescence localization of the Na^+/glucose symporter in uninduced (**A**) and HMBA-induced (**B**) LLC-PK_1 cell cultures. Confluent monolayers were maintained for 2 weeks in the presence or absence of 5 mM HMBA, and then immunofluorescence staining was carried out using MAb 15E1.2.

augmented by chemical inducers of differentiation by an unknown mechanism, and antagonized by tumor promoters [12]. Results from the present study indicate that induction of the cotransport type in LLC-PK_1 cells represents a committment of an increased number of cells in the population to express the symporter.

HMBA effects on differentiated gene expression have been extensively studied in nonepithelial cell types such as murine erythroleukemia cells [20] and neuroblastoma [21]. The mechanism of HMBA action in increasing differentiated gene expression in renal epithelial monolayers is not understood. Changes in DNA methylation [22,23], polyamines [24], chromatin structure [25], protein kinase C [26], and increased intracellular Ca^{2+} [27] have been proposed to mediate HMBA stimulation of erythroid differentiation. In renal epithelial cultures, HMBA treatment is followed after a 24-h lag by an elevation in intracellular cyclic AMP levels [28], increased transepithelial resistance [28], and an inhibition of the transport efficiency of the Na^+, K^+, ATPase leading to an elevation of intracellular Na^+ [29,30]. Similar effects of HMBA on the sodium pump and Na^+ levels have been previously noted in murine erythroleukemia cells [31]. The relevence of these ionic changes to the biological effects of HMBA in renal cells is underscored by the observation that variant renal epithelial cells, which are not inducible by HMBA, are also refractory to HMBA inhibition of the sodium pump [32].

Both facilitative [33] and cotransport types [34] of glucose transporters are inducible by glucose deprivation; the facilitative type accumulates because of an alteration of its half-life [35], but the mechanism of accumulation of the cotransport type is unknown. It is very unlikely that the symporter increase triggered by HMBA is due to glucose deprivation since induction is carried out with frequent medium change in high-glucose medium and HMBA stimulates rather than inhibits glucose uptake. Fur-

thermore, variant LLC-PK_1 clones that are refractory to induction of trehalase, an apical enzymatic activity coregulated with the glucose symporter, after growth on glucose-limiting medium still show normal induction by HMBA [36].

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