Cloning and functional expression of an SGLT-1-like protein from the Xenopus laevis intestine

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Cloning and functional expression of an SGLT-1-like protein from the Xenopus laevis intestine. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1251–G1259, 1999.—A cDNA encoding an Na+-glucose cotransporter type 1 (SGLT-1)-like protein was cloned from the Xenopus laevis intestine by the 5'- and 3'-rapid amplification of cDNA ends method. The deduced amino acid sequence was 673 residues long, with a predicted mass of 74.1 kDa and 52–53% identity to mammalian SGLT-1s. This gene was expressed in the small intestine and kidney, reflecting a tissue distribution similar to that of SGLT-1. The function of the protein was studied using the two-microelectrode voltage-clamp technique after injection of cRNA into Xenopus laevis oocytes. Perfusion with myo-inositol elicited about twofold larger inward currents than perfusion with d-glucose. The order of the substrate specificity was myo-inositol > d-glucose > D-galactose ≈ α-methyl-D-glucoside. The current induced by myo-inositol increased with membrane hyperpolarization and depended on external myo-inositol and Na+: the apparent Michaelis-Menten constant was 0.25 ± 0.07 (SD) mM with myo-inositol, whereas the apparent concentration for half-maximal activation was 12.5 ± 1.0 mM and the Hill coefficient was 1.6 ± 0.1 with Na+. In conclusion, the cloned protein shares features with both SGLT-1 and the Na+-myo-inositol cotransporter.

sodium-glucose cotransporter; sodium-myo-inositol cotransporter; Xenopus laevis oocytes; voltage clamp

The sodium-glucose cotransporter (SGLT) was first cloned from the rabbit intestine in 1987 (9). To date, more than five SGLT s have been isolated from various mammals, and their function has been extensively studied by biochemical and electrophysiological methods in heterologous expression systems (mainly Xenopus laevis oocytes) (see reviews in Refs. 11, 36, 37). In the intestine and kidney, SGLT exists in the apical membrane of epithelial cells facing the lumen and takes up glucose into the cell against a glucose concentration gradient using the Na+ electrochemical potential difference between the inside and the outside of the cell.

Mammalian SGLTs are subdivided into the SGLT-1 and SGLT-2 types according to their transport properties (11). Small amounts of SGLT activity also exist in Xenopus oocytes (34), but the properties of Xenopus SGLT are slightly different from those of mammalian SGLTs (35). For example, neither Xenopus SGLT nor SGLT-2 transports galactose (24), but the transport stoichiometry of Xenopus SGLT is 2 Na+:1 glucose, the same as that of the mammalian SGLT-1 (35). These facts suggest that Xenopus SGLT is, in a sense, a natural chimera combining the properties of mammalian SGLT-1 and SGLT-2. Therefore, to study the structure-function relationships of SGLT, we decided to clone Xenopus SGLT and chose the intestine as an RNA source because we hypothesized that SGLT must be highly expressed in that tissue.

The cloned protein, which we named Xenopus SGLT-1-like protein or xSGLT1L,1 however, preferred myo-inositol to glucose as a substrate when expressed in Xenopus oocytes. Nevertheless, our results, including amino acid sequence, transport properties, and the expression pattern in various tissues, showed that the protein was more similar to SGLT-1 than to the Na+-myo-inositol cotransporter (SMIT), another member of the SGLT cotransporter family (32).

EXPERIMENTAL PROCEDURES

Cloning of xSGLT1L cDNA. Total RNA was extracted from the Xenopus laevis small intestine by the acid guanidinium thiocyanate-phenol-chloroform method (3), and poly(A)+ RNA was purified with an mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). A 409-bp DNA fragment was obtained by RT-PCR using the following degenerated primers (13): sense (P1), 5'-CTCTCTGTGTTGCCAGYATATYG-3'; anti-sense (P2), 5'-GCTGCTAGKGTGCRYTGTTGATAC3'. The PCR product was inserted into T vector (22), which was derived from pBluescript II SK(−) (Stratagene) after digestion by EcoRI. Further extension of the cDNA toward the 3' end was carried out using the 3' system for rapid amplification of cDNA ends (RACE; Gibco BRL) and nested primer sets P3 (5'-CTGCTGGGT-CACCAGTGCAGGATCC-3') and P4 (5'-AACAAGTCGTGACATGGATGTTG-3'), according to the manufacturer’s instruction manual. LA Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) was used for PCR because its fidelity is about fivefold higher than that of the usual Taq (personal communication from Takara Shuzo). Similarly, the 5' end of the cDNA was obtained by the 5'-RACE method using the following primers: P5 (5'-GCCAAGGAAGATCG-

1 The nucleotide sequence reported in this paper has been deposited in the DDBJ-EMBL-GenBank databases with the accession no. AB008225.

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GAAGTGACCGGGTCCC-3' and P6 (5'-TTCCCAGAATGAG-TATTGGGAATGGCGTGG-3'). The DNA fragments made by the 5'- and 3'-RACE method were isolated and inserted into the T vector. To eliminate PCR errors, two or more clones isolated independently were sequenced and compared, and those with a common sequence were selected. The full-length cDNA was constructed by ligation of the 5' and the 3' cDNAs obtained by RACE at the PstI site in the P2 primer region.

Preparation of cRNA. cRNA was synthesized from a linearized plasmid containing the xSGLT1L cDNA using T7 RNA polymerase and an mCAP mRNA capping kit (Stratagene) according to the manufacturer's instruction manual. The transcript was dissolved in diethyl pyrocarbonate (DEPC)-treated water at 0.5 mg/ml.

Expression of xSGLT1L in oocytes. Xenopus oocytes were prepared as described elsewhere (24). Briefly, a piece of the ovary from a frog was incubated at 20°C for 1 h in the presence of 1 mg/ml of collagenase (type I, Sigma) in Barth's solution, and, subsequently, the oocytes in stages V–VI were defolliculated manually. The composition of Barth's solution was 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.91 mM CaCl2, 10 mM HEPES-NaOH, pH 7.4, and 0.01 mg/ml of penicillin and streptomycin. The defolliculated oocytes were incubated overnight at 20°C in Barth's solution, and viable oocytes were injected with 40 nl of cRNA solution (0.5 mg/ml) or DEPC-treated water with a glass micropipette (4). The oocytes were incubated in Barth's solution at 20°C, with daily medium changes, until use.

Electrophysiological methods. Oocytes were subjected to electrical measurements by the two-microelectrode voltage-clamp technique with a DPV-15D amplifier (Dia Medical System, Tokyo, Japan). Data acquisition and membrane voltage control were performed using pCLAMP version 6 software (Axon Instruments). The intracellular voltage record-
... and current electrodes were filled with 3 M KCl (resistance of 1.0–1.5 MΩ), and the bath electrode was a low-resistance agar bridge (28). The oocytes were perfused with a solution containing (in mM) 88 NaCl, 2 KCl, 1.8 CaCl₂, and 10 HEPES-NaOH, pH 7.4 (24). In the Na⁺-free solution, the Na⁺ was replaced with choline, and the pH was adjusted with KOH in the perfusion buffer. Voltage-clamp measurements were performed at room temperature (20–25°C) between 3 and 18 days after the injection.

Northern blot analysis. Ten micrograms of total RNAs from the various Xenopus laevis tissues were electrophoresed in a formaldehyde-agarose gel, transferred to a nylon membrane, and subjected to hybridization. The probe, the cDNA fragment from the Pst I site in the P2 primer region to the 3′ end of the cDNA, was labeled with [α-32P]dCTP by the random priming method using a BcaBEST labeling kit (Takara Shuzo). Hybridization was performed at 42°C in a solution containing 50% formamide, 5× sodium chloride-sodium phosphate-EDTA (SSPE), 5× Denhardt’s solution, and 0.5% SDS in the presence of 0.2 mg/ml of denatured salmon sperm DNA. The membrane was washed three times for 15 min in 2× SSPE–0.1% SDS at room temperature, then washed twice for 30 min in 0.2× SSPE–0.1% SDS at 65°C, and subjected to autoradiography.

RESULTS

As shown in Fig. 1, the cDNA for xSGLT1L was cloned and the nucleotide and deduced amino acid sequences were determined. This open reading frame (ORF) was confirmed by the fact that there was a stop codon (TGA) 48 nt upstream of the first methionine of xSGLT1L in the same frame (data not shown). This clone possessed a poly(dA) tail of only 14 nt downstream of 5′-ATTAAA-3′. On the basis of the observation that possession of a long poly(A)† tail resulted in a high level of expression of cRNA in Xenopus oocytes (19), 49 nt of poly(dA) tail were artificially added downstream of the 3′ end of the cDNA clone. Another clone containing 80 nt of 5′-untranslated sequence...
failed to be expressed into xSGLT1L in oocytes (data not shown). In this clone, there was another initiation codon 17 nt upstream of the first methionine of xSGLT1L (data not shown) and this frame stopped at nt 87 (TAG). On the other hand, the truncated clone with only 10 nt of the 5'-untranslated region was strongly expressed into xSGLT1L in oocytes. In the course of cloning, we obtained a short 3'-RACE product when the P4 primer was used as the anti-sense (P4' primer in Fig. 1A) as well as the sense primer. In the P4' primer region, 18 of 30 nt were matched, so we used the P4 primer for priming of reverse transcription in the 5'-RACE method. xSGLT1L consisted of 673 amino acids (74.1 kDa), and the predicted amino acid sequence shared 53 and 46% identity with rabbit SGLT-1 (9) and canine SMIT (16), respectively (Fig. 1B). The hydrophobicity plots of the three transporters were very similar (data not shown). By analogy with the model of 14 membrane-spanning regions proposed for SGLT-1 (26, 31, 32), we identified 14 putative membrane-spanning regions in xSGLT1L and hypothesized that both termini were located outside the cell (Fig. 1A). The protein also contained potential N-glycosylation and phosphorylation sites.

Expression of xSGLT1L in cRNA-injected oocytes was examined by the two-microelectrode voltage-clamp technique. Perfusion with myo-inositol elicited large inward currents in cRNA-injected oocytes, but no current was observed in control (noninjected or water-injected) oocytes (Fig. 2 and see Fig. 6A). The maximum current induced by myo-inositol was 395 nA when oocytes were voltage clamped at −60 mV (data not shown). The peak amplitude of sugar-induced current in each cRNA-injected oocyte was normalized relative to the myo-inositol-induced current (Fig. 2C). The results indicated that the substrate preference of xSGLT1L was myo-inositol > D-glucose > D-galactose ≈ α-methyl-D-glucoside (α-MG).

The steady-state currents elicited by xSGLT1L expressed in oocytes decreased with the membrane potential in the range from −150 to 50 mV (Fig. 3C). When large stepwise changes of voltage were applied to cRNA-injected oocytes, membrane currents declined exponentially to the steady state in the absence of myo-inositol (Fig. 3A). This pre-steady-state current was not observed in the presence of myo-inositol (Fig. 3B) or in control (noninjected or water-injected) oocytes (data not shown).

Currents elicited in cRNA-injected oocytes perfused with myo-inositol obeyed the Michaelis-Menten equation (Fig. 4A). The apparent Michaelis-Menten constant (Km) for myo-inositol was 0.25 ± 0.07 (SD) mM (n = 7). Figure 4B shows that the currents induced by myo-inositol at various Na+ concentrations fitted the Hill equation. The Hill coefficient was 1.6 ± 0.1 (n = 6), and the apparent concentration for half-maximal activation (K0.5) was 12.5 ± 1.0 mM. These results suggest that xSGLT1L transports two Na+ per one myo-inositol molecule. Phlorizin, a competitive inhibitor of SGLT and SMIT (7, 8, 14, 19, 25), competitively inhibited xSGLT1L (Fig. 4C), and the apparent inhibition constant (Ki) was 7.3 ± 2.3 μM (n = 7).

xSGLT1L also showed Michaelis-Menten-type kinetics with external D-glucose. The apparent Km was 6.3 ± 1.2 (SD) mM, as determined in five xSGLT1L-expressing oocytes from two batches (data not shown). This value was 25-fold greater than that with myo-inositol. It is reported that SGLT-1 works as a Na+ uniporter in the absence of sugar and generates an inward current (20, 33). This is called the Na+ leak; it repre-
sents ∼20% (or less) of the maximum sugar-induced current and is inhibited by phlorizin, like the sugar-induced current. We therefore perfused the oocytes expressing xSGLT1L with 0.5 mM phlorizin. The baseline current, however, was not changed, or was changed very little, by this treatment (Fig. 5).

To evaluate the physiological function of xSGLT1L, we injected poly(A)^+ RNA derived from the Xenopus laevis small intestine into oocytes and measured the membrane currents. In control (noninjected) oocytes, L-alanine, D-glucose, and α-MG induced small inward currents, whereas myo-inositol did not (Fig. 6, A and C). The D-glucose and α-MG responses were probably due to the endogenous SGLT in Xenopus oocytes (24, 34, 35), and the L-alanine response might have been caused by the endogenous amino acid transporter (5). After the poly(A)^+ RNA injection, the responses elicited by L-alanine and D-glucose became larger than the responses in the control oocytes (Fig. 6, B and C), but myo-inositol induced no response in RNA-injected oocytes.

Northern blot analysis revealed that an xSGLT1L mRNA of ∼3.3 kb was abundantly expressed in the small intestine and kidney (Fig. 7).
The amino acid sequence of xSGLT1L shared 52–53% identity with those of human (10), rabbit (9), and rat (17) SGLT-1s, 50% identity with that of human SGLT-2 (14), and 45–46% identity with those of human (2), canine (16), and bovine (21) SMITs. Many amino acids important for the SGLT-1 function were conserved in xSGLT1L, including Asn-246, a potential N-glycosylation site, and Arg-421, which is thought to be involved in trafficking SGLT-1 to the plasma membrane (18). In addition, 15 of 19 sites that were point mutated in SGLT-1 from patients suffering from glucose-galactose malabsorption (23) were also conserved: the sites were Asp-27, Arg-134, Ser-158, Ala-165, Trp-270, Cys-286, Gin-289, Arg-294, Ala-298, Arg-373, Ala-382, Phe-399, Gly-420, Val-464, and Arg-493.

However, xSGLT1L preferred myo-inositol to D-glucose as a substrate when expressed in Xenopus oocytes, and it showed very low activities toward D-galactose as a substrate when expressed in Xenopus oocytes.
and α-MG, <12% of that demonstrated toward myo-inositol. α-MG is well known as a specific substrate for SGLT but not for facilitated glucose transporters (11, 14, 19, 36). Therefore, we named the transporter cloned in this study Xenopus SGLT-1-like protein or xSGLT1L. Its substrate specificity was more similar to that of SMIT (7) than to the specificities of SGLT-1 (7) and SGLT-2 (14, 19). Hereafter, we discuss points of similarity and difference among xSGLT1L, SGLT-1, and SMIT (Table 1).

xSGLT1L transports two Na⁺ per one organic solute, as do SMIT (7) and SGLT-1 (11, 12, 36), whereas

<table>
<thead>
<tr>
<th>Protein and Substrate</th>
<th>xSGLT1L, myo-inositol</th>
<th>SGLT-1, D-glucose</th>
<th>SMIT, myo-inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kᵣ, mM</td>
<td>0.3</td>
<td>0.05–0.5* (12)</td>
<td>0.05 (7)</td>
</tr>
<tr>
<td>Coupling ratio (Na⁺ to substrate)</td>
<td>2:1 (11, 12, 36)</td>
<td>2:1 (7)</td>
<td></td>
</tr>
<tr>
<td>K₀.₅ for Na⁺, mM</td>
<td>2:1</td>
<td>13</td>
<td>75 (7)</td>
</tr>
<tr>
<td>Pre-steady-state current</td>
<td>Yes</td>
<td>Yes (8, 25)</td>
<td>Yes (7)</td>
</tr>
<tr>
<td>Na⁺ leak</td>
<td>No</td>
<td>Yes (20, 33)</td>
<td>No (7)</td>
</tr>
<tr>
<td>Kᵣ for phlorizin, µM</td>
<td>7</td>
<td>0.03–1.4 (12)</td>
<td>55 (7)</td>
</tr>
<tr>
<td>Transcription in</td>
<td>Small intestine</td>
<td>Yes (11)</td>
<td>No (16)</td>
</tr>
<tr>
<td>Kidney</td>
<td>Yes (8, 25)</td>
<td>Yes (11)</td>
<td>Yes (2, 16)</td>
</tr>
<tr>
<td>Brain</td>
<td>No (27)</td>
<td>Yes (27)</td>
<td>Yes (2, 16)</td>
</tr>
</tbody>
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| Numbers in parentheses denote references: xSGLT1L, Xenopus Na⁺-glucose cotransporter type 1 (SGLT-1)-like protein; SMIT, Na⁺-myo-inositol cotransporter. Kᵣ, Michaelis-Menten constant; K₀.₅, concentration for half-maximal activation; Kᵣ, inhibition constant. *Value is for α-methyl-D-glucoside.
0.05–0.5 mM, and competitive inhibition by phlorizin (7, 12). These common features may be due to the sequence homology among these three transporters, especially in the NH₂-terminal half. On the other hand, their different substrate specificities may be explained by the difference in the COOH-terminal half, where the homology is relatively low. This is consistent with the recent report that the last five transmembrane helices form the sugar pathway through SGLT-1 (26).

In the absence of myo-inositol, xSGLT1L showed a pre-steady-state current after stepwise changes of the membrane potential (Fig. 3A). Similar results were observed for SGLT-1 and SMIT expressed in Xenopus oocytes, and the current has been attributed to the charge movements due to Na⁺ binding-dissociation and a conformational change of the transporters (7, 8, 25). This fact also suggests functional similarity between xSGLT1L and mammalian homologs.

SGLT-1 generates the so-called Na⁺ leak, which is an inward current carried by Na⁺ in the absence of sugar (20, 33), whereas SMIT does not (7). Because the leak is inhibited by phlorizin, the data in Fig. 5 imply that xSGLT1L produces negligible Na⁺ leak, if any. In this respect, xSGLT1L resembles SMIT rather than SGLT-1.

Northern blot analysis showed that xSGLT1L mRNA was expressed in the small intestine and kidney. The size of the mRNA was estimated at ~3.3 kb, which was ~0.8 kb longer than our cDNA clone. This difference, the 5'-untranslated region of the 3.3-kb xSGLT1L mRNA may be relatively long because one cDNA clone derived from 5'-RACE had a 5'-untranslated region of ~190 bp (data not shown).

SGLT-1 is strongly expressed in the small intestine, at a much lower level in the kidney, and very weakly in the lung and liver (11). In addition, the protein is expressed in the brain (27). On the other hand, SMIT is expressed in various tissues, such as kidney, brain, placenta, pancreas, heart, skeletal muscle, and lung (2), but not in the ileal mucosa (16). Accordingly, the main difference between SGLT-1 and SMIT is expression in the small intestine. The Northern analysis shown in Fig. 7, therefore, indicates that the expression pattern of xSGLT1L is more similar to that of SGLT-1 than to that of SMIT.

SGLT-1 and SMIT also differ in terms of the structure of their genomic DNAs. The coding region of SMIT mRNA resides within one exon (2, 29), whereas SGLT-1 mRNA is composed of 15 exons (30). It is thought that the SMIT gene might have been retrotransposed from the SGLT-1 gene (21, 29). In preliminary work, we performed PCR on the Xenopus laevis genome using primers P4–P7 (Fig. 1A), and the results suggested that the region between P4 and P6 could not be mapped into one exon (data not shown). This fact strengthened our conclusion that the cloned protein is not SMIT but a member of the SGLT-1 subfamily. Therefore, investigating the genomic structure of xSGLT1L should shed light on the evolution of the SGLT family.

xSGLT1L appears to be different from the endogenous SGLT in Xenopus oocytes (24, 34, 35), the transporter we first aimed to clone, because expression of xSGLT1L was not observed in the ovary with the use of Northern analysis (Fig. 7C). Furthermore, noninjected oocytes occasionally showed small inward currents when D-glucose or α-MG was applied, but no response was elicited by myo-inositol (Fig. 6A). This fact implies that the inward current was not due to xSGLT1L but rather to the endogenous SGLT.

Does xSGLT1L play a role in nutrient absorption, like SGLT-1, in the small intestine? To address this question, we recorded membrane currents from oocytes injected with poly(A)⁺ RNA purified from the small intestine of Xenopus laevis. However, the poly(A)⁺ RNA-injected oocytes produced no current when perfused with myo-inositol. On the other hand, the response induced by D-glucose or L-alanine was significantly increased, which suggested the expression of their transporters in the small intestine. It is still unclear why the expression of xSGLT1L was not observed. However, as noted above, there is another initiation codon 17 nt upstream of the first methionine of xSGLT1L, and the protein was not expressed in oocytes injected with cRNA containing this region. Because this ATG resides within the sequence 5'-TGGC TAATGA-3', it probably does not function as an initiation codon (15); however, translation of xSGLT1L might be repressed by this sequence in oocytes. Alternatively, xSGLT1L might have been derived from Xenopus SGLT mRNA by a mutation (or mutations) that occurred during RACE cloning, and, as a result, the substrate specificity of the SGLT might have been drastically changed. Although we confirmed the sequence of the open reading frame (ORF) using at least two independent clones (in 25% of the ORF by using two and in 75% of the ORF by using three or more independent clones), we still might not have been able to detect a mutation (or mutations), especially if it occurred in an early cycle of PCR. However, the production of simultaneous changes in the substrate specificity and the Na⁺ leak by a mutation (or mutations) is unlikely, so xSGLT1L cDNA is very unlikely to be a PCR artifact. More experiments are needed to elucidate the physiological function of xSGLT1L.

In conclusion, the protein cloned in this study shares properties with both SGLT-1 and SMIT: whereas the substrate specificity and the absence of the Na⁺ leak resemble the properties of SMIT, other kinetic parameters, the tissue distribution of transcription and the genomic organization, indicate a close resemblance to SGLT-1.

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