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Molecular evidence for two renal Na^+/glucose cotransporters

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Previous studies have shown that two kinetically and genetically distinct Na^+/glucose cotransporters exist in mammalian kidney. We have recently cloned and sequenced one of the rabbit renal Na^+/glucose cotransporters (SGLT1) and have found that it is identical in sequence to the intestinal Na^+/glucose cotransporter. Northern blots showed that SGLT1 mRNA was found predominantly in the outer medulla of rabbit kidney. Injection of mRNA from outer medulla and outer cortex into *Xenopus* oocytes resulted in equal expression of Na^+-dependent sugar uptake, indicating that the outer cortex sample contained mRNA encoding both SGLT1 and a second Na^+/glucose cotransporter. Western blots using antipeptide antibodies against SGLT1 showed that the SGLT1 protein is more abundant in outer medulla than outer cortex. However, brush border membrane vesicles prepared from outer cortex had a greater capacity for Na^+-dependent glucose transport, indicating the presence of a second transporter in the vesicles from outer cortex. It appears that the cloned renal Na^+/glucose cotransporter, SGLT1, is the 'high affinity, low capacity' transporter found predominantly in outer medulla. There is evidence that a second transporter, the 'low affinity, high capacity' transporter, is in outer cortex. Finally, the cDNA and protein sequences of the two renal Na^+/glucose cotransporters are predicted to differ by more than 20%.

Introduction

The uptake of glucose in the rabbit kidney appears to be handled by at least two different Na^+/glucose cotransporters. Studies by Barfuss and Schafer [1] and Turner and Moran [2,3] have shown that these transporters differ in their kinetic properties and in their spatial distribution in the kidney. A low affinity transporter with a 1:1 Na/glucose coupling stoichiometry is found in the outer cortex, and a high-affinity transporter with a 2:1 coupling stoichiometry is found in the outer medulla. Lately, however, these studies have been questioned by authors who claim that there is only a single renal Na^+/glucose cotransporter [4].

We have recently isolated a partial-length cDNA encoding a renal Na^+/glucose cotransporter [5] which is identical in amino acid sequence to the rabbit intestinal Na^+/glucose cotransporter, SGLT1 [6] (see also Ref. 7). While the kinetic properties of the cloned transporter show similarities to both transporters of Turner and Moran [2,3], SGLT1 has a low K_m for D-glucose (0.1 mM) and a Na^+ Hill coefficient of 1.7 [8], in common with the high-affinity transporter of the outer medulla. Our initial localization studies of the cloned renal SGLT1 suggested that it was found in cortex, leading to some confusion about the identity of the cloned transporter relative to the kinetic studies of Turner and Moran [2,3]. However, the samples used in our initial Northern [5] and Western [9] blots came from whole cortex, which also included the outer stripe of the medulla.

In this paper we have combined several approaches to determine the localization of the cloned renal SGLT1. We show that the cloned renal SGLT1 is found predominantly in the outer medulla and provide evidence for the existence of a second renal Na^+/glucose cotransporter.

Methods

Kidney dissection. New Zealand white rabbits weighing 2–2.5 kg were killed with an i.v. injection of 50 mg/kg sodium pentobarbital. For each rabbit, one kidney was used for preparation of RNA while the other kidney was used for preparation of brush border membrane vesicles. The kidneys were perfused with ice-cold 0.9% NaCl, 10 mM Na-phosphate (pH 7.4). They were cut in half longitudinally on a glass plate over ice and then dissected with a clean razor blade.

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Most of the inner medulla was removed and discarded. The outer and inner stripes of the medulla and portions of the inner cortex were shaved away and these formed the samples called Outer Medulla. The remaining 1–2 mm of cortex was saved as Outer Cortex *.

RNA preparation. The samples used for preparation of RNA were immediately frozen in liquid nitrogen and then stored until use, up to one week, at −80°C. The RNA was prepared by CsCl gradient centrifugation [10] using a modified homogenization buffer [11]. Poly(A$^+$) RNA was selected by oligo(dT) chromatography [12] but LiCl replaced NaCl in all solutions. Before the final precipitation, the mRNA was centrifuged through a Costar 0.45 μm filter to remove any particulates from the oligo(dT) column that would otherwise clog an oocyte injection needle. RNA samples were stored at −80°C.

Northern blot. The mRNA from each sample was separated in a 1% agarose gel containing 0.66 M formaldehyde [13], transferred to reinforced nitrocellulose filters (Duralose, Stratagene), and fixed to the filters by UV crosslinking (Stratalinker, Stratagene). Filters were prehybridized at least six hours at 42°C in 50% formamide, 5 × SSC [13], 3 × Denhart's [13], 25 mM sodium phosphate buffer (pH 6.5), 0.2% sodium dodecyl sulfate (SDS), 10% Dextran sulfate, and 250 μg/ml Prehybe-HS (Lofstrand Labs). Purified cDNA consisting of the coding region of the rabbit intestinal Na$^+$/glucose cotransporter (described in Ref. 5) was labelled with ^{32}P-dCTP using an Oligolabelling kit (Pharmacia) and used as the probe. The blots were hybridized at 42°C overnight. Washes were as follows: 15 min at room temperature in 5 × SSC, 0.1% SDS, 0.05% sarkosyl; 15 min at 60°C in 5 × SSC, 0.1% SDS, 0.05% sarkosyl; three 15 min washes at 60°C in 0.1 × SSC, 0.1% SDS [5]. Autoradiography was carried out at −80°C and autoradiograms were scanned with a Hoefer GS300 densitometer.

Oocyte injections. Stage V and VI oocytes [14] from *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were dissected and defolliculated as described previously [15]. They were injected with 50 nl of water or mRNA (1 μg/μl) one day following their isolation. Uptakes were measured five days later.

Oocyte uptake experiments. The uptake of methyl α-D-[U-^{14}C]glucopyranoside (αMDG) or L-[^{3}H]alanine into oocytes was measured as described in Refs. 8 and 15. Oocytes were incubated in a solution containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes-Tris (pH 7.5) and either 50 μM ^{14}C-αMDG or 100 μM L-[^{3}H]alanine. Uptakes were measured for 1 h (αMDG) or 10 min (alanine). Choline Cl replaced NaCl in the wash solution.

Membrane preparation. Brush border membrane vesicles (BBMV) were prepared by a $MgCl_2$ precipitation method (Ref. 16 and Shirazi-Beechey, personal communication) *. Briefly, the dissected kidneys were homogenized using a Polytron (Brinkmann) in 300 mM mannitol, 1 mM EDTA, 20 mM Mes-Tris (pH 6.5), 0.1 mM phenylmethylsulfonyl fluoride. $MgCl_2$ was added to 10 mM and the samples were mixed 20 min. The samples were centrifuged four times at 6000 × g for 10 min each, taking the supernatant each time. The membranes were pelleted twice at 35 000 × g for 30 min, and resuspended each time in 200 mM mannitol, 50 mM KCl, 10 mM Hepes-Tris (pH 7.4). Protein concentrations were measured by Bio-Rad assay. BBMV were used for uptake experiments immediately after preparation.

Western blots. Western blot analysis was used to identify the Na$^+$/glucose cotransporter in renal brush border membranes [9] using two antibodies raised to peptides selected from different regions of the intestinal Na$^+$/glucose cotransporter. Ab-E was raised against amino acids 402–420 and Ab-C was raised against amino acids 604–615. Specific binding of the antibodies was determined from the difference between binding in the presence and absence of the peptide to which the antibody was raised. Western blots were scanned with a Hoefer GS300 densitometer.

Transport experiments using BBMV. The uptake of glucose into brush border membrane vesicles was measured using a rapid filtration assay [17]. Transport solutions contained 100 mM NaCl or cholineCl, 50 mM KCl, 10 mM Hepes-Tris (pH 7.4), 25 μg/ml valinomycin, and 50 μM–20 mM D-[^{3}H]glucose. Initial rates of transport were estimated from 3 s incubations at 20–22°C. Uptake was stopped with 1 ml ice-cold stop buffer containing 100 mM NaCl, 50 mM KCl, 10 mM Hepes-Tris (pH 7.4). The reaction mixture was filtered through a 0.45 μm nitrocellulose filter (BA85, Schleicher and Schuell) and washed with 4 ml stop buffer. The radioactivity retained on the filter was measured by liquid scintillation counting.

Results

The hybridization of the SGLT1 Na$^+$/glucose cotransporter cDNA to renal and intestinal mRNA at high stringency is shown in Fig. 1. As reported in previous studies [5,6], the predominant hybridization

* The entire cortex of a rabbit kidney is about 3 mm thick and the dissection done by Turner and Moran [2], using a microtome, took only the outermost 0.5 mm of the cortex. In our study, the kidneys had to be dissected as quickly as possible to prevent RNA degradation, thus precluding a detailed dissection.

* This was the same procedure used to purify the renal BBMV, donated by S. Shirazi-Beechey, used in our previous study [9].

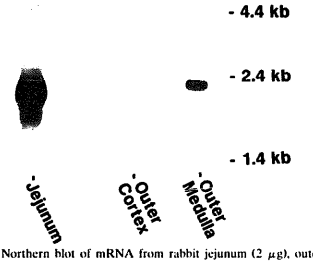

Fig. 1. Northern blot of mRNA from rabbit jejunum (2 μg), outer cortex (5 μg), and outer medulla (5 μg). The blot was probed at high stringency using cDNA encoding SGLT1 as a probe. The position of size standards is shown at right.

signal in mRNA from jejunum was a single band at approximately 2.3 kb. Poly(A$^+$) RNA prepared from outer cortex and outer medulla of rabbit kidney also had single hybridization signals at 2.3 kb (Fig. 1). Densitometric scans showed that the hybridization intensity was three times greater in mRNA from outer medulla than from outer cortex.

The mRNA from the same preparation as that used in the Northern blot was then injected into *Xenopus* oocytes and the expression of Na^+-dependent glucose transport was measured using α-methyl-D-glucopyranoside (αMDG) as a substrate (Fig. 2). While the Northern blot showed that mRNA encoding SGLT1 was more abundant in outer medulla than outer cortex, there was approximately equal expression of αMDG uptake from both sources of mRNA. This was seen at αMDG concentrations of 50 μM (Fig. 2) and 1 mM (data not shown).

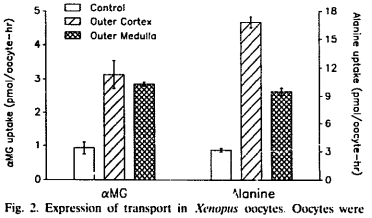

Fig. 2. Expression of transport in *Xenopus* oocytes. Oocytes were injected with water (control), or with mRNA prepared from outer cortex and outer medulla and transport was measured five days later. The left side of the figure shows expression of α-methyl-D-glucopyranoside (αMDG) uptake and the expression of L-alanine uptake is shown on the right.

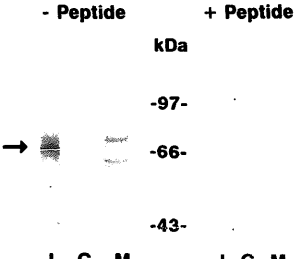

Fig. 3. Western blot of rabbit brush border membrane vesicles from jejunum (J), outer cortex (C), and outer medulla (M) using an antipeptide antibody against amino acids 402–420 of SGLT1. Each lane contained 20 μg protein. The proteins were separated in 8% SDS-PAGE gels. The position of size standards is shown in the center. Immunoreactivity was measured in the absence (– peptide) and presence (+ peptide) of the peptide used to raise the antibody in order to determine specific binding of the antibody. The arrow indicates specific binding to a protein at approx. 70 kDa.

The uptake of L-alanine in these oocytes was measured as an indicator of mRNA integrity (Fig. 2), since a previous study [15] showed that oocytes injected with mRNA from whole renal cortex expressed L-alanine uptake. The expression of L-alanine transport was greater in ooeytes injected with mRNA from outer cortex than outer medulla. Our previous study [15] showed that approximately half the L-alanine uptake expressed from whole cortex was Na^+-dependent.

Fig. 3 shows a Western blot of renal BBMV from outer cortex and outer medulla using an antipeptide antibody against amino acids 402–420 of SGLT1 [9]. A second antipeptide antibody, against amino acids 604–615, gave identical blockable signals (data not shown). Only the bands blocked by the peptide used to raise the antibody are considered to represent specific antibody binding (see Ref. 9). As seen previously for rabbit intestinal and renal BBMV [9], specific binding of the antibodies was seen at approx. 70 kDa in jejunum, outer cortex and outer medulla (Fig. 3). Densitometric scans of the Western blots indicated that abundance of the transporter in outer medulla was 3.5-fold greater than in outer cortex, as was seen for mRNA abundance in the Northern blot of Fig. 1. In jejunum the transporter migrates as a broad band, most likely representing a single glycosylated protein (see also Ref. 9). In the renal samples the signal is split into two bands (Fig. 3), due to the presence of an abundant renal protein which comigrates with the transporter at a size of approx. 70 kDa (results of Ponceau staining of blots not shown).

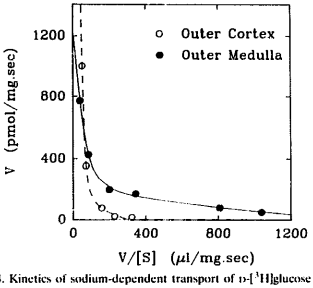

Fig. 4. Kinetics of sodium-dependent transport of D-[^{3}H]glucose (50 μM–20 mM) in brush border membrane vesicles prepared from outer cortex and outer medulla. The sodium-dependent component of transport was determined from the difference of uptake in Na^+ and choline-containing buffers. Three second uptakes were measured. The data represent the means of triplicate determinations. The kinetic parameters for outer cortex are: $K_{m1} = 0.34 \pm 0.16$ mM, $V_{max1} = 82 \pm 23$ pmol/mg per s; $K_{m2} = 68 \pm 21$ mM, $V_{max2} = 4058 \pm 856$ pmol/mg per s. The kinetic parameters for outer medulla are: $K_{m1} = 0.11 \pm 0.03$ mM, $V_{max1} = 153 \pm 17$ pmol/mg per s; $K_{m2} = 14 \pm 3$ mM, $V_{max2} = 1066 \pm 64$ pmol/mg per s. The data were fitted to a two-component Michaelis-Menten model using the Sigmaplot 4.0 (Jandel Scientific) computer program.

Brush border membrane vesicles from the same preparation as those used in the Western blot of Fig. 3 were analyzed for kinetics of Na^+-dependent glucose transport (Fig. 4). There were clear differences in kinetics between vesicles prepared from outer cortex and outer medulla, although, as expected from the dissection (see Methods), both preparations showed evidence of two saturable pathways. As was seen in previous studies [2,3] there was a 'low affinity, high capacity' pathway and a 'high affinity, low capacity' pathway. The transport of glucose via the high-affinity pathway ($K_m \approx 0.11$–0.34 mM) * was 2-fold greater in vesicles from outer medulla than outer cortex and followed the distribution of SGLT1 protein abundance seen in Western blots (Fig. 3). Interestingly, this distribution of SGLT1 transport and protein abundance paralleled the SGLT1 mRNA distribution (Fig. 1). In contrast, the Na^+/glucose transport capacity (V_{max}) of the low-affinity pathway ($K_m \approx 14$–68 mM) predominated in vesicles from outer cortex, and was 4-fold higher in outer cortex than in outer medulla.

* We do not distinguish between the K_m values for the same pathway in outer cortex and outer medulla (e.g. 0.11 vs. 0.34 mM), and consider the differences to be a consequence of curve-fitting.

Discussion

Our results support the hypothesis proposed by Barfuss and Shafer [1], and Turner and Moran [2,3], that the rabbit kidney contains two kinetically different Na^+/glucose cotransporters found in different parts of the kidney. Previously, we showed that one of the renal transporters is identical in amino acid sequence to the intestinal Na^+/glucose cotransporter, SGLT1 [5]. The results reported here indicate that the cloned renal transporter is the 'high affinity' transporter found predominantly in the outer medulla. This study also provides evidence that a second renal Na^+/glucose cotransporter must exist, and that it is different in sequence from SGLT1. These results verify genetic studies predicting two renal Na^+/glucose cotransporters, one of which is identical to the intestinal transporter [18].

The cloned renal Na^+/glucose cotransporter, SGLT1, appears to be the 'high affinity, low capacity' transporter of the outer medulla, described by previous studies [1,2]. The mRNA encoding the SGLT1 transporter and the protein recognized by two antipeptide antibodies against SGLT1 were found predominantly in the outer medulla. Furthermore, the K_m for glucose of the cloned transporter [8] was remarkably similar to the K_m of the high-affinity transporter in rabbit renal brush border membrane vesicles (Fig. 4).

We believe that a second renal Na^+/glucose cotransporter must exist in the outer cortex for the following reasons. First, there was greater expression of glucose transport in oocytes injected with outer cortex mRNA than would be predicted from the relative abundance of SGLT1 mRNA in outer cortex (Figs. 1 and 2). Second, the maximal rate of glucose transport in brush border membrane vesicles from outer cortex exceeded the transporter abundance estimated from Western blots. While the antibodies indicated that the outer medulla contained 3.5-fold more SGLT1 protein than outer cortex, there was a 3.4-fold greater capacity (V_{max}) of Na^+-dependent glucose uptake in vesicles from outer cortex.

Since the SGLT1 cDNA did not appear to hybridize with the mRNA encoding the second Na^+/glucose cotransporter under high stringency conditions, we can conclude that the mRNA sequences of the two Na^+/glucose cotransporters are not very similar and probably differ by more than 20%. The human intestinal SGLT1 cDNA has 82% identity with the rabbit SGLT1 sequence [19] and a strong hybridization signal was seen in a Northern blot at high stringency [5]. The two renal Na^+/glucose cotransporters also must differ in protein sequence. The antipeptide antibodies used in this study recognize two different portions of SGLT1. Since there was no apparent cross-reactivity with the second Na^+/glucose cotransporter, the protein se-

quences at the two extramembranous loops from which the peptides were derived must be quite different. The human intestinal SGLT1 shares 84% amino acid identity with the rabbit SGLT1 [19] and is detected in Western blots using the same antibodies [9].

A cautionary note must be made about the use of single antipeptide antibodies to study the distribution of cloned transport proteins. In a recent study of rat kidney which used an antipeptide antibody against amino acids 564–575 of the rabbit SGLT1 [20], it was concluded that the renal Na^+/glucose cotransporter was in the brush border of S1, S2 and S3 segments of rat proximal tubule. The differences between this study and ours could be explained by species differences or by the existence of a family of renal transporters closely related in sequence to SGLT1 [21]. At a minimum, two different antipeptide antibodies should be employed to reduce the chance of cross-reactivity between different, but related, proteins.

We conclude that the cloned renal Na^+/glucose cotransporter, SGLT1, is the 'high affinity' transporter found predominantly in the outer medulla. Furthermore, there must be a second renal Na^+/glucose cotransporter, a low affinity, high capacity system, which has a different sequence than SGLT1 and which is found predominantly in the outer cortex.

Acknowledgements

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References

1 Barfuss, D. and Schafer, J. (1981) Am. J. Physiol. 240, F322–F332.
2 Turner, R.J. and Moran, A. (1982) Am. J. Physiol. 242, F406–F414.
3 Turner, R.J. and Moran, A. (1982) J. Membr. Biol. 70, 37–45.
4 Koepsell, H., Fritzsch, G., Korn, K. and Madrala, A. (1990) J. Membr. Biol. 114, 113–132.
5 Coady, M.J., Pajor, A.M. and Wright, E.M. (1990) Am. J. Physiol. 259, C605–C610.
6 Hediger, M.H., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 330, 379–381.
7 Morrison, A.I., Panayotova-Heiermann, M., Feigl, G., Scholermann, B. and Kinne, R.K.H. (1991) Biochim. Biophys. Acta 1089, 121–123.
8 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.
9 Hirayama, B.A., Wong, H.C., Smith, C.D., Hagenbuch, B.A., Hediger, M.A. and Wright, E.M. (1991) Am. J. Physiol. 261, C296–C304.
10 Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D, Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) (1990) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York.
11 Chomczynski, P. and Sachhi, N. (1987) Anal. Biochem. 162, 156–159.
12 Jacobson, A. (1987) Methods Enzymol. 152, 254–261.
13 Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology, Elsevier Science, New York.
14 Dumont, J.N. (1972) J. Morphol. 136, 153–180.
15 Coady, M.J., Pajor, A.M., Toloza, E.M. and Wright, E.M. (1990) Arch. Biochem. Biophys. 265, 73–81.
16 Hilden, S.A., Johns, C.A., Guggino, W.B. and Madias, N.E. (1989) Biochim. Biophys. Acta 983, 77–81.
17 Wright, S.H, Hirayama, B., Kaunitz, J.D., Kippen, I. and Wright, E.M. (1983) J. Biol. Chem. 258, 5456–5462.
18 Elsas, L.J., Hillman, R.E., Patterson, J.H. and Rosenberg, L.E. (1970). J. Clin. Invest. 49, 576–585.
19 Hediger, M.H., Turk, E. and Wright, E.M. (1989) Proc. Natl. Acad. Sci. USA 86, 5748–5752.
20 Takata, K., Kasahara, T., Kasahara, M., Ezaki, O. and Hirano, H. (1991) J. Histochem. Cytochem. 39, 287–298.
21 Pajor, A.M. and Wright, E.M. (1992) J. Biol. Chem. 267, 3557–3560.