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Targeting of recombinant Na^+/glucose cotransporter (SGLT1) to the apical membrane

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A full-length Na^+/glucose cotransporter cDNA (SGLT1) from rabbit intestine was subcloned into the pMAMneo mammalian expression vector and transfected by Ca^{2+} precipitation into Madin–Darby canine kidney (MDCK) cells. Stable MDCK transfectants isolated after clonal isolation and selection in G418 exhibited dexamethasone-inducible Na^+/glucose cotransport activity under regulation of the MMTV promoter of the vector. Transfectants expressed the recombinant 75 kDa Na^+/glucose cotransporter subunit as shown by Western blot, and SGLT1 mRNA as shown by Northern blot, but these were undetectable in untransfected MDCK cells. Over 93% of total recombinant transport activity was targeted to the apical membrane. This indicates that the primary amino acid sequence of SGLT1 contains the information necessary to target this transporter to the apical membrane.

Sodium/glucose cotransport; Transfection; Membrane polarity; Madin–Darby canine kidney cell

1. INTRODUCTION

The Na^+/glucose cotransporter (SGLT1) is a secondary active transporter expressed in the apical membranes of kidney proximal tubule and intestine. SGLT1 is a member of a multi-gene family of Na^+-coupled cotransporters [1–3] and has no sequence homology with the gene family of Na^+-independent facilitative glucose transporters [4]. At present, there is no information available concerning the signals necessary to correctly target SGLT1 to its apical membrane destination. While the sorting to the apical membrane of viral glycoproteins [5,6] and of certain enzymes which are targeted via their C-terminal linkage to a glycosyl–phosphatidyl–inositol (GPI) anchor [7] has received considerable study, almost nothing is known about how other types of apical membrane proteins, such as transporters, which exhibit multiple transmembrane segments, are targeted. Transporters sequenced thus far, such as SGLT1, and a member of its gene family, SNST1, the Na^+/nucleoside cotransporter [2], typically are predicted to have 11 transmembrane segments and are devoid of GPI linkages. While SGLT1 is glycosylated on Asn-248, this does not appear to play a role in either targeting or functional activity, based on site-directed mutagenesis at this locus and expression of the mutant mRNA in *Xenopus* oocytes [8], as well as analysis of native Na^+/glucose cotransport activity in tunicamycin-treated LLC-PK_1 cells. (Wu and Lever, submitted) SGLT1 appears to lack a large cleavable signal sequence, as shown by in vitro translation in microsomes [8].

We have stably transfected Madin–Darby canine kidney (MDCK) cells with rabbit intestinal SGLT1 cDNA under regulation of the dexamethasone-inducible MMTV promoter. Recombinant SGLT1 cDNA has previously been transiently expressed in a number of non-polarized cell types and has been shown by kinetic and electrophysiological criteria to encode a transporter with the characteristics of the classical Na^+/glucose cotransporter [9,10]. The MDCK cell line is a polarized renal epithelial cell line which has been used to study mechanisms involved in the biogenesis of membrane polarity in epithelial cells [6,11]. It is an ideal host cell for these studies since it is not of proximal tubule origin and therefore does not express endogenous SGLT1.

Our results demonstrate that information necessary for proper targeting of transport-competant SGLT1 to the apical membrane is encoded within its primary amino acid sequence.

2. MATERIALS AND METHODS

MDCK clone 4, isolated from the parental MDCK cell line as reported previously [13], was grown in a 50:50 mixture of Ham's F12:Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum, 5 mM glutamine, 0.12% sodium bicarbonate and 15 mM HEPES, pH 7, in an atmosphere of 5% CO_2 in air. Transfectant cell lines were maintained in this medium supplemented with 500 μg/ml G418. For polarity studies, cells were grown in 4.9 cm^2 Cyclopore-PET polyethylene terephthalate track-etch membrane filter cups (Falcon, #3090; Becton-Dickenson, Lincoln Park, NJ). Transepithelial resistance of filter-grown monolayers was measured using

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Abbreviations: dex, dexamethasone; MDCK, Madin–Darby canine kidney; αMGP, α-D-methylglucopyranoside

an epithelial voltohmmeter (EVOM; World Precision Instruments Inc., New Haven, CT). Hexamethylene bisacetamide was obtained from Aldrich, Milwaukee, WI. Dexamethasone and G418 were from Sigma, St. Louis, MO.

A full-length rabbit intestinal SGLT1 inserted in pBluescript KS, pMJC424 [1] was the generous gift of Dr. Ernest Wright, UCLA. A 2.4 kb *Xho*I/*Xba*I restriction fragment from pMJC424 was ligated to the *Nhe*I/*Xho*I site of the pMAMneo vector (Clontech, Palo Alto, CA) in an orientation permitting expression of the open reading frame. The resulting pMAMneo-SGLT1 plasmid DNA was purified by polyethylene glycol precipitation [14] before use in transfection experiments, and the construct was confirmed by Southern blot and double-stranded DNA sequencing.

For isolation of stably transfected cell lines, MDCK cells plated at 2×10^6 cells per 6 cm dish were harvested by trypsinization after 24 h and then transfected with 20 μg plasmid DNA using the Ca^{2+}-precipitation method of Graeve et al. [15]. Following the glycerol step, cells were plated onto a 10 cm dish, grown to confluence and then split 1:6 in medium containing 500 μg/ml G418. Resistant colonies were harvested and cloned by limiting dilution in 96-well microtiter plates in the presence of 500 μg/ml G418.

Total RNA was extracted from cell cultures using the guanidine isothiocyanate procedure and poly(A)$^+$ RNA was then isolated after two purifications on an oligo(dT)-cellulose column [16]. Poly(A)$^+$ RNA was denatured and fractionated on a 1% agarose gel containing formaldehyde [14]. The RNA was then transferred to a Duralon-UV membrane and UV-crosslinked using a Stratalinker1800, both obtained from Stratagene, La Jolla, CA. An SGLT1 probe was prepared from pMJC424 by preparing a 442 bp N-terminal *Bam*H1 fragment. The filters were prehybridized for 2 h and then hybridized overnight at 42°C with 1×10^6 cpm/ml of probe labeled by the random-primer method in a solution containing 50% formamide, 5 × SSPE (20 × SSPE = 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA-Na_2, pH 7.4), 1 × PE (1 × PE = 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% Ficoll, 5 mM EDTA, 0.2% polyvinylpyrrolidone) and 150 μg/ml denatured salmon sperm DNA. Blots were washed in successive changes of 0.1 × SSC, 0.1% SDS for 15 min at room temperature, 15 min at 42°C, and 15 min at 55°C. Filters were then exposed overnight, unless stated otherwise, at −80°C to X-OMAT AR films (Kodak, Rochester, NY) with double intensifying screens.

For transport assay, transfectant cells were plated at a confluent cell density and, unless stated otherwise, recombinant gene expression under regulation of the MMTV promoter of the vector was induced by the addition of 2 μM dexamethasone (dex) for 24 h before transport assay. Maximal levels of SGLT1 expression were observed after a 24 h induction period. Cell monolayers on 35 mm dishes were assayed for Na$^+$-dependent αMGP uptake activity as described by Peng and Lever [17]. Protein content of cell monolayers was determined as described previously [18].

Western blot analysis was carried out using monolayers solubilized in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonylfluoride (PMSF), 1 μg/ml aprotinin, 1% Triton X-100 [14] and harvested with a rubber policeman. The lysate was centrifuged at 12,000 × *g* for 2 min at 4°C. Supernatants (300 μg protein) were resolved by SDS-PAGE (10% acrylamide) overnight at 7.5 mA constant current. Proteins and pre-stained molecular weight markers (Bio-Rad, Richmond, CA) were electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) at room temperature using an ABN Model SD 1000 electrotransfer unit. The filter was incubated in a blocking solution containing 5% non-fat milk in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Tween 20 for 2 h at room temperature. The filter was then incubated with a polyclonal antibody against the pig renal Na$^+$/glucose cotransporter at 1:3,000 dilution in blocking solution overnight at 4°C and washed 6 times with Tris-buffered saline (TBS) containing 0.01% Tween 20. It was next incubated with horseradish peroxidase-conjugated secondary antibody at 1:300 dilution for 3 h at room temperature and then washed 6 times with TBS. Detection was carried out using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) with an exposure time of 10 s.

3. RESULTS AND DISCUSSION

Stable MDCK clones, obtained by G418 selection after calcium phosphate-mediated transfection with pMAM neo-SGLT1 DNA, were assayed as confluent monolayers for expression of SGLT1 activity using Na$^+$-dependent uptake of the non-metabolizable analog, α-methylglucopyranoside (αMGP). αMGP is specifically transported by Na$^+$-dependent glucose cotransporters, including SGLT1, and is not recognized by facilitative glucose transporters present in the basolateral membrane [18]. SGLT1 transport activities of 3 transfectant clones, representing high, medium and low levels of expression, are shown in Table I. Na$^+$-dependent glucose uptake activity was sensitive to inhibition by 10 μM phlorizin, a specific inhibitor of Na$^+$/glucose cotransporters (Table I) and was stable to at least 4 weeks passage in the presence of 500 μg/ml G418; but a gradual decline in activity was noted after more prolonged culture. The lowest level of SGLT1 activity shown, 4 nmol/h/mg protein, is similar to the native level of cotransporter expression in confluent, differentiated LLC-PK_1 cells, a porcine renal epithelial cell line with properties of renal proximal tubule [19]. SGLT1 transport activity was not detectable in untransfected MDCK cells (not shown), as expected based on their presumed collecting duct or distal tubule origin [20]. The highest level of SGLT1 activity shown represents a 15-fold increase over the native level of expression in LLC-PK_1 cells.

Fig. 1 shows the result of Northern blot analysis of poly(A)$^+$ RNA isolated from each of the 3 transfectant clones listed in Table I. The SGLT1 probe detected a 2.4 kb mRNA species corresponding to the transcript encoded by the symporter cDNA insert plus a 3.2 kb species which represented a transcript containing this

Table I

Na$^+$/glucose cotransport activity of MDCK cell transfectants

Transfectant	Transport activity (nmol/h/mg protein)	
	Control	+Phlorizin
3H12	60.9 ± 1.37	8.96 ± 0.88
8A1	8.16 ± 0.06	4.38 ± 0.16
5E1	4.08 ± 0.43	2.07 ± 0.06

Confluent monolayers of transfectant clones were assayed for αMGP uptake activity in the presence or absence of 10 μM phlorizin. Recombinant gene expression was induced with 2 μM dexamethasone for 24 h before transport assay. Na$^+$-dependent uptake is shown after subtraction from values obtained in the absence of Na$^+$. Values are means ± S.E.M. of triplicate determinations.

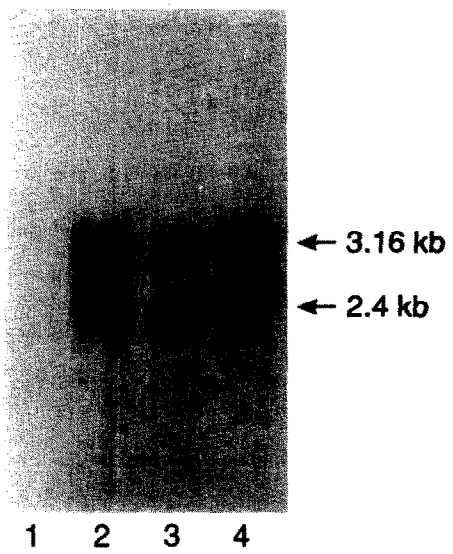

Fig. 1. Expression of SGLT1 mRNA in transfectants. Samples (1 μg) of poly(A)$^+$ RNA from each of the indicated cell types were resolved by formaldehyde–agarose gel electrophoresis and transferred to Duralon-UV filters. Blots were hybridized with a 400 bp N-terminal fragment prepared from SGLT1 plasmid pMJC424 and labeled by nick-translation. Lane 1, MDCK cells; lane 2, transfectant 3H12; lane 3, transfectant 5E1; lane 4, transfectant 8A1.

insert plus a poly(A) tail encoded by the vector. Among the 3 transfectants, levels of SGLT1 mRNA were approximately proportional to the levels of transport activity. No hybriziation to poly(A)$^+$ RNA from untransfected MDCK cells was detected (Fig. 1, lane 1), as predicted from the absence of expression of this transporter in MDCK cells.

Furthermore, no SGLT1 expression could be detected in untransfected cells by PCR analysis (not shown). By comparison, the rabbit intestinal SGLT1 probe detected transcripts of 2.2 and 3.9 kb in LLC-PK$_1$ cells, representing native symporter expression [21].

Fig. 2 illustrates the result of Western blot analysis of total cell protein extracted from transfectant 3H12. A polyclonal antibody [22] prepared against the purified porcine renal Na$^+$/glucose cotransporter 75 kDa subunit [23] was used for immunodetection. This antibody cross-reacted with the 75 kDa recombinant rabbit intestinal SGLT1 protein expressed after dex induction of the transfectant. In the absence of dex induction, levels of this protein in 3H12 cells were greatly diminished. Treatment of cultures during the final 24 h with 1 μM tunicamycin, a concentration sufficient to block incorporation of [^{3}H]glucosamine into protein, dit not alter the apparent mobility of the immunostained band. Treatment with tunicamycin was associated with increased levels of the 75 kDa SGLT1 subunit in either the presence or absence of dex. Tunicamycin treatment also caused a reproducible increase in native SGLT1 transport activity in LLC-PK$_1$ cell (Wu and Lever, submitted). The mechanism of this stimulation by tunicamycin is not understood. No immunostaining of a 75 kDa band was detected in untransfected MDCK cells (Fig. 2).

Monolayers of transfectant 3H12 were assayed for polarity of αMGP uptake activity expressed at 24 h after induction of recombinant gene expression by 2 μM dex. Cells were grown on Cyclopore-PET filter units such that a polarized monolayer was formed with the basolateral surface in contact with the filter [11]. Polarity of transport was assessed by addition of labeled substrate to either the apical or basolateral surface. The transepithelial resistance of each monolayer was greater than 150 $\Omega \cdot cm^{-2}$, indicating that the monolayer was intact and retained effective tight junction barriers to the passage of small molecules by a paracellular route. The apical membrane surface exhibited a Na$^+$-dependent αMGP uptake activity of 13.3 ± 0.8 nmol/h/mg protein (n = 3), whereas the basolateral surface had a transport activity of 1.0 ± 0.2 nmol/h/mg protein (n = 3), after subtraction of mean Na$^+$-independent uptake values of 0.2 and 0.5 nmol/h/mg, respectively. The observed 13- fold ratio of apical:basolateral cotransport activity was lower than the 100-fold ratio observed for expression of the native cotransporter in LLC-PK$_1$ cells [24], but this difference may partially reflect differences in surface areas of the two membrane domains in these different cell lines. Phlorizin is a specific, high-affinity competitive inhibitor of Na$^+$/glucose cotransport activity [25]. In the presence of 10 μM phlorizin and Na$^+$, apical αMGP uptake was reduced by 85% to 1.9 ± 0.1 nmol/ h/mg (n = 3), whereas basolateral uptake of 0.1 ± 0.04 nmol/h/mg (n = 3) represented 90% inhibition by phlorizin. The total level of apical glucose cotransport activity of filter-grown monolayers was comparable to the value of 15.2 ± 0.3 nmol αMGP/h/mg protein (n = 3) observed for parallel cultures of 3H12 monolayers grown on plastic culture dishes. Dome formation was evident in transfectant monolayers grown on plastic, further indicating that transfectants were not impaired in tight junction formation and transepithelial fluid transport processes.

Morphometric analysis of MDCK monolayers grown on nitrocellulose filters indicated that the basolateral membrane area was 7.6-fold greater than that of the apical domain [26], therefore, on a surface area basis, the density of SGLT1 cotransporters in the apical membrane greatly exceeds that on the basolateral surface. Our results indicate that 93% of total recombinant SGLT1 transport activity is targeted to the apical membrane in MDCK cells. Due to the low levels of expression of recombinant rabbit SGLT1, we were unable to rule out by immunological criteria the possibility that an inactive transporter is also present on the basolateral membrane. However, transport activity measurements clearly demonstrate that the primary amino acid sequence of the SGLT1 transporter subunit contains the information necessary for functional transporter to

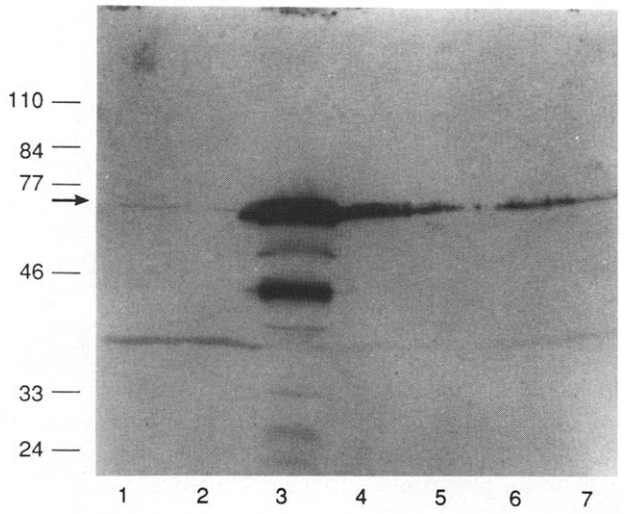

Fig. 2. Immunodetection of Na^+/glucose cotransporter subunit in transfectant clones. Monolayers on 100 mm dishes were treated with 5 mM hexamethylene bisacetamide for 12 days. Where stated, 2 μM dexamethasone (dex) was added during the final 24 h before cells were harvested for Western blot analysis. Lane 1, MDCK cells; lane 2, MDCK cells plus dex; lane 3, LLC-PK_1 cells; lane 4, transfectant 3H12 plus tunicamycin plus dex; lane 5, transfectant 3H12 plus dex; lane 6, transfectant 3H12 plus tunicamycin; lane 7, transfectant 3H12. Tunicamycin, 1 μM, was added where stated. The arrow indicates the position of the 75 kDa Na^+/glucose cotransporter subunit.

reach its proper cellular destination in polarized epithelial cells.

This represents the first demonstration of polarized sorting of a recombinant mammalian cotransporter. DNAs for several brush border membrane hydrolases of kidney proximal tubule [27–29] and human CD8 glycoprotein [30] were also sorted to the apical membrane when expressed in MDCK cell transfectants, suggesting that an apical sorting signal may reside in their cDNA sequence. In the case of aminopeptidase N, Noren and Sjostrom [31] provided evidence for an apical sorting signal located in the catalytic headgroup of the enzyme.

Another significant finding from this study is the observation that recombinant rabbit intestinal SGLT1 expressed in MDCK cells cross-reacted with antibody prepared against a 75 kDa Na^+/glucose cotransporter subunit purified to homogeneity from pig kidney. This is the first evidence linking the 75 kDa protein purified by conventional fractionation and reconstitution of activity [23] with the primary sequence of SGLT1 cDNA encoding the transport activity [1]. SGLT1 is expressed with the identical sequence in both kidney and intestine of the rabbit [32]. Based on the cDNA sequence of a partial cDNA clone, pig kidney SGLT1 is predicted to exhibit 87% amino acid similarity with SGLT1 from rabbit intestine [21], therefore, the observed cross-reactivity between our antibody to the pig kidney transporter subunit and the recombinant rabbit intestinal protein is not unexpected.

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REFERENCES

[1] Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 330, 379–381.
[2] Pajor, A.M. and Wright, E.M. (1992) J. Biol. Chem. 267, 3557–3560.
[3] Kong, C.-T., Yet, S.-F. and Lever, J.E. (1993) J. Biol. Chem. 268, 1509–1512.
[4] Gould, G.W. and Bell, G.I. (1990) Trends Biochem. Sci. 15, 18–23.
[5] Rodriquez-Boulan, E. and Pendergast, M. (1980) Cell 20, 45–44.
[6] Simons, K. and Fuller, S.D. (1985) Annu. Rev. Cell Biol. 1, 243–288.
[7] Ferguson, M.A.J. and William, A.F. (1988) Annu. Rev. Biochem. 57, 285–320.
[8] Hediger, M.A., Mendlein, J., Lee, H.S. and Wright, E.M. (1991) Biochim. Biophys. Acta 1064, 360–364.
[9] Birnir, B., Lee, H.S., Hediger, M.A. and Wright, E.M. (1990) Biochim. Biophys. Acta 1048, 100–104.
[10] Ikeda, T.S., Hwang, E.S., Coady, M.J., Hirayama, B.A., Hediger, M.A. and Wright, E.M. (1989) J. Membr. Biol. 110, 87–95.
[11] Cereijido, M., Ehrenfeld, J., Meza, I. and Martinez-Palomo, A. (1980) J. Membr. Biol. 52, 147–159.
[12] Vega-Salas, D.E., Salas, P.J.I. and Rodriguez-Boulan, E. (1987) J. Cell Biol. 104, 905–916.
[13] Thomas, S.R., Schultz, S.G. and Lever, J.E. (1982) J. Cell. Physiol. 113, 427–432.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Press, NY.
[15] Graeve, L., Drickamer, K. and Rodriguez-Boulan, E. (1989) J. Cell. Biol. 109, 2809–2815.
[16] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology, Elsevier, N.Y.
[17] Peng, H. and Lever, J.E. (1992) J. Cell. Physiol. (in press).
[18] Lever, J.E. (1987) Methods Enzymol. 146A, 376–383.
[19] Amsler, K. and Cook, J.S. (1982) Am. J. Physiol. 242, C94–C101.
[20] Handler, J.S., Perkins, F.M. and Johnson, J.P. (1980) Am. J. Physiol. 238, F1–F9.
[21] Ohta, T., Isselbacher, K.J. and Rhoads, D.B. (1990) Mol. Cell. Biol. 10, 6491–6499.
[22] Wiley, L.M., Lever, J.E., Pape, C. and Kidder, G.M. (1991) Dev. Biol. 143, 149–161.
[23] Wu, J.-S.R. and Lever, J.E. (1987) Biochemistry 26, 5958–5962.
[24] Rabito, C.A. (1981) Biochim. Biophys. Acta 649, 286–296.
[25] Semenza, G., Kessler, M., Hosang, M., Weber, J. and Schmidt, U. (1984) Biochim. Biophys. Acta 779, 343–379.
[26] von Bonsdorff, C.-H., Fuller, S.D. and Simons, K. (1985) EMBO J. 4, 2781–2792.
[27] Jalal, F., Lemay, G., Zollinger, M., Berteloot, A., Boileau, G. and Crine, P. (1991) J. Biol. Chem. 266, 19826–19832.
[28] Casanova, J.E., Mishumi, Y., Ikehara, Y., Hubbard, A.L. and Mostov, K.E. (1991) J. Biol. Chem. 266, 24428–24432.
[29] Nelson, W.J. (1992) Regulation of cell surface polarity from bacteria to mammals, Science 258, 948–955.
[30] Migliaccio, G., Zurzolo, C., Nitsch, L., Obici, S., Lotti, L.V., Torrisi, M.R., Pascale, M.C., Leone, A. and Bonatti, S. (1990) Eur. J. Cell. Biol. 52, 291–296.
[31] Noren, O. and Sjostrom, H. (1992) FEBS Lett. 308, 14–17.
[32] Coady, M.J., Pajor, A.M. and Wright, E.M. (1990) Am. J. Physiol. 259, C605–C610.