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Identification of two unique polypeptides from dog kidney outer cortex and outer medulla that exhibit different Na^+/D-glucose cotransport functional properties

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The cloned Na^+/D-glucose cotransporter SGLT1 and an additional recently isolated human kidney cDNA Hu14/K15 belong to a family of similar cotransport proteins including the Na^+-dependent nucleoside and Na^+-dependent *myo*-inositol carrier SMIT1. For the present study we used two different polyclonal antibodies raised against the amino acid sequence 402–420 (Ab-E) and 565–574 (Ab-P) of SGLT1 to probe brush-border membrane fractions from different regions (outer cortex → outer medulla) of dog kidney. In Western blots both Ab-E and Ab-P react specifically (peptide blockable) with two distinct bands migrating on SDS-PAGE under reducing conditions at 75.5 kDa and 72.5 kDa. The higher molecular mass polypeptide is greatly enriched (13:1) in outer cortex and diminishes progressively towards outer medulla, whereas the lower molecular mass band is barely detectable in outer cortex but is enriched in outer medulla (4:1). Brush-border membrane vesicles (BBMV) prepared from the same outer cortical and outer medullary regions that were probed with Ab-E and Ab-P exhibit strikingly different Na^+/D-glucose functional transport behavior. The Na^+/D-glucose cotransport activity in outer cortical BBMV is a low-affinity system with $K_m = 5.98 \pm 1.01$ mM, $V_{max} = 13.05 \pm 0.55$ nmol/mg protein per min, and with 1:1 Na^+:D-glucose stoichiometry. Outer medulla BBMV exhibit high-affinity $K_m = 0.27 \pm 0.03$ mM $V_{max} = 0.97 \pm 0.04$ nmol/mg protein per min and 2:1 Na^+:D-glucose stoichiometry. Comparison of SGLT1, Hu14/K15, SNST1 and SMIT indicates that Ab-E could cross react with all four, but Ab-P would recognize SGLT1, Hu14/K15, SNST1 but not SMIT. Also SNST1 is not expressed in outer cortex. Based on currently available sequence data, and its marked enrichment in outer cortex, the 75.5 kDa band is a likely candidate protein responsible for low-affinity and 1:1 Na^+:D-glucose stoichiometric Na^+/D-glucose cotransport activity (Hu14/K15) while the minor 72.5 kDa band in outer cortex is probably SGLT1. In outer medulla, the predominant band recognized by both Ab-E and Ab-P is the 72.5 kDa protein and this could be either SGLT1 or SNST1.

Introduction

Five years ago Hediger et al. [2] isolated a cDNA clone for a Na^+/D-glucose cotransporter (SGLT1) from rabbit small intestine. Expression of SGLT1 [4] revealed that this cotransporter exhibits high-affinity (K_m 0.1 mM) kinetic behavior and 2:1 Na^+/D-glucose stoichiometry. Coady et al. [1] subsequently isolated a cDNA clone from rabbit kidney which had greater than 99% homology with SGLT1.

Since 1978, however, it has been known that Na^+-dependent D-glucose transport in brush-border membrane vesicles (BBMV) prepared from whole cortex of both dog and human kidney exhibit curvilinear kinetics which is most simply explained on the basis of independent high- and low-affinity sites [15,16]. Turner and Moran [13,14] later demonstrated the presence of a high-affinity, Na^+-dependent glucose cotransport system in outer medulla of rabbit kidney, with 2:1 Na^+/D-glucose stoichiometry, and a distinct low-affinity system with a 1:1 Na^+/D-glucose stoichiometry in the outer cortex. On the other hand, Koepsell et al. [5] have presented evidence that the complex kinetics of Na^+/D-glucose cotransport, and Na^+-dependent phlorizin binding observed in isolated renal cortical BBMV can be explained by a single-carrier model. Pajor et al. [9] have reported evidence for the expression in *Xenopus* oocytes of two renal Na^+/D-glucose cotransporters with different kinetic behavior, but the protein involved in low-affinity Na^+-glucose cotransport from the outer cortex was not biochemically defined. Recently, the cDNA for a related Na^+-glucose cotransporter has been isolated from human kidney [17]. Apparently this isoform, designated Hu14/K15, exhibits low-affinity kinetic behavior when expressed in *Xenopus* oocytes (Hediger, personal communication).

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In the present study we have utilized two polyclonal antibodies (Ab-E and Ab-P) prepared against peptides synthesized from two specific sequences in SGLT1. SGLT1 and Hu14/K15 are very similar with respect to the two peptide sequences used to raise Ab-E and Ab-P [17]. Comparison of these regions with the currently available sequence data from other members of this family of cotransporters reveals that there is strong homology with the Na^+ nucleoside cotransporter SNST1 [6] in both regions but SNST1 is not expressed in outer cortex. The Na^+ *myo*-inositol carrier, SMIT1 [8] also exhibits significant homology over the 402–420 sequence but is completely different in the 565–574 region. With this information we reasoned that if both Ab-E and Ab-P specifically react in immunoblots with the same protein band in outer cortex, then using the criteria proposed by Hirayama et al. [3] this band must be either SGLT1 or Hu14/K15.

The experimental questions we then sought to answer were (i) is there a difference in regional expression in the kidney with respect to the two specific proteins recognized by Ab-E and Ab-P and (ii) if present does the regional difference in expression correlate with a functional difference in Na^+/D-glucose transport activity? Consequently we probed BBMV prepared from different regions of the dog kidney: outer medulla → outer cortex. At the same time we studied the kinetics, Na^+/D-glucose stoichiometry, and phlorizin inhibition of the Na^+/D-glucose uptake in outer medulla and outer cortex in the same BBMV preparations. Our results show that the high-affinity Na^+/D-glucose carrier with 2:1 Na^+:D-glucose stoichiometry is present in BBMV from prepared outer medulla and that this functional activity is associated with a fourfold enrichment of a 72.5 kDa protein specifically recognized by both Ab-E and Ab-P. However, in BBMV prepared from outer cortex, only low-affinity Na^+/D-glucose transport activity is found with 1:1 Na^+:D-glucose stoichiometry and this activity correlates with a 13-fold enrichment of a slightly larger protein of estimated molecular mass 75.5 kDa, also specifically recognized by both antibodies. These results strongly suggest that the 75.5 kDa protein, highly enriched in outer cortex is Hu14/K15 and that it is likely the protein responsible for low-affinity 1:1 Na^+:D-glucose stoichiometric, Na^+/D-glucose cotransport activity.

Methods

Membrane preparations

Kidneys from mongrel dogs of both genders were surgically removed while under sodium pentobarbital anesthesia. The bisected kidney was placed in ice-cold heparinized normal saline (0.9%) to intensify the appearance of regions on the cut surface for dissection.

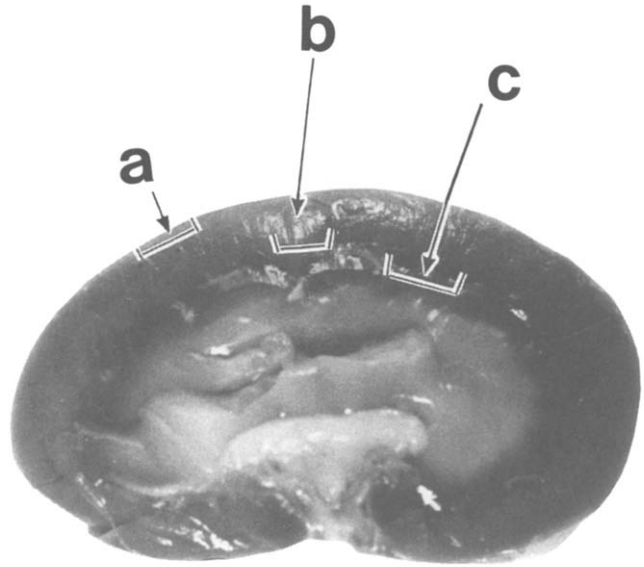

Fig. 1. Bisected kidney from a normal mongrel dog illustrating the three regions where tissue was taken for the preparation of brush-border membranes. Outer cortical sections (a) were ≤ 1.0 mm thick and the average yield per dog was 8 g. Sections from inner (mid) cortex (b) were dissected and measured approx. 2 mm in thickness from the surface of the kidney (after removal of the outer cortex). Yield from this section averaged 12 g. The outer strip (c) of the medulla (approx. 1 mm) yielded 5 g of tissue.

Fig. 1 is a photograph of a bisected dog kidney defining the regions as outer cortex, mid cortex or outer medulla. With fine dissecting scissors, the outer medullary tissue (5–7 g/dog) was first carefully dissected out and lifted off the renal column of Bertin in a continuous strip. The outer medullary strip was carefully trimmed off of surrounding remains of cortical tissue and any papillary tissue and then finely minced.

Outer cortical tissue (7–10 g/dog) was obtained from the kidney halves after the removal of the outer medulla. The kidney halves were divided into roughly 1-inch squares and fitted onto a Stadie-Riggs microtome. Outer cortical slices of ≃ 0.5–1 mm thick were sectioned out from the kidney surface. All tissue dissection was carried out on ice or at 4°C.

BBM vesicles were prepared separately from both outer cortical and outer medullary tissue in parallel. Briefly, minced tissue was suspended in ice-cold isolation medium (10 mM triethanolamine HCl, 250 mM sucrose (pH 7.6)) to a final dilution of 10 ml/g. The tissue was then homogenized and filtered. The resulting 'homogenate' was subjected to differential centrifugation yielding a crude membrane fraction. After resuspension in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-Tris, pH 7.4 (Buffer A), a $MgCl_2$ precipitation followed.

Differential centrifugation carried out on the supernatant resulted in a brush-border membrane fraction which was vesiculated by passing through a 25-G nee-

dle once and a 30-G needle twice and finally stored in liquid nitrogen.

Purity of preparations were routinely monitored by assaying the activity of enzymes known to be characteristic of brush-border microvilli, basal lateral membranes and mitochondria, namely, alkaline phosphatase, Na^+/K^+-ATPase, and succinate dehydrogenase. Protein concentrations were determined using the Bio-Rad protein assay kit.

The preparation of membranes used for biochemical characterization was identical to that used for functional studies with the exception that all buffers during isolation and purification contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 mM ethylenediaminetetraacetate (EDTA), and 0.5 mM benzamidine.

On the day of uptake experiments, frozen aliquots of BBMV were incubated at 37°C for 30 min, suspended in the appropriate buffer for the experiment and centrifuged at 20000 rpm for 20 min. The pellet was resuspended to the required volume, at a protein concentration of 1–2 mg/ml, and repassed through 25-G and 30-G needles.

Vesicle uptake measurements

All glucose transport studies were performed with the standard Millipore filtration technique. 10-s initial uptake experiments of D-glucose in both outer cortical and outer medullary BBM vesicles under Na^+ gradient condition of 40 mM ($[Na^+]_o > [Na^+]_i$) and in the absence of the transmembrane electrical potential differences were carried out to estimate the initial rate of uptake and to determine V_{max} and K_m. We have established that the flux of 1 mM D-glucose for outer cortical preparation and 0.1 mM D-glucose for outer medullary preparation is linear with time for up to 12 s at 25°C over a broad range of glucose concentrations. All experiments were done in quadruplicates at 25°C. The vesicles were suspended in Buffer A300 (10 mM Tris-Hepes, 300 mM mannitol (pH 7.4)) with 100 mM KSCN and 12.5 μg valinomycin/mg vesicle protein. The incubation medium was Buffer A (100 mM Tris-Hepes, 100 mM mannitol (pH 7.4)) with 100 mM KSCN and 40 mM NaCl or 40 mM choline chloride (final concentrations).

Effect of phlorizin inhibition on kinetics of initial Na^+-dependent D-glucose uptake in the outer cortical and outer medullary BBM vesicles was evaluated in two ways. In the first instance, phlorizin at appropriate concentration was included in the incubation medium and 10-s D-glucose uptake assays were carried out as described above. Since under these conditions there is insufficient time for phlorizin to have reached maximal equilibrium binding, we also carried out a separate protocol as described below. Vesicles were preincubated in Buffer A containing various concentrations of phlorizin and 12.5 μg valinomycin/mg vesicle protein with either 40 mM NaCl and 25 mM KCl or 25 mM KCl only. As a control, vesicles with no phlorizin preincubation but otherwise similar buffer were included. The incubation medium was Buffer A with either 140 mM NaCl and 25 mM KCl or 25 mM KCl and D-glucose over a concentration range of 0.1–10 mM for the outer cortical BBM vesicles and 0.025–1.0 mM for the outer medullary BBM vesicles and in the presence of same concentration of phlorizin for the 'experimental' vesicles. The effect of phlorizin inhibition was observed by comparing the Na^+-dependent component of initial uptake in the absence and presence of phlorizin.

The procedure for uptake measurements was as follows. A 50-μl aliquot of suspended vesicles (1–2 mg/ml) preincubated in appropriate buffer at 25°C was combined with an 100-μl aliquot of incubation medium containing radiolabeled ligands and other constituents as required at time zero. After an appropriate time, the reaction was terminated by the addition of 1.35 ml of ice-cold stop solution (10 mM Tris-Hepes, 300 mM NaCl, 300 μM phlorizin, 200 mM mannitol) and Millipore filtration (HAWP 0.45 μM) and further washed by 3×1.35 ml of the same solution. The filter was then transferred to Ready Protein (Beckman) and counted.

Data analysis

D-Glucose uptake was corrected for non-specific binding and trapping by membranes and filters by subtracting away simultaneously measured 'uptake' of tracer L-glucose. The sodium-dependent component of the resulting stereospecific glucose flux was calculated by subtracting the uptake in the absence of Na^+ from the uptake in its presence. The empirical data from transport/kinetic studies are analyzed by ENZFITTER (Elsevier Biosoft, Cambridge, UK) a non-linear regression data analysis program and a best-fit curve is calculated according to the Michaelis-Menten kinetics equation for K_m and V_{max} determination in the outer cortical and outer medullary BBMV, respectively. The data are weighted simply, and the program's built-in robust weighting eliminates 'outliers'. The fitted data can subsequently be transformed into a linear form to the Lineweaver-Burk, Eadie-Hofstee and Hanes plots as desired.

In the Na/glucose stoichiometry experiment of the outer medullary BBMV, the Na^+:glucose stoichiometry is calculated by a non-linear regression analysis of the data to the Allosteric Kinetics (Hill) equation:

$$\text{Flux} = V_{max} \cdot S^n / (K + S^n)$$

where K is $K_{0.5}^n$.

As for the outer cortical Na^+:glucose stoichiometry experiment, the lines are linear regression least-squares fits to the experimental data yielding slope and x-intercept. Details of the experimental protocols for carrying out the activation method are given in the figure legends.

Peptide synthesis

A peptide derived from a region of the cloned rabbit intestinal Na^+/glucose cotransporter SGLT1 consisting of amino acid sequence 565–574 was synthesized by Integrated Protein Technologies. The peptide was constructed on a Pharmacia Biolynx Automated Peptide Synthesizer and was purified by reversed-phase HPLC. This peptide was subsequently used for polyclonal antibody production.

Polyclonal antibody production

The synthesized peptide was conjugated to bovine serum albumin with gluteraldehyde and injected subcutaneously into three rabbits, by standard techniques. Rabbits were boosted every 4 weeks and test bleeds were performed 7–10 days after an injection. When an appropriate titre was reached, sera were collected and frozen for processing. IgG fractions were isolated and purified on Protein A-Sepharose (Pharmacia) using a buffer system which ensured optimal binding and neutral pH elution (Pierce – The Immuno Pure Gentle Ag/Ab Buffer System). The final purified antibody (P1) was stored at −20°C at a protein concentration of 2.5 mg/ml.

Antibody E (Ab-E) used in this study (raised against amino acid sequence 402–420 of SGLT1) was kindly provided by Dr. Ernest Wright's laboratory and details of its production and purification are available in Ref. 3.

SDS-polyacrylamide gel electrophoresis

SDS gel electrophoresis was carried out on either 8% or 10% polyacrylamide minigels, under reducing conditions, according to the method of Laemmli [7]. Protein loads varied according to experiment, and gels were stained either with Coomassie blue or silver stained (Pierce Silver Stain Kit). Molecular mass standards (Bio-Rad) were run in parallel in each case.

Western blot analysis

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham) by the method of Towbin et al. [12]. Molecular mass markers were visualized by staining with Amido black and transfer efficiency was determined by staining the gel after with Coomassie blue. Remaining active sites on the membrane were blocked with PBS containing 0.5% non-fat dry milk and 0.05% Tween-20 (polyoxyethylenesorbitan monolaurate) for 1 h at room temperature on a flat bed shaker. Blots were then incubated with the selected primary antibody at dilutions of 1:250 to 1:1000, overnight at 4°C, with continuous agitation. Membranes were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad) for 2 h at room temperature. After thorough washing, blots were developed using an enhanced chemiluminescence detection system (Amersham-ECL Western Blotting Detection System). Briefly, reagents were mixed 1:1 for a final volume sufficient to cover the membrane. Blots were exposed for one minute at room temperature, drained, wrapped

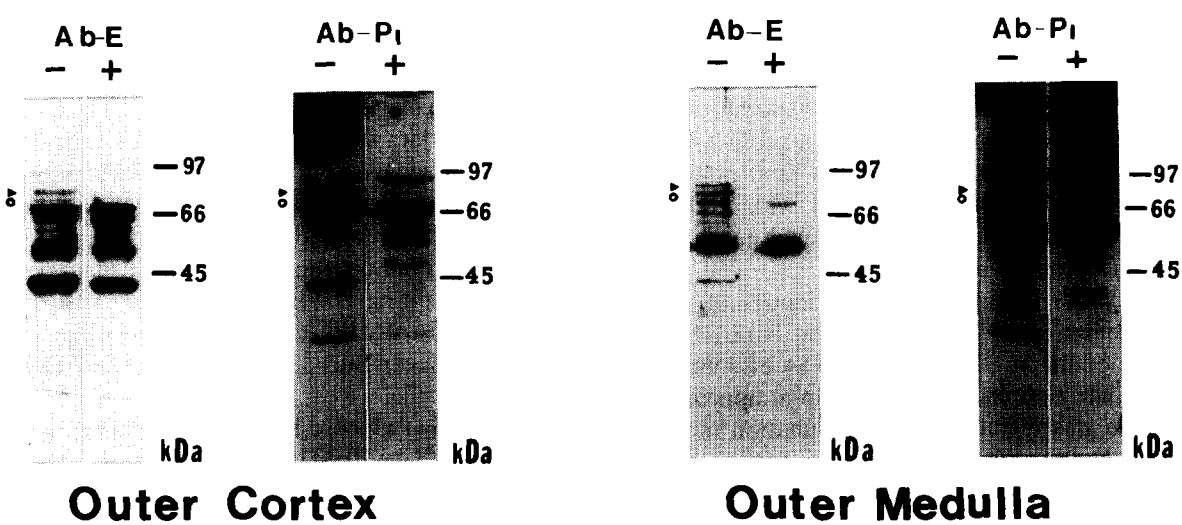

Fig. 2. Western blot analysis of dog brush-border membranes prepared from either outer cortex or outer medulla (as described in Methods). Each lane contained identical protein loads (10 μg) and were resolved on 8% SDS-PAGE minigels. After transfer to nitrocellulose, membrane strips were incubated with either Ab-E or Ab-P at a 1:1000 dilution (−). Specific (blockable) bands were identified by incubating duplicate membrane strips with each antibody after preabsorption with its corresponding peptide (+). Molecular mass standards were run in parallel and are indicated on each panel. Position of specific bands at 75.5 kDa (►) and 72.5 kDa (○) are shown.

in saran wrap and placed in a film cassette. Under proper dark room conditions, autoradiography film was placed on top of the blots and exposure was from 60 s to 1 h. Film was developed in a Kodak M-35A X-OMAT Processor. In all cases, duplicate blots were run whereby primary antibodies were preabsorbed with their relevant peptide. Peptide blockable bands were therefore determined to have specific immunoreactivity. Preabsorption was for 1 h at 37°C with antibody dilutions containing a final peptide concentration of 0.5 μg/ml. Films were scanned with a Hoefer GS-300 Transmittance/ Reflectance Scanning Densitometer and analyzed on a Mac II using Mac Integrator.

Results

Immunolocalization of peptide specific antibodies in dog kidney

Fig. 2 shows Western blots of intact BBMV from outer medulla and outer cortex probed with Ab-E and

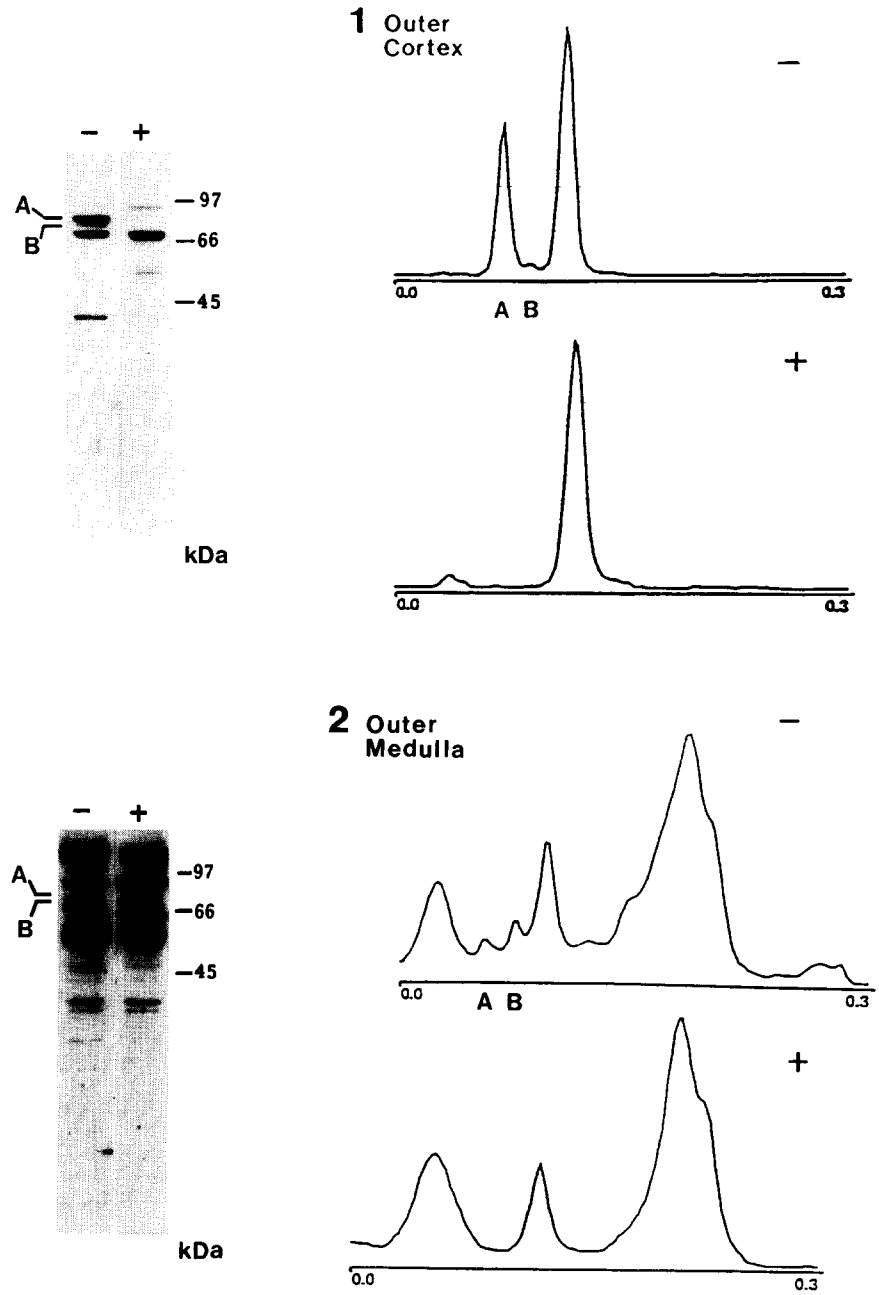

Fig. 3. Densitometric scans of Western blots of dog brush-border membranes prepared from outer cortex (Panel 1) and outer medulla (Panel 2). Upper portion of each tracing demonstrates Ab-P1 staining (−) without peptide blocking. Lower portions are scans of blots incubated in the presence of peptide (+). The difference between the two represent the location of specific (blockable) bands, which are indicated by A (75.5 kDa) and B (72.5 kDa). The relative intensity of the higher molecular mass band to the lower, in outer cortex, is 13:1; whereas in the outer medulla, the lower molecular mass band is approx. 4-fold greater than the higher.

Ab-P. In outer cortex both Ab-E and Ab-P react specifically (peptide blockable) with a prominently staining band migrating at approx. 75.5 kDa. In addition Ab-E and Ab-P react faintly but specifically with a minor band at approx. 72.5 kDa. By comparison in outer medulla, the 75.5 kDa band stains much less intensively but the 72.5 kDa band is now significantly enriched. Occasionally (but not consistently) we observe specifically staining lower molecular mass bands that we and others [3] have interpreted as representing proteolytic fragments.

The epitopes recognized by Ab-E and Ab-P are from two different regions of SGLT1, and since the recently cloned kidney cDNA with similarity to SGLT1 (Hu14/K15) is identical at these regions (Ref. 17, and Hediger, personal communication) then consistent with the criteria proposed in Ref. 3 the 75.5 kDa and 72.5 kDa bands could represent either SGLT1 or Hu14/K15.

Evidence supporting the conclusion that these two bands are indeed different proteins is provided by densitometry of the immunoblots (Fig. 3). Inspection of Fig. 3 reveals that antibody P reacts specifically (ie. is peptide blockable) with two distinct polypeptides (75.5 kDa and 72.5 kDa). In outer cortex the ratio of 75.5/72.5 is 13:1 whereas in outer medulla it is 1:4. This *disproportionate* distribution of these two bands obtained from two different regions of the kidney means that the lower molecular mass band at 72.5 kDa cannot simply be a proteolytic fragment or a different glycosylation product of the 75.5 kDa band. The simplest interpretation for the minor presence of the 72.5 kDa protein in outer cortex and minor presence of the 75.5 kDa protein in outer medulla is that the BBMV

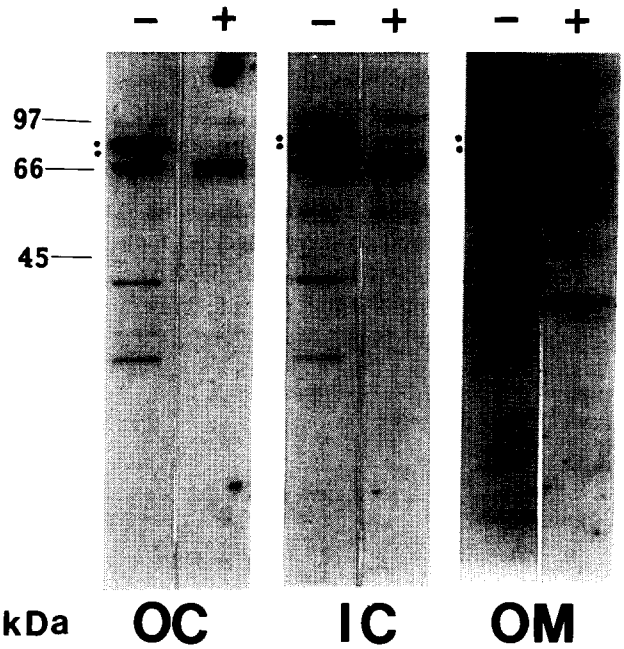

Fig. 4. Western blot analysis of dog brush-border membranes prepared from three regions in the kidney; outer cortex (OC), inner cortex (IC), and outer medulla (OM). Each one contained equal protein loads and were run out on the same 10% minigel. Membrane strips were probed with antibody P1 either without (−) or with (+) peptide absorption. Molecular mass markers are shown at left and specific immunoreactive bands are indicated (•).

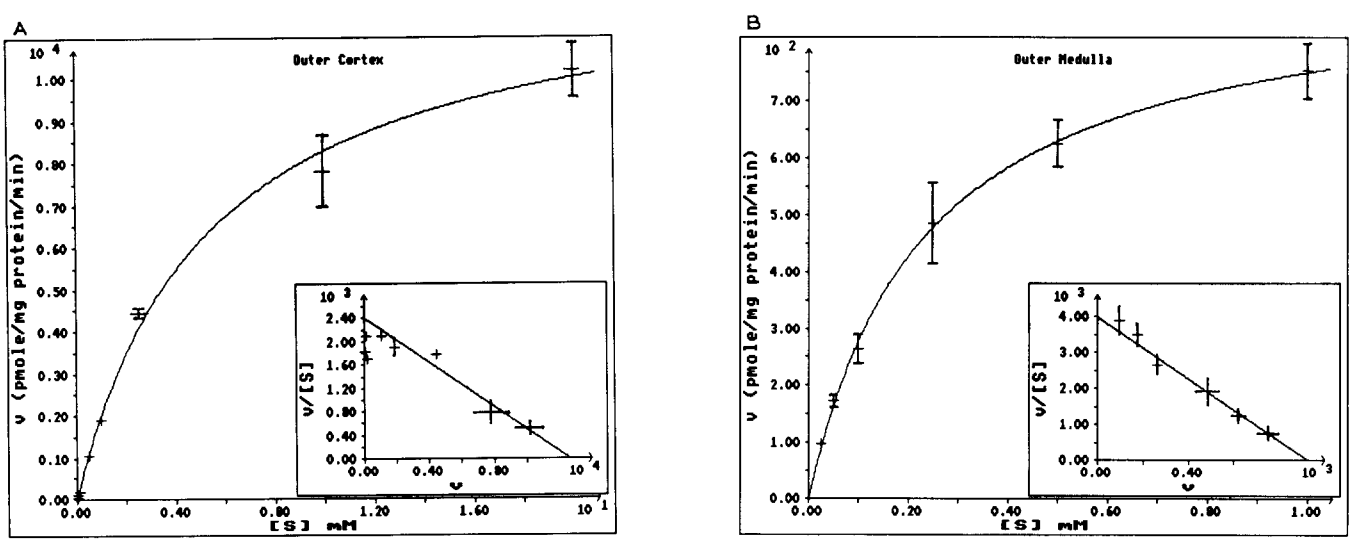

Fig. 5. Na^+-dependent glucose flux into outer cortical (A) and outer medullary (B) BBMV as a function of glucose concentration. Vesicles were preincubated in Buffer A300 with 100 mM KSCN and valinomycin. Incubation medium was Buffer A containing 100 mM KSCN with either 40 mM NaCl or 40 mM choline chloride (final concentrations) and D-glucose over a concentration range of 0.025–20.0 mM (outer cortical preparation) and 0.025–2.5 mM (outer medullary preparation). Uptake was measured after 10 s. The Na^+-dependent component of D-glucose flux was obtained by subtracting the uptakes in the presence of choline at each concentration from those found in the presence of sodium. The figure shows a hyperbolic plot of v vs. [S], where v is the D-glucose flux and [S] is D-glucose concentration. A non-linear regression analysis curve fit yields $K_m = 5.98 \pm 1.01$ mM, $V_{max} = 13.05 \pm 0.55$ nmol/mg protein per min for outer cortex and $K_m = 0.27 \pm 0.03$ mM $V_{max} = 0.97 \pm 0.04$ nmol/mg protein per min for outer medulla. (Inset) Eadie-Hofstee plot of the same data.

from outer cortex contain a 'contaminant' from outer medulla and conversely that the BBMV from outer medulla contain a 'contaminant' from outer cortex.

To further support this conclusion, we prepared BBMV from three separate regions of the kidney: outer medulla, inner (or mid) cortex, and outer cortex (see Fig. 1) and screened for the presence of an immunoreactive polypeptide using antibody P (Fig. 4). As shown in Fig. 4, the 72.5 kDa band is virtually absent from both outer and mid cortical regions defined in Fig. 1 and only becomes prominent in outer medulla. Conversely the 75.5 kDa protein which is highly enriched in the outer cortex and mid cortex region is markedly diminished in outer medulla.

Again, the simplest interpretation is that the 75.5 kDa polypeptide is associated with the nephron segment most abundant in outer cortex, i.e., the proximal convoluted tubule, while the 72.5 kDa protein is associated with the segment most enriched in outer medulla (and not present in outer cortex), i.e., proximal straight tubule and ascending or descending limb loop of Henle.

Kinetics of Na^+/D-glucose cotransport in outer medulla and outer cortex BBMV

Figs. 5A and 5B show the Eadie-Hofstee plots for Na^+/D-glucose cotransport measured in the absence of membrane potential, in the presence of a Na^+ gradient, over a concentration range from 0 to 20 mM. The data from outer cortex (where the 75.5 kDa band is enriched 13:1) indicate the existence of a single low-affinity Na^+/D-glucose cotransporter $K_m = 5.98 \pm 1.01$ mM, $V_{max} = 13.05 \pm 0.55$ nmol/mg protein per min ($n = 2$, $\pm$S.D.). On the other hand, Na^+-dependent D-glucose uptake in BBMV prepared from outer medulla is associated with a single high-affinity carrier $K_m = 0.27 \pm 0.03$ mM $V_{max} = 0.97 \pm 0.04$ nmol/mg protein per min ($n = 3$, $\pm$S.D.).

Figs. 6A and 6B demonstrate that phlorizin is a competitive inhibitor of both the outer cortex and outer medulla transporters and that the K_i for both systems is approximately the same, i.e., for outer medulla $K_i = 0.87$ μM for outer cortex 0.89 μM at 25°C. It is important to note that the data in Figs. 6A and 6B are obtained at 10 s which means that phlorizin binding equilibrium has not yet been obtained (see Ref. 10). To check whether this made any difference, we carried out a separate protocol in which vesicles were preincubated with phlorizin to achieve equilibrium binding. Then 10-s Na^+/D-glucose uptake was measured using appropriate incubation medium containing the same concentrations of phlorizin. There was no change in the kinetics of phlorizin inhibition except that the apparent K_i decreased from 0.87 μM to 0.4 μM (data not shown).

Na^+:D-glucose stoichiometry was measured using the activation method. As shown in Fig. 7 and accompanying legend, the Hill coefficient n, for the high-affinity Na^+/D-glucose cotransporter from outer medulla

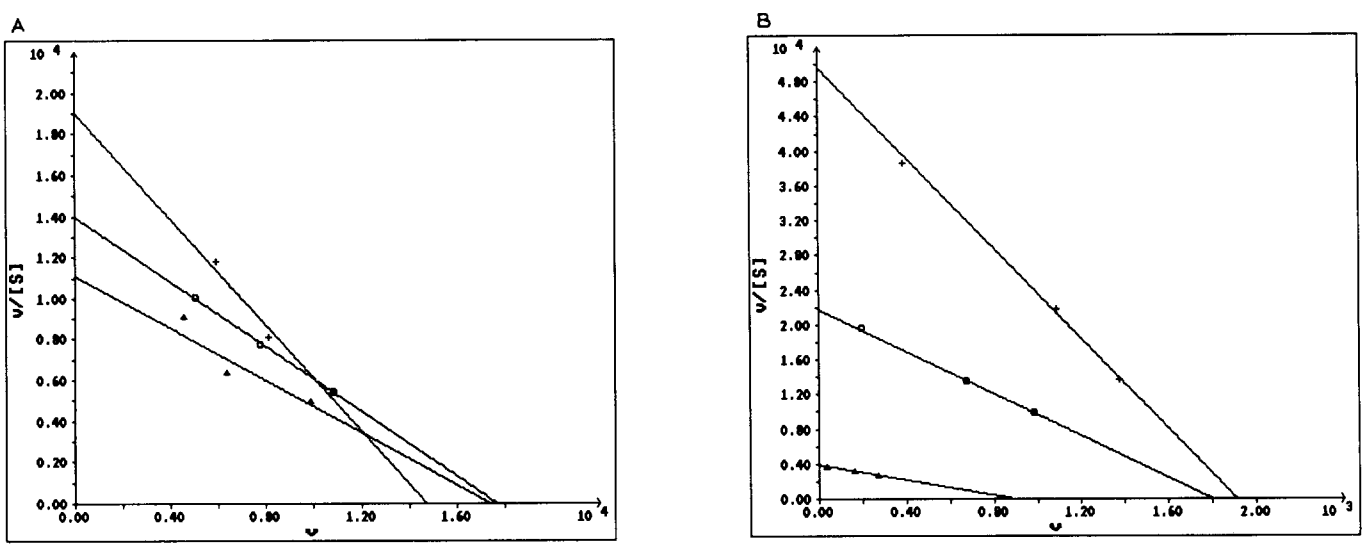

Fig. 6. Effect of phlorizin on the kinetics of initial Na^+-dependent D-glucose flux in outer cortical (A) and outer medullary (B) BBMV shown as an Eadie-Hofstee plot. Vesicles were preincubated in Buffer A300. The incubation medium was Buffer A with either 150 mM NaSCN or 150 mM KSCN and D-glucose over a concentration range of 0.5–2.0 mM (outer cortical preparation) and 0.01–0.1 mM (outer medullary preparation). Na^+-dependent component of initial uptake was obtained by subtracting away at each concentration uptake measured in the presence of K^+ from that found in the presence of Na alone or with the addition of various concentrations of phlorizin. (A) Outer cortical preparation. Uptake in the presence of Na^+ and the absence (+) or in the presence of 0.5 μM (○) or 1 μM (△) phlorizin. K_i is 0.885 μM. (B) Outer medullary preparation. Uptake in the presence of Na^+, the absence (+) or in the presence of 1.0 μM (○), 10 μM (△) phlorizin. $K_i = 0.87$ μM.

is $n = 2.03$ which implies a 2:1 Na^+:D-glucose stoichiometry. By contrast, the data shown in Fig. 8 for outer cortex, indicate that a linear Hill plot is obtained with $n = 1.03$, i.e., a 1:1 Na^+:D-glucose stoichiometry.

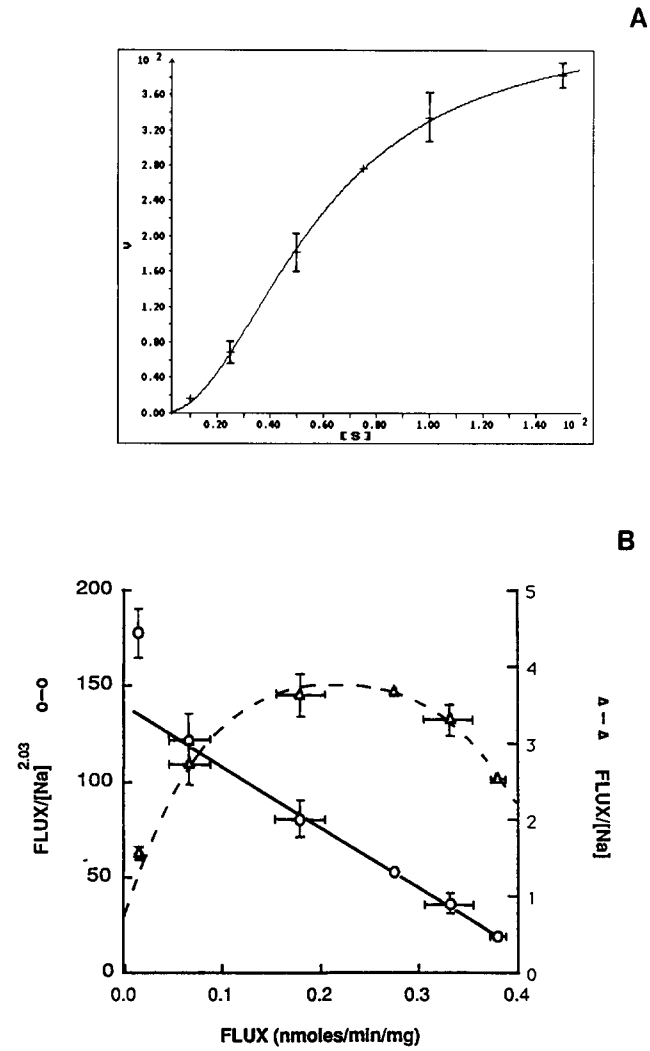

Fig. 7. Na^+-dependent stereospecific D-glucose flux as a function of Na^+ concentration (activation method) in the outer medullary preparation. Vesicles were prepared in Buffer A containing 100 mM KSCN, 600 mM mannitol and valinomycin (12.5 μg/mg protein). Incubation medium was Buffer AK, 0.1 mM D- and L-glucose, plus 0–200 mM NaCl with choline replacing sodium isosmotically. Uptake was measured after 3 s of incubation at 25°C. (A) A plot of v vs. [S], where v is flux in pmol/mg per min and [S] is Na^+ concentration in mM. (B) Plots of flux/$[Na^+]$ vs. flux (△ and dashed line) and flux/$[Na^+]^{2.03}$ vs. flux (○ and solid line). The data in panel A were fitted to the allosteric kinetics Hill-type equation by non-linear regression analysis. The result was $V_{max} = 0.441 \pm 0.001$ nmol/mg protein per min, $K_{0.5} = 58.2$ mM (see Data analysis) and $n = 2.03 \pm 0.002$. In panel B, the non-linear dashed line was obtained by a polynomial fit of order 4. The solid line through the data points of flux/$[Na^+]^{2.03}$ vs. flux was calculated from the theoretical fit obtained from the non-linear regression analysis (above). The units of $[Na^+]$ are mol per litre.

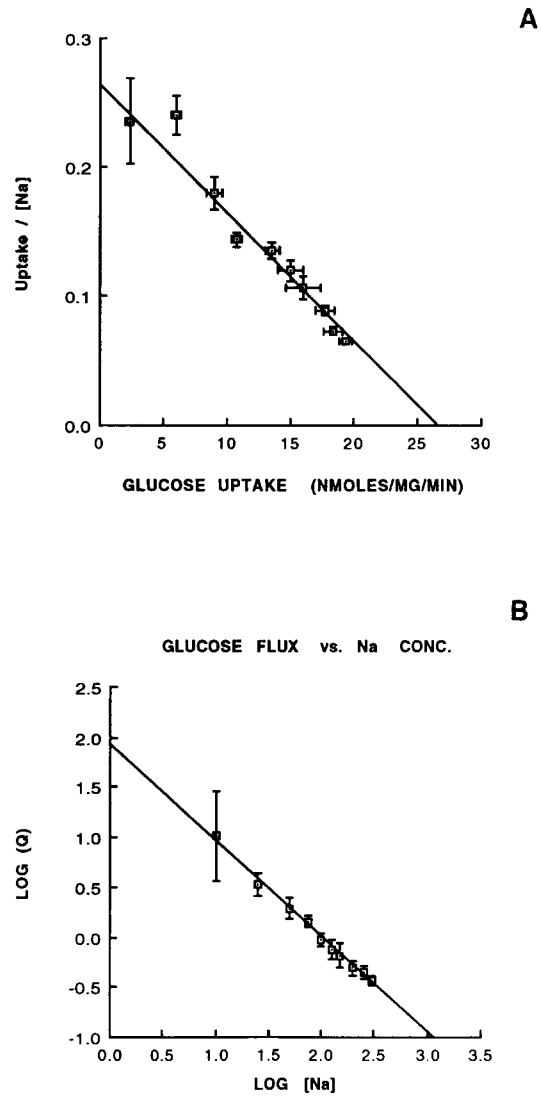

Fig. 8. Na^+:glucose stoichiometry of the outer cortical BBM D-glucose transporter determined by the activation method. Stereospecific Na^+-dependent component of initial D-glucose flux measured as a function of Na^+ concentration in mM. Vesicles were preincubated in Buffer AK with 200 mM mannitol plus valinomycin. The incubation medium was Buffer AK, 1 mM D-glucose, 1 mM L-glucose, plus 0–400 mM NaCl with choline replacing sodium isosmotically. Uptake was measured after 3 s incubation at 25°C. (A) Scatchard-type plot of data. Least-squares fit to the data yields slope ($K_{0.5}$) 101.1 mM and x-intercept (F_∞, the initial flux at saturating Na^+ concentration) 26.47 nmol/mg protein per min, $r = 0.984$. (B) Hill (log-log) plot of data. Q is given as $(F_\infty - F)/F$, (F being the initial flux rate at any given Na^+ concentration and F_∞ being the x-intercept of the Scatchard plot in Fig. 4A. Least-squares fit yields slope of (stoichiometry) 1.03 and x-intercept ($K_{0.5}$) 100.7 mM, $r = 0.995$.

Discussion

Up to the present there has been controversy as to the existence of a distinct low-affinity Na^+/D-glucose carrier in the kidney in addition to a high-affinity

Na^+/D-glucose cotransporter SGLT1. Recently Wells et al. [17] have apparently successfully cloned an additional Na^+/D-glucose cotransporter called Hu14/K15, from human kidney, a protein with significant homology to SGLT1 and which when expressed in frog oocytes yields low-affinity Na^+-dependent glucose transport (Hediger, personal communication). There is reason to believe that Hu14/K15 might indeed be the low-affinity Na^+/glucose carrier expressed in the proximal convoluted tubule in rabbit, dog, and man, but so far as indicated, Hu14/K15 has only been expressed in oocytes.

In the present studies employing a strategy for identification of renal Na^+/glucose carriers similar to that used for the intestinal transporter [3] we used antibodies raised against peptides from two different regions of SGLT1: 402–420 (Ab-E) and 565–574 (Ab-P), to probe different regions of dog kidney.

As indicated in the introduction of this paper, SGLT1 and Hu14/K15 are very similar in the regions specified by the peptide sequences 402–420 and 565–574. From currently available information the only other member of the family of Na^+ cotransporters that is sufficiently homologous in these regions that it could be recognized by both Ab-E and Ab-P is SNST1. But SNST1 is not expressed in outer cortex [6]. Therefore Ab-E and Ab-P in outer cortex must be recognizing either SGLT1 or Hu14/K15. Because the 75.5 kDa band is enriched in the outer cortex about 13 times compared to the 72.5 kDa band, and because brush-border membrane vesicles from outer cortex prepared from the same fraction used in the immunoblots exhibit only low-affinity kinetic characteristics and 1:1 Na^+:glucose stoichiometry, we believe that the 75.5 kDa band is the protein most likely to be responsible for the low-affinity 1:1 Na^+:D-glucose stoichiometric Na^+/D-glucose cotransport activity.

The lower molecular mass 72.5 kDa band, which is only present as a minor band in outer cortex is most likely SGLT1. In outer medulla, since both Ab-E and Ab-P react with the 72.5 kDa, the candidate proteins recognized are SGLT1 and SNST1. Pajor et al. [9] found that Ab-E specifically reacted with a 70 kDa band in rabbit kidney (close to the mobility of the 72.5 kDa band in our study) that was about 3.5-times enriched in outer medulla compared to outer cortex – a result very similar to the 4-times enrichment we obtained, shown in Fig. 3. Moreover, Ref. 9 also reports that hybridization of SGLT1 cDNA to renal outer medulla was three times greater compared to outer cortex. Although the 72.5 kDa band identified by immunoblotting in the present study could in principle be either SGLT1 or SNST1, it is clear that any Na^+/glucose cotransporter in this region can only be associated with high-affinity 2:1 Na^+:D-glucose stoichiometric cotransport activity, i.e., SGLT1 [1].

The kinetic parameters for the Na^+/glucose transporter in the dog are very similar to those reported by Turner and Moran [13,14] for the rabbit. But it is worth noting that in the present study both outer cortical and outer medullary Na^+/D-glucose cotransporters are competitively inhibited by phlorizin. The K_i for phlorizin inhibition of the outer cortical protein is somewhat lower than that for the outer medulla, although the phlorizin sensitivity of the two Na^+/D-glucose cotransporters is not as different as was apparently found in rabbit kidney [14]. Whether these differences in phlorizin inhibition reflect species differences in the Na^+/glucose carrier sequence remains to be determined.

Finally, in a recent paper [11], the rat kidney distribution of a single antipeptide antibody against amino acids 564–575 of rabbit SGLT1 (i.e., identical to Ab-P in the present study) concluded that the renal Na^+/D-glucose cotransporter was located on the brush-border membrane of S1, S2 and S3 segments of the proximal tubule. Based on our findings using an identical Ab-P as well as Ab-E, we believe that the results of Ref. 14 can best be interpreted as reflecting the presence of at least two different Na^+/D-glucose carriers, Hu14/K15 in the most proximal segments and SGLT1 in the most distal regions of the proximal tubules.

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